John C. Stavridis

Oxidation: The Cornerstone of Carcinogenesis

Oxidation and Tobacco Smoke Carcinogenesis. A Relationship Between Cause and Effect

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This book is a tribute to the memory of
my teacher C. D. Gardikas.
Who taught me to respect academic ethics
to show deference to the scientific community
to love medical students.

Foreword

The aim and scope of this book is to provide readers with a comprehensive understanding of the elements of carcinogenesis.

Oxygen and its metabolite, the reactive oxygen species (ROS) are often quite toxic, and under certain circumstances, carcinogenic. Free radicals and the toxic metabolites of oxygen have been implicated in the cell injury processes. This assault fosters the development of a number of efficient intracellular biochemical mechanisms of defense against oxidant injury. Whenever the rate of endogenous and/or exogenous oxidant generation exceeds the endogenous antioxidant capabilities, cell injury is sustained.

Although the ROS can play an important role in normal cell functions, their high concentration during the oxidative challenge by tobacco smoke constituents can induce cell damage.

Different substances present in both the gaseous phase and solid phase of tobacco smoke may cause apoptosis to the bronchoepithelial cells and certain forms of cancer. The common pathway of the cell injury underlying these varied conditions is the destruction of cell membrane proteins and nucleic acids through oxidation.

This book is unique in that it correlates lung carcinogenesis with oxidation and smoking; it includes a detailed analysis of the oxidative and toxic components of cigarette smoke and the molecular effect these components create in the process of oxidation and lung cancer. The subject is of such great practical importance that its serious study is almost obligatory.

The reader wishing to master the subject must work earnestly and seriously, and I hope this book will serve as a reliable guide in his/her important studies. Once he/she has mastered the principles, the more difficult aspects of the subjects encountered in research or at the bedside will become easy to grasp.

On some points I have decided that the advantages of simplicity and clarity in an instructive text outweigh the advantages and elegance of strict accuracy and complicated logic.

Preface

"Oxidations and lung carcinogenesis in relation with tobacco smoke" has stimulated sufficient interest to justify this first edition. In a sense, a textbook of such an important subject which is written for the first time makes this undertaking very interesting and useful.

When I decided to publish this venture, I was motivated primarily to collect the most up-to-date and established knowledge in the field. Nothing comparable has been attempted in the area of lung carcinogenesis during the past forty years. During this time, there has been a rapid expansion of knowledge. Many of these advances have occurred in our understanding of the mechanisms in lung carcinogenesis.

Although many components of tobacco smoke are known to be toxic and carcinogenic, little is known of the dose-response relations, the specific carcinogens, the pathogenic mechanisms, or the interrelationship between the many components of tobacco smoke with enough precision to make scientifically reliable quantitative judgments about the risk factors associated with the use of the tobacco products. Evidence of long-term studies should be a requirement for a conclusion.

In the range of exposures involved in smoking there is a relationship between oxidative stress and the incidence of cancer and the other pulmonary and tobacco-related illnesses. Among the different toxic effects of cigarette smoke on human tissues, the oxidation of structural and functional molecules and modulation of cell turnover are of paramount importance.

The reactive oxygen species (ROS) and ROS-generating compounds play a key role in tobacco smoke-induced biological lesions, contributing to lipid peroxidation, protein oxidation and DNA damage.

It may soon be possible to tie in our knowledge of lung carcinogenesis with practical application perspectives. The material presented is relevant not only to the researchers in the various individual areas of biology and/or molecular biology but also to the clinical oncologist, the practitioner, the teacher and senior student, by adding a little to their knowledge, by recalling facts which have slipped their memory, or by stimulating them to seek further information in the literature.

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Every chapter and its bibliography have been critically scrutinized and corrected by my colleague Dr. G. Baltatzis, who gave unsparingly of his time and intellect throughout the course of the preparation of this book.

I am especially grateful to my secretary, Ms. Evi Glykou who handled correspondence, kept tract of chapters, and typed the whole text.

Contents

1 Oxygen and Nitrogen Free Radicals 1
Oxygen: The Molecule Which is Sovereign in Oxidative Reactions 1
Peroxides 1
Free Radicals 3
What do We Call Free Radicals? 3
Variations of the Term "Free Radicals" 3
Free Radical Categories 4
Oxygen Free Radicals 4
The Role of Oxidative Substances in the Physiological Function of Cells 5
Toxicity Mechanisms of Oxygen Free Radicals 5
Cellular Sources of Free Radicals 6
Production of Endogenous Free Radicals 7
Different Types of Oxygen Free Radicals 7
Biological Reactions of Free Radicals 8
Superoxide Dismutase (SOD) and Oxygen Free Radicals 8
The Role of Iron 8
Chemical Compounds that Cause Damage in the Biological Systems via Free Radical Production 9
Microsomal System of Electron Transfer 9
Soluble Oxidase Enzymes 9
Transition Metal Ions 10
Free Radicals and Calcium Homeostasis 10
Xanthine Oxidase (XO), Source of Free Radicals 10
Xanthine Oxidase (XO) Effect on the Cell Membrane 11
Biological Molecules as Targets of Free Radicals 11
Lipid Peroxidation 12
Peroxidation of Polyunsaturated Fatty Acids 12
Formation of Carbonyl Compounds 14
Peroxidation of Arachidonic Acid 14
Protein Peroxidation 15
Protein Oxidation Products 15
DNA Peroxidation 15

Protection of DNA by Metallothionein 16
Nitrogen Free Radicals 17
Nitric Oxide as a Free Radical 17
Nitric Oxide (NO) Toxicity 18
Toxicity of Nitric Oxide Present in the Gas Phase of Cigarette Smoke 20
Cellular Protection from Nitric Oxide (NO) 20
Peroxynitrite ($ONOO^-$) 21
Reactions of $ONOO^-$ with Components of the Mitochondrial Electron Chain 22
Toxicity Mechanisms of $ONOO^-$ via Endogenous CO_2 in the Mitochondria 23
The Role of Mitochondrial Creatine Kinase (MCK) in the Protection Against Oxidative Stress 23
Signaling of Programmed Cell Death by Peroxynitrite 24
References 25

2 Oxidative Stress 31
The Role of Oxygen in Oxidative Stress 31
Reactive Oxygen Species in Oxidative Stress 32
Enzyme Role in Oxidative Stress 33
Oxidation of Proteins in Oxidative Stress 34
Degradation and Substitution of Oxidised Proteins in the Mitochondria Cytoplasm and Nucleus 34
Direct Repair Systems of Oxidative Damage 35
Indirect Repair Systems: Elimination of Damage 36
Lipid Peroxidation in Oxidative Stress 36
DNA Peroxidation in Oxidative Stress 37
Endogenous DNA Damage 39
DNA Oxidation 39
DNA Methylation 39
Mechanisms of DNA Oxidative Damage 40
Inducible Systems of Defense and Repairing Against Oxidative Stress . . . 41
Gene Expression as a Response to the Oxidative Stress 41
Oxidative Damage of DNA in Cancer: Role of Lipid Peroxidation and 8-oxoGUa 42
ROS Activity in Mitochondria 43
Antioxidative Enzymes in Saliva 43
References 44

3 Oxidative Damage of Genome DNA: Repair Mechanisms 49
Reconstitution of DNA Damage 50
The Role of DNA-polymerase ß in the Repair Mechanism of DNA Damage 50
DNA Damage-Repairing Pathways — Reconstitution of Genomic DNA . 50
Introduction 50
The Role of Glycosylases in BER Reconstruction of DNA Damage . 51

Double Strand DNA Damage . . . 51
The Role of Methyl-Transferase in Reconstitution of DNA Damage 52
Reconstitution of DNA Damage by Base Excision Repair (BER) . . . 52
Reconstitution of DNA Damage with Excision of the Oxidized DNA Bases by the Specific Glycosylases NEIL 1 and NEIL 2 . . . 53
Reconstitution of DNA Damage by Nucleotide Excision Repair (NER) . . . 54
Reconstitution of the DNA Lesions Caused by Mismatch Repair (MMR) . . . 55
Reconstitution of DNA damage by Nucleotide Pool Sanitation . . . 55
Role of the Mut Proteins in the DNA Repair Processes . . . 56
Restoration of Oxidized Pyrimidine . . . 57
Direct Restoration of DNA Damage . . . 57
Reconstitution of Double-Strand Breaks by Homologous Recombination (HR) and Nonhomologous End Joining (NHEJ) . . 57
Oxidation Damage of DNA: A Causal Factor of Cancer Development 58
Overexpression of Enzymes Which Participate in the Reconstitution of Endogenous Damage . . . 59
References . . . 60

4 Mitochondria: Structure, Function and Relationship with Carcinogenesis . . . 63
Mitochondrial Contribution to Oxidative Processes . . . 63
Pumping of H^+ to the Outer Part of the Mitochondrion, Induced by the Electron Transport Chain . . . 63
ATP Production . . . 64
Respiration-dependent Calcium Transport to the Mitochondria . . . 65
Intramitochondrial Ca^{2+} Cycling . . . 65
Mitochondrial Permeability Transition . . . 66
Components of the MPT Pore . . . 66
Role of Cyclophilin-D . . . 67
General Remarks . . . 67
Role of Calcium in the Function of the PT Pore . . . 68
Role of Adenine Nucleotides and Pi . . . 68
Evidence of Increased Mitochondrial Permeability (MP) . . . 69
Rupture of the Outer Mitochondrial Membrane Due to Swelling: The Influence of the Irreversible Transitional Permeability . . . 69
Mitochondrial Membrane Potential . . . 69
Mitochondrial Matrix pH . . . 70
Mitochondria and Accidental Programmed Cell Death (PCD) . . . 70
Elaboration on Apoptosis and Necrosis . . . 70
Mitochondrial Potential ($\Delta\Psi m$) and Programmed Cell Death (PCD) 71
Release of Cytochrome c in the Cytosol During Apoptosis . . . 71
Synthesis and the Role of Cytochrome c . . . 71
Roles of Bid, Bax and Bcl-2 . . . 72

Caspases as Mediators of Apoptosis . . . 72
Alterations of Mitochondrial Functions that Constitute Initiators of Apoptosis . . . 74
Gene Regulation of Apoptosis . . . 74
Oxidative Stress and the Mitochondria . . . 75
Respiratory Chain Function During Oxidative Stress . . . 75
Conditions of Elevated $O_2^{-\cdot}$ Production or Shortage of Antioxidants 76
Function of the Pores During Oxidative Stress . . . 76
Opening of the Mitochondrial Membrane Pores During Oxidative Stress . . . 77
Role of Ca^{2+} in Pore Opening During Oxidative Stress . . . 77
Lipid Peroxidation of the Mitochondrial Membrane . . . 78
Oxidative Stress and the Mechanisms of Apoptosis . . . 78
ROS, Bcl-2 and Programmed Cell Death . . . 79
Oxidative Modifications of the Mitochondrial DNA Bases . . . 80
Oxidative Damage of Mitochondrial RNAs . . . 81
Nitric Oxide (NO) Toxicity in the Mitochondria . . . 81
Nitric Oxide (NO) Action in Mitochondrial Respiration . . . 81
Long-term Activity of NO Causes Production of $ONOO^-$ . . . 82
Nitric Oxide in the Mitochondria Causes Formation of the ROS and RNS and Mediates MPT . . . 83
Nitric Oxide Activity on MPT . . . 83
Pathophysiological Function of NO . . . 84
Activation of Apoptosis by NO . . . 84
Anti-apoptotic Activity of NO . . . 85
Cellular Necrosis by NO . . . 86
DNA Damage by NO . . . 86
NO-induced Apoptosis . . . 87
Mitochondria and Carcinogenesis . . . 88
References . . . 89

5 Oxidative Activation of Transcription Factor Pathways by Cigarette Smoke Oxidants . . . 97
Activation of the Transcription Factor NFkB . . . 97
Structure and Function of NFkB . . . 97
Control of lkBa by Tyrosine Phosphorylation . . . 98
Cross-talk Between NFkB and MAP Kinases . . . 98
Activation of NFkB During Oxidative Stress: Role of the Antioxidative Inhibitors . . . 100
Effect of Cigarette Smoke Oxidants on NFkB: Activation of Intracellular Adhesion Molecule-1 (ICAM-1) . . . 100
Oxidative/Anti-oxidative Interventions in the Activation Mechanisms of NFkB and AP-1 . . . 101
NFkB Regulation by Nitric Oxide (NO) . . . 101
Mechanism of Oxidative Substance Action on NFkB Regulation . . . 102

Oxidative Stress Through Receptor Molecules 102
NFkB and Immune Function 103
Reactive Oxygen Species (ROS) and Mechanisms of Immune Cell Activation 103
Ras Gene Activation 103
Oxidative Signals and Redox Pathways in T-cells 104
Oxidation of the Transcription Factor ERK 105
Phosphoinositide Pathway Activation 105
Role of Protein Kinase C (PKC) 105
Structure and Function of the PKC Molecule 106
Intracellular Distribution of PKC 107
Effect of Oxidation on the Structure of the PKC Molecule 107
Effect of Oxidation on Membrane Bound PKC 108
Effect of Oxidative Stress on PKC Function 108
Phospholipase Activity 109
Activation of the Phosphoinositide Pathway from Cigarette Smoke 110
Acetaldehyde 110
Reactive Oxygen Species 110
Anaphylotoxin C5a 110
Malondealdehyde-acetaldehyde Combination 111
Hydroxyl Radicals 111
References 112

6 Cell Protection Mechanisms from Oxidation 117
Definitions and Interpretations 117
Reductive and Oxidative Agents: Definition and Interpretation 117
Antioxidant and Pro-oxidant Substances: Definition and Interpretation 117
Antioxidative Substances 118
Antioxidative Substances Which the Cell Acquires from the Environment 119
Antioxidative Effect and Toxicity of Flavonoids 120
Antioxidative Enzymes and Proteins 120
Reduced Glutathione in Broncho-epithelial Cells 122
Smoking and the Protective Role of Reduced Glutathione 123
Glutathione Transporting Pumps (GSSG) During Oxidative Stress 124
References 125

7 Role of DNA Adducts in Carcinogenesis 127
Central Role of DNA Adducts in the Mechanisms of Carcinogenesis 127
Endogenously-produced DNA Adducts 128
DNA Adducts and Nitrosamines 128
Oncogenesis and Metabolism of 4-(methyl-nitrosamino)-1-(3-pyridil)-1-butanol (NNK) and its Metabolites 128

DNA Adducts and Lipid Peroxidation 130
Aromatic Amines and Amides 130
O^4-alkyl-thymines 131
Cyclic Adducts of the Nucleic Acids 131
Significance of the Adduct Determination 131
Cancer Biomarkers 131
Protein Adducts 132
Excretion of Metabolites in Urine 132
Other Biomarkers 133
References 133

8 Transmolecular Reactions of Genetic Toxicity: Alkylation and Methylation of DNA 137
Nucleophilia and Genotoxicity of Some Chemical Substances 137
Alkylating Agents 137
DNA Methylation 138
Methylation in Eukaryotes 139
DNA Sub-methylation in Cancer 139
Local DNA Hypermethylation 140
DNA Methylation Mechanisms in Neoplasmic Cells 141
Role of DNA-methyl-transferase (DNA-MTase) Activity Alterations 141
Evidence that Increased DNA-MTase Activity Plays a Decisive Role in Tumor Evolution 141
Other Risk Factors Related to Tumor Evolution Which Play an Important Role in the Hyper-methylation of CpG Islets 142
Mutational Changes in the Gene Promoter Regions Constitute the Hypermethylation Background of the CpG Islets 142
In Normal Cells, Regional Factors are Associated with the Defence of CpG Islets Which are Altered in Neoplastic Cells 142
References 143

9 Toxicity and Carcinogenicity of Hydrocarbons 145
Isoprene 145
Properties of Isoprene Metabolism 145
Molecular Toxicological Behaviour of Isoprene 145
Pharmacokinetics of Isoprene 146
Mutational Activity of Isoprene 146
Studies on Isoprene Carcinogenesis 146
Isoprene Toxicity in the Gaseous Phase of Cigarette Smoke 146
Toxicity of Isoprene Photochemical Products 147
Butadiene 147
Stereochemistry of Butadiene 148
Metabolism of Butadiene 148
Mutagenic Activity of Butadiene 149
Mutations of the DNA Bases 149

Loss of Heterozygosity in B_6C_3Fi Mice 150
References 150

10 Toxicity and Carcinogenicity of the Aromatic Hydrocarbons 153
Benzene 153
Metabolism and Distribution of Benzene in Tissues 153
Toxicity Mechanisms 154
Clinical Significance of Benzene Toxicity 155
Biomarkers Which Detect the Exposure to Benzene 156
Toluene 156
Toxicity Mechanisms 157
References 158

11 Toxicity and Carcinogenicity of Aldehydes 161
Acrolein 161
Toxicity of Acrolein 162
Effect of Acrolein on the Broncho-epithelial Cells 163
Effects of Acrolein on the Liver and Pulmonary Cells 163
Acrolein, Glutathione and Apoptosis 164
Acrolein and Specific Genes Which are Related to Cell Proliferation and Apoptosis 164
Acrolein and NF-kB 164
Acrolein and AP-1, Fos and Jun 165
Acrolein and p53 165
Acrolein and C-myc 165
Gene Expression Following Exposure to Acrolein 166
Acetaldehyde 166
Acting Mechanism 166
Metabolism 167
Mutagenesis 167
Chemical and in vivo Reactions 167
Conclusion 168
Formaldehyde 168
Action Mechanism 168
Metabolism 169
Carcinogenic Activity 169
Mutagenic Activity 169
Propionaldehyde 169
Combined Activities of Aldehydes 170
Conclusion 170
References 170

12 Toxicity and Carcinogenicity of the Nitriles 175
Acrylonitrile 175
Toxicity of Acrylonitrile in Humans 177
Aliphatic Nitriles 177

Methacrylonitrile . . . 177
References . . . 178

13 Toxicity and Carcinogenicity of the Eterocyclic Organic Compounds . 181
Furan: Toxicity and Carcinogenicity . . . 181
References . . . 181

14 Toxicity and Carcinogenicity of Metals . . . 183
Toxicity of Arsenic . . . 183
Mechanisms of Gene Damage . . . 184
Toxicity Mechanisms of Arsenic Compounds . . . 184
DNA Methylation by Arsenic . . . 185
Effect of Arsenic on the Cellular Cycle . . . 186
Conclusions . . . 186
Toxicity of Mercury . . . 186
Toxicity of Cadmium . . . 187
Cadmium Genotoxicity . . . 187
Gene Deregulation and Information Transfer . . . 188
Genes that Control Glutathione and Thiol Proteins . . . 188
K-ras and P53 Genes . . . 189
Transcription Factors . . . 189
Translation Factors . . . 189
Suggested Genotoxicity Mechanism . . . 189
Mechanisms Which Alter Gene Expression in the Presence of Cadmium . . . 190
Disruption of E-cadherin-mediated Cell Adhesion . . . 191
Cadmium and Apoptosis . . . 191
Suppression of DNA Repair by Cadmium . . . 192
Reactive Oxygen Species (ROS) and Cellular Antioxidant System . . 192
Cadmium and Emphysema in Smokers . . . 192
Cadmium Synergy with Other Metals . . . 193
Nickel Toxicity . . . 193
Chromium Toxicity . . . 194
Reduction of Cr (VI) by Reducing Enzymes, Organelles and Intact Cells . . . 194
Production of Free Radicals . . . 195
Production of Thiol Radical (GS$^{\cdot}$) . . . 195
Production of Hydroxyl Radicals by Cr (V) Reactions . . . 195
Production of Hydroxyl Radicals by Cr (IV) Reactions . . . 196
Production of Hydroxyl Radicals by the Reaction of Cr (III) . . . 196
NF-kB Activation . . . 197
AP-1 Activation . . . 197
p53 Activation . . . 197
Tyrosine Phosphorylation . . . 198
Apoptosis . . . 199
Termination of the Cell Cycle . . . 201

The Portrait of Gene Expression . . . 201
Conclusion . . . 201
Lead Toxicity . . . 202
Metals as Signals for the Transcription of Information into Nuclear DNA . 202
Iron Toxicity . . . 203
Biological Oxidation and the Role of Iron in Oxidative Stress . . . 203
Iron as a Possible Cause for Intracellular 8-oxoGua Accumulation . . 204
References . . . 205

15 Oxidative Profile of Cigarette Smoke and Lung Cancer . . . 211
ROS and Gene Toxicity . . . 213
Oxidation Affects Various Cellular Functions . . . 213
Mechanisms . . . 214
Mitochondrial DNA Damage by Oxidation . . . 214
Role of the P450 Enzymes in the Toxicity/Carcinogenicity of Certain Cigarette Smoke Chemicals . . . 215
Conclusion . . . 216
References . . . 217

16 Smoking and Lung Cancer . . . 219
Pre-neoplastic Alterations in the Smoker's Lung . . . 219
Pre-neoplastic Alterations . . . 219
DNA Adducts Due to Active Substances in Tobacco Smoke . . . 221
DNA Adducts and Carcinogenesis in Smokers . . . 222
Genes Frequently Implicated in the Carcinogenic Mechanisms in Smokers . . . 223
K-ras Genes and O^6-methyl-guanine-DNA-methyltransferase (MGMT) in Lung Cancer . . . 223
K-ras Gene Mutation and the Role of Glutathione-s-transferse M1 (GSTM1) in the Incidence of Lung Cancer . . . 224
Mutational Prototype of the Exons 1 and 2 of K-ras Gene . . . 224
Role of DNA-methyl-transferase (DNA MTase) in Cancer Development . . . 225
Activity of Tobacco Smoke Oxidative Substances on Growth Factor Receptors . . . 225
p53 Gene, Pulmonary Cancer and Smoking . . . 226
Transcriptional Characteristics of p53 . . . 226
DNA-adducts, P53 Gene and Pulmonary Cancer . . . 227
Mutational Template . . . 228
Frequency of Mutagenesis by Anti-diol-epoxide of Benzopyrene . . . 229
Cellular Enzymic Protection from the Metabolic Products of B[α]P 230
The Correlation of p53 Gene Mutations with the Histological Image of Pulmonary Cancer . . . 230
p53 Gene Activation . . . 230
Repair or Apoptotic Function of the p53 Gene . . . 231

Role of p53 in Tumour Formation 231
Activity Mechanisms of p53 232
Epilogue 232
References 234

Index 241

Chapter 1
Oxygen and Nitrogen Free Radicals

Oxygen: The Molecule Which is Sovereign in Oxidative Reactions

Oxygen is an essential life element. Not counting hydrocarbons, there is a greater diversity of molecules with oxygen than with carbon. Dioxygen or diatomic oxygen (O_2) is a diradical in its ground state with low solubility in aqueous solutions, that reacts in a kinetically peculiar fashion in oxidation reactions, and forms toxic byproducts as it gets reduced. As a result, all life forms evolved ways to deal with these problems, including ways to increase its solubility (with dioxygen binding and transport proteins), and enzymes (that could activate it kinetically and also detoxify oxygen by-products). It is important to understand the properties of dioxygen since oxidation reactions using it, power not only our bodies but our entire civilization.

The traditional Lewis structure of ground state dioxygen (Fig. 1.1) is not easily constructed since the electrons are added in pairs, and dioxygen is a biradical (a fact that also explains its paramagnetic behaviour). The two unpaired oxygens of the molecule, each have a spin state of 1/2 for a total resultant spin S of 1, making ground state oxygen a triplet $(2S+1) = 3$. Six of the second cell electrons are distributed among the sigma molecular orbitals (two each in σ_{2s}, σ^*_{2s}, and σ_{2p},) and six among the pi molecular orbitals (two each in two different π_{2p} orbitals, and one electron each in two different π^*_{2p}), so the net number of electrons in bonding orbitals is 4, giving a bond order (or number of 2). In contrast it is easy to write the Lewis structure of singlet, excited state oxygen, since all electrons can be viewed as paired, with two net bonds (1 sigma, 1 pi) connecting the atoms of oxygen (Fig. 1.1).

Peroxides

When oxygen oxidizes organic molecules, it itself is reduced (Fig. 1.2). Addition of electrons one at a time to the molecular orbitals of ground state dioxygen produces the step-wise reduction products of oxygen. With the addition of one electron, superoxide is formed. The addition of one more electron, produces peroxide. If two more electrons are added, the number of electrons in bonding and antibonding are

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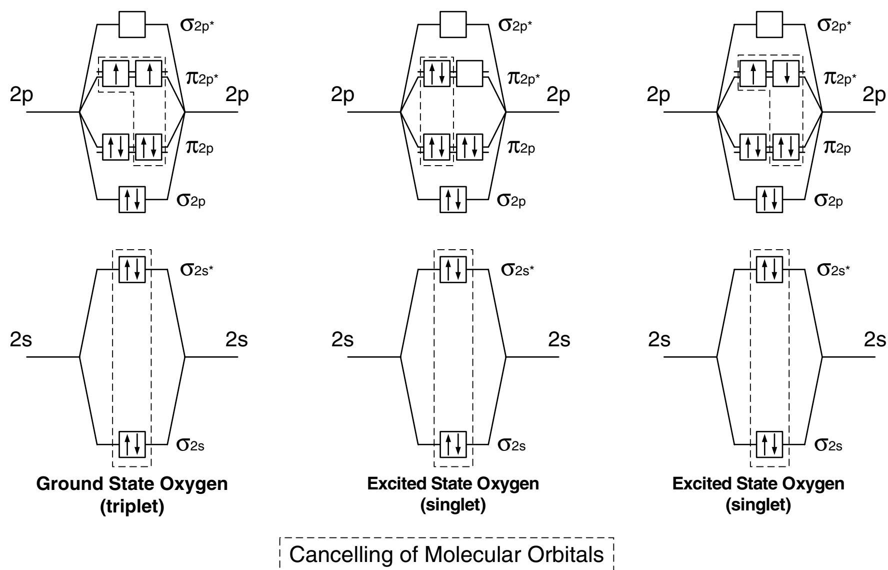


Fig. 1.1 Molecular orbitals of Oxygen

identical and as a result two separated oxides are formed since no bonds connect the atoms any more. Each of these species can react with protons to produce species such as HO_2, H_2O_2 and H_2O. It is the first two reactive products of dioxygen that make it potentially toxic.

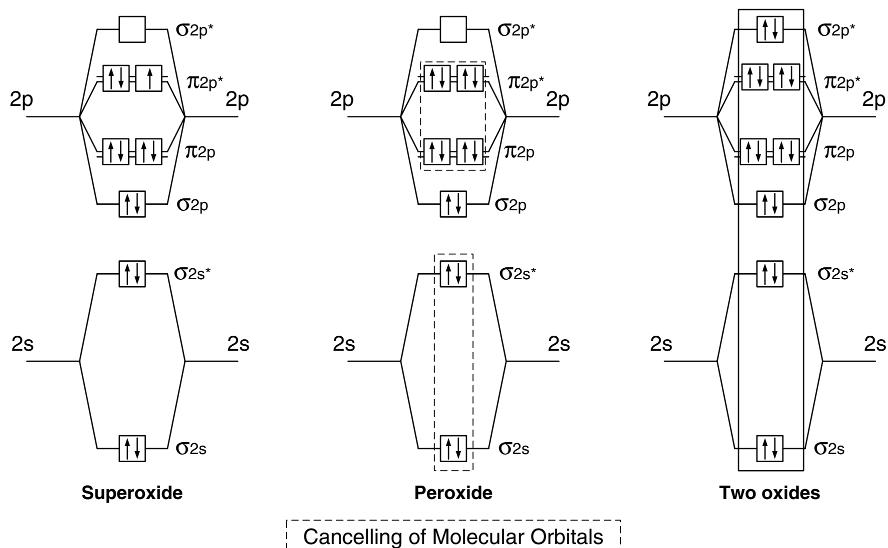


Fig. 1.2 Dioxygen reduction species

Three important kinds of peroxides can be generally found in oxidative reactions: superoxide radical, hydrogen peroxide and hydroxyl radical ($O_2^{-\cdot}$, H_2O_2 and $^{\cdot}OH$, respectively). There is broad knowledge concerning the possibility of conversion of one kind to another [1]. One electron reduction of molecular oxygen to a superoxide radical is not thermodynamically favored [2]. This "spin" limitation is counteracted by the reaction of molecular oxygen with a paramagnetic center that will contribute an electron to the exchanged pair. Transition metals, like Fe or Cu, usually have unpaired electrons and are excellent catalysts for O_2 reduction. A well proven source of H_2O_2 derived from O_2 is the procedure of the transport of electrons into the mitochondria and its dissociation is a source of $^{\cdot}OH$. Hydroxyl radicals can be dispersed at a distance 5–10 times longer than a molecular diameter (2–3Å) [3] and they are responsible for most of the damage reported in a variety of biological systems. Many components of complexes I, II and III in the mitochondria have thermodynamic properties compatible with the reduction of O_2 to $O_2^{-\cdot}$ [4–6].

Free Radicals

What do We Call Free Radicals?

The molecules that have one or more unpaired electrons in their outer orbital are called free radicals. For example, the anion superoxide free radical ($O_2^{-\cdot}$) is an oxygen molecule in which one electron has been added. It is known that molecules that are more stable have electrons arranged in their outer orbital in such a way that every electron is conjugated with one of the opposite spin. This arrangement stabilizes the molecule (it lowers its energy content). The presence of non-conjugated electrons leads to a higher energy content of the molecules and as a result, most of them are unstable and therefore more reactive.

The high reactivity of free radicals and their subsequent inclination to initiate oxidation-reduction chain reactions are the quintessence of their extremely high toxicity and their central role in tissue damage (Fig. 1.3).

Variations of the Term "Free Radicals"

Free radicals are an independent chemical species with one or two unpaired electrons. The literature refers to them by different names, such as: oxy-radicals, oxygen-free radicals, nitrogen-free radicals and different combinations of these terms. Nowadays, the term reactive oxygen species (ROS) is preferred to those mentioned above, since species like single oxygen, hydrogen peroxide, hypochlorous acid, peroxide, hydroperoxide and epoxy-metabolites of endogenous lipids, have active functional groups that contain oxygen but they are not radicals and they do not necessarily react with tissues following a free radical mechanism. In addition, it has to be underlined that the superoxide radical behaves under certain circumstances as a reducing rather than as an oxidative substance.

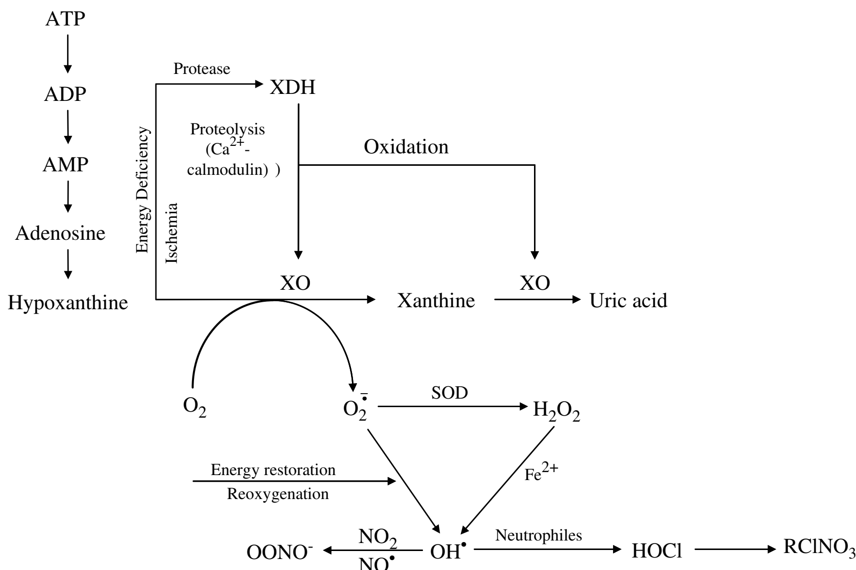


Fig. 1.3 Oxidative damage of tissue cells via xanthine oxidase mechanism

Both chemical forms of $O_2^{-\cdot}$ are important, since endogenous reactions in biological systems take place in either an aqueous or non-aqueous environment. The different chemistry renders an ability to either give or gain an electron, which is equivalent to the behavior of gaining or losing a proton, respectively; this is important in the chain of events that link O_2 with H_2O. When $O_2^{-\cdot}$ accepts protons, hydroperoxy radicals are produced ($O_2^{-\cdot} + H^+ \rightarrow HOO$ which are more active than $O_2^{-\cdot}$).

Free Radical Categories

There are two major free radical groups:

A. Oxygen-free Radicals
B. Nitrogen-free Radicals

Oxygen Free Radicals

The reactive oxygen species (toxic oxygen metabolites) are continuously produced since they are natural byproducts of cell metabolism.

Toxic oxygen metabolites play a role in tissue damage caused by a variety of reasons. Procedures that take place in various biological systems in response to free radicals are important in tissue damage. Such damage develops whenever the balance between the rate of free radical production and the cell's ability to endogenously eliminate them is disturbed.

The Role of Oxidative Substances in the Physiological Function of Cells

1. Oxidative cell metabolism for energy production
2. Arahidonic acid cascade
3. Microorganism execution via phagocytosis
4. Enzymic elimination of xenobiotics
5. Ovulation

Toxicity Mechanisms of Oxygen Free Radicals

After exposure to free radical action, tissues are damaged in a variety of ways. Lipids, proteins and DNA are the targets of free radicals.

Lipid Damage

The most serious damage is the lipid peroxidation of:

1. polyunsaturated fatty acids
2. arachidonic acid

Protein Damage

Peroxidation is also the most serious protein damage. The loss of the sulfhydryl groups as well as the resulting changes in amino acids, which lead to carbonyl-group formation, are of great importance. Oxidized proteins become the most sensitive to proteolysis. When the rate of damage of a protein is higher than the rate of its synthesis, then, cell damage appears.

DNA Damage

Poly-(ADP-ribose)-polymerase is a key enzyme implicated in the peroxidation of DNA. It is activated during the procedure of DNA damage by peroxidation. As soon as the enzyme is activated it starts using large amounts of NAD in order to repair the DNA damage caused by peroxidation. NAD consumption reduces the ability of the cell to produce ATP and the cell is led to energy failure and there are differences in the calcium distribution among the different cell compartments; this subsequently leads to cell death.

The mechanisms by which oxygen toxic metabolites participate in tissue damage have been extensively studied by Granger and collaborators [7] who used the small intestine of the cat as their experimental system.

Characteristic damages of microcirculation that were noticed in the intestine at the end of an artificial ischemic period, were for example, increased transparency of the capillaries (an hour post-ischemia) and overall necrosis of the intestinal mucosa

(3 hours post-ischemia); these were noticeably improved after the administration of the appropriate substances before the re-oxygenation of tissues, such as:

A. Catalase to neutralize H_2O_2
B. DMSO or mannitol to neutralize $^{\cdot}OH$ [8]
C. Deferoxamine or transferrin to prevent radical production via Fe^{3+} isolation [9]
D. Allopurinol, to inhibit $O_2^{-\cdot}$ production from xanthine oxidase [10, 11]
E. Superoxide dismutase (SOD) to neutralize $O_2^{-\cdot}$ [12].

In this way, Granger showed that the initiation and the further advance of tissue damage were mainly caused by the radicals $O_2^{-\cdot}$, $^{\cdot}OH$, and H_2O_2 and that NO initiated damage when the homeostatic mechanisms of the cell were not able to neutralize the oxidative charge.

Cellular Sources of Free Radicals

Sources that produce free radicals in the cell are:

Protoplasm

- Xanthine oxidase
- Hemoglobin
- Riboflavin
- Catecholamines

Mitochondria

- Electron transport system

Lysosomes

- Myeloperoxidase
- Metal ions (transition metals) - $Fe^{++(+)}$, $Cu^{+(+)}$

Endoplasmic reticulum

- Mixed function oxidase system of electron transport
- Cytochromes P450 and b5

Peroxisomes

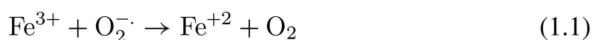
- Oxidases
- Flavoproteins

Production of Endogenous Free Radicals

Oxygen free radicals are produced during many biological processes [13–16]. The components of the electron transport chain which transfer reducing analogues to cytochrome oxidase, are the oxidation substrates of O_2 and produce superoxide radicals ($O_2^{-\cdot}$). A considerable number of electrons from the respiratory chain are diverted to this route [13, 15]. In order to counteract this problem, the cell develops defensive mechanisms, such as the presence of superoxide dismutase (SOD) which catalyzes $O^{-\cdot}$ conversion to hydrogen peroxide (H_2O_2), and catalases and peroxidases, which convert hydrogen peroxide to water. Hydrogen peroxide that escapes this conversion reacts with the reduced transition metals (Fe^{2+} or Cu^{+}) and produces hydroxyl radicals ($^{\cdot}OH$), the oxidative factor that most directly damages DNA [17, 18]. Hydroxyl radicals react vigorously with macromolecules, but there are defensive mechanisms in the form of micromolecules, such as glutathione [19] and alpha-tocopherol molecules that block the chain reactions of free radicals [20].

Different Types of Oxygen Free Radicals

The products of reduction of one, two or three electrons, such as the superoxide radical, hydrogen peroxide and the hydroxyl radical, respectively, are found in various biological systems. In addition, there are carbon-centric radicals which are produced when a hydrogen atom is detached from an unsaturated bond of a fatty acid during lipid peroxidation, as well as nitrogen- and sulfur-centric radicals that initiate various kinds of damage. Transition metal ions are important for free radical production. They have the ability to move electrons and this is the basis of free radical toxicity. For example, the superoxide anion and a transition metal such as Fe can be extremely toxic due to hydroxyl radical production. This route, known as the Haber-Weiss reaction, is Fenton-type chemistry:



$$Fe^{3+} + O_2^{-\cdot} \rightarrow Fe^{+2} + O_2 \quad (1.1)$$

$$Fe^{2+} + H_2O_2 \rightarrow {}^{\cdot}OH + OH^{-} \quad (1.2)$$

Transition metal complexes play two roles in biological systems: (a) they are protective against O_2 and they are sensitizing to $O_2^{-\cdot}$ toxicity. The development of one or the other role depends on the properties of the metal ligands as well as on the steady state concentration of the active O_2 product.

Oxygen dominance in biological systems shows that oxygen-centric radicals are the most abundant. However, organic molecules contain other atoms that may exist in free radical form and play a role in tissue damage. Thiol radicals are sulfur-centric and are formed from thiol compounds (such as glutathione), and from the dissociation of the disulfide bonds of proteins. These radicals are active and can be connected to oxygen and produce additional radicals [21].

Table 1.1 Examples of free radical reactions

Electron Transport	$O_2^{-\cdot} + Fe^{3+} \rightarrow Fe^{2+} + O_2$
Disproportonation	$2R–(CH_2)_n–CH_2–CH_2X \rightarrow R–(CH_2)_n–CH_2–CH_2X + R–(CH_2)_n–CH{=}CHX$

Biological Reactions of Free Radicals

There are fundamental reaction mechanisms which free radicals follow when they act in biological systems (Table 1.1). These reactions involve biological molecules, such as DNA, proteins and lipids. The cells have developed defensive mechanisms to protect themselves and repair the damage caused by oxidation. These mechanisms include superoxide dismutase, catalase, the glutathione system, vitamin E, ascorbic acid, uric acid and probably many additional substances, such as lipases to remove oxidized fatty acids and DNA repairing enzymes and proteases for the degradation of the damaged proteins.

Superoxide Dismutase (SOD) and Oxygen Free Radicals

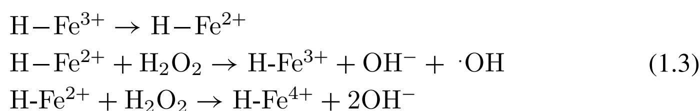
The Role of Iron

A superoxide radical is produced by one electron reduction of oxygen in aerobic organisms, during their physiological functions and pathological conditions, such as the activation of neutrophile granules, irradiation and the rehematosis of ischemia.

All aerobic organisms produce superoxide dismutase, an enzyme that converts superoxide ($O_2^{-\cdot}$) to hydrogen peroxide (H_2O_2).

Dismutase molecules that contain copper or zinc (Cu, Zn-SOD) are mostly present in the cellular protoplasm, whereas dismutase molecules that contain manganese (Mn-SOD) are mainly present in the mitochondria. Diethyl-disulfo-carbamate (DDC) inhibits Cu-SOD and Zn-SOD by displacing Cu and Zn ions that are present in the active center of the enzyme.

Selective inhibition of SOD by DDC results in cell damage by H_2O_2. Cellular iron contributes to the damage by catalyzing the production of molecules more potent than $O_2^{-\cdot}$ and H_2O_2. Iron is stored in cells in the form of Fe^{3+}, bound both to heme and to ferritin. Fe^{3+} that is bound to heme and mostly to ferritin (L) has to be reduced to Fe^{2+} in order to be able to react with H_2O_2 and form peroxy-complexes which produce $\cdot$OH radicals, or it has to be oxidized to higher states:



$$
\begin{aligned}
&H-Fe^{3+} \rightarrow H-Fe^{2+} \\
&H-Fe^{2+} + H_2O_2 \rightarrow H\text{-}Fe^{3+} + OH^- + \cdot OH \qquad (1.3)\\
&H\text{-}Fe^{2+} + H_2O_2 \rightarrow H\text{-}Fe^{4+} + 2OH^-
\end{aligned}
$$

Deferoxamine protects the cells from H_2O_2 action, since it neutralizes cellular iron without damaging the endogenous antioxidant factors, although neutralization

of iron cannot protect cells from non-oxidative toxic factors. It has been proven recently that in cells where endogenous SOD is inhibited by DDC, factors that neutralize iron prevent H_2O_2 damage. Sensitization against H_2O_2 caused by DDC is due to the action of the reactive oxygen species (ROS). In addition, cellular iron plays an essential role in damaging via the ROS, even in cells where SOD is inhibited. The two precursor substances, $O_2^{-\cdot}$ and H_2O_2, are less toxic to the cells when iron is absent. Consequently, among the substances that can reduce cellular iron, converting it from Fe^{3+} to Fe^{2+} it is probably $O_2^{-\cdot}$ that reacts with Fe^{3+} and releases Fe^{2+}.

Exposure to H_2O_2 in vitro causes a fast and significant decrease in cellular ATP levels, resulting in the accumulation of its degradation products, such as hypoxanthine. Hypoxanthine is the substrate of xanthine oxidase (XO) for $O_2^{-\cdot}$ production.

Chemical Compounds that Cause Damage in the Biological Systems via Free Radical Production

There are various chemical compounds which, acting like free radicals, can damage the various biological systems. For example, ethanol is related to a significant increase in superoxide radical ($O_2^{-\cdot}$) concentration in the liver mitochondria [22]. Carbon tetrachloride (CCl_4) is a model compound that damages the liver via free radicals. Carbon tetrachloride promotes a reducing reaction with cytochrome P450E1 and produces the trichloro-methyl radical ($CCl_3^{\cdot}$) which, in the presence of oxygen, is converted to the superoxide trichloro-methyl radical ($^{\cdot}OOCCl_3$) [23, 24]. An element that causes damage via free radical formation is iron. Nitrile-triacetate iron (Fe-NTA) for example, causes oxidative damage to DNA and lipid peroxidation [25]. Xenobiotic substances cause extensive damage following a free radical mechanism (redox cycling compounds). These substances are reduced and form free radicals after accepting a single electron. The formed free radicals can subsequently give their unpaired electron to O_2, resulting in $O_2^{-\cdot}$ radical anions.

Microsomal System of Electron Transfer

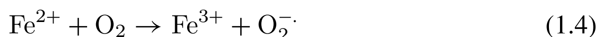
The endoplasmic reticulum membrane system contains a family of oxidizing enzymes with a mixed function and which aim to oxidize various xenobiotics. Oxidation of comparatively inactive substrates by mono-oxygenase enzymes requires electron sparing from NAD(P)H for the production of a partly reduced oxygen species. During this electron transport, the resulting electron escape leads to damage of the surrounding microstructures.

Soluble Oxidase Enzymes

Soluble oxidase enzymes oxidize endogenous as well as exogenous substrates. Among these enzymes, the best studied is xanthine oxidase, which directly reduces oxygen molecules to peroxide and hydrogen peroxide and perhaps to hydroxyl radicals.

Transition Metal Ions

One electron reduction of molecular oxygen (O_2) to produce the superoxide radical ($O_2^{-\cdot}$) is not thermodynamically favored. However, transition metals (Fe, Cu) usually have unpaired electrons and are considered to be great catalysts for molecular oxygen reduction:



$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{-\cdot} \quad (1.4)$$

Transition metal ions such as iron and copper, facilitate electron transport in biological macromolecules (lipids, proteins and DNA). In addition, they catalyze the degradation of existing organic superoxides. Transition metal ions are important for promoting many toxic reactions that are caused by free radicals. For example, superoxide anion is comparatively inactive in an aqueous solution. However, in the presence of hydrogen peroxide and of one transition metal, the very active hydroxyl radical is produced ($^{\cdot}OH$) (Fenton reaction). The final outcome of these reactions is cellular damage. In various biological systems, oxidative damages caused by metals can be prevented by specific proteins (chelating proteins). Some of these proteins, for example, metallothioneins, are not specific, whereas others like ferritin, are very specific. The overall activity of protein-chelators fulfils the biological need of a system not to have such metals in its fluids. It is obviously important to preserve bound metal ions at low concentration levels in the blood plasma (e.g. iron), where the ability of transferrin to bind iron is more than three-fold the amount of iron transferred normally to the tissue cells from the position where it was absorbed, especially to erythropoietin cells. For obvious reasons, under physiological conditions, there are no free metal ions in body fluids. Bound iron levels are very high in the brain tissue. When iron is released from its bound form, after an appropriate stimulus, damage is caused very rapidly to the polyunsaturated fatty acids in the brain, since in the cerebrospinal fluid there is no efficient iron-binding capacity.

Free Radicals and Calcium Homeostasis

Intracellular calcium levels are, under physiological conditions, 10,000 times lower than the levels in extracellular fluids. Any disturbance in Ca^{2+} transport can seriously damage cellular function. Calcium-ATPase enzymes contain important thiol groups that can be inactivated by the ROS [26, 27]. Damage caused by the ROS can reduce cell energy and its ability to maintain the density gradient of Ca^{2+} and other ions. It is clear that free radicals are able to disturb calcium homeostasis [28–32].

Xanthine Oxidase (XO), Source of Free Radicals

Xanthine oxidase is a biological source of free radicals. It is released together with its substrate, purines, in the peripheral blood when various lesions, such as organ transplantation or heavy surgery occur, especially in the liver, intestine, lungs and large vessels.

More than 90% of tissue XO exists in the form of xanthine dehydrogenase, an enzyme that uses nicotinamide-adenino-nucleotide (NAD) as an electron acceptor and results in preventing electron transport to molecular oxygen and the subsequent formation of hydrogen peroxide and superoxide radicals. Under ischemic conditions, xanthine-dehydrogenase is converted to xanthine-oxidase, which uses oxygen as an electron acceptor and results in formation of the superoxide radical ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2). The conversion of xanthine dehydrogenase to xanthine oxidase depends either on the oxidation of its sulfhydryl groups or on proteolysis via Ca^{2+} and calmodulin. In low energy conditions such as hypoxia or ischemia, a protease that exists in its inactive form in the cytoplasm is activated due to lack of energy from the cells (Fig. 1.3); this is in order to prevent calcium transport from the positions in which it is restricted. As soon as the protease is activated, it catalyzes xanthine-dehydrogenase conversion to xanthine-oxidase. Both xanthine and hypoxanthine are purine substrates of xanthine oxidase. In low energy states, two things happen in the tissues: (a) the xanthine-oxidase enzyme is produced as described above; it coexists with one of its two substrates (xanthine and hypoxanthine) and acting on them it produces a superoxide ion. (b) The molecular oxygen produced from the hypoxia state, is the substrate of xanthine oxidase and in an explosive way, peroxide radicals and hydrogen peroxide are produced.

Xanthine Oxidase (XO) Effect on the Cell Membrane

On the cell membrane there are xanthine oxidase receptors which mostly consist of glucosaminoglycans. When XO is associated with glucosaminoglycans on the cellular surface, especially endothelial cells, it produces reactive oxygen molecules intracellularly. The reaction between XO and glucosaminoglycans increases XO concentration on the cellular surface. This XO concentration is a few thousand times higher than that of the plasma. Consequently, $O_2^{-\cdot}$ and H_2O_2 production rates and cytotoxicity are increased. In this way, pathophysiological procedures such as increased vessel permeability, vessel tone lesion and activation of response to inflammation, are sequentially initiated in the endothelial cells. Circulating XO causes oxidative damage via $O_2^{-\cdot}$ and H_2O_2 even in positions distant from the site where they were produced, thus contributing to damage of the vital organs of the human organism. However, a part of oxygen radicals produced from the presence of XO is diffused inside the blood cells, particularly in the erythrocytes and they are neutralized from superoxide-dismutase, glutathione-peroxidase and catalase. Other protective mechanisms include plasma antioxidants and superoxide-dismutase which are associated with endothelial cells.

Biological Molecules as Targets of Free Radicals

When tissues are exposed to the free radical effect they are damaged. The targets of the free radicals are lipids, proteins and DNA (Fig. 1.4). The especially strong

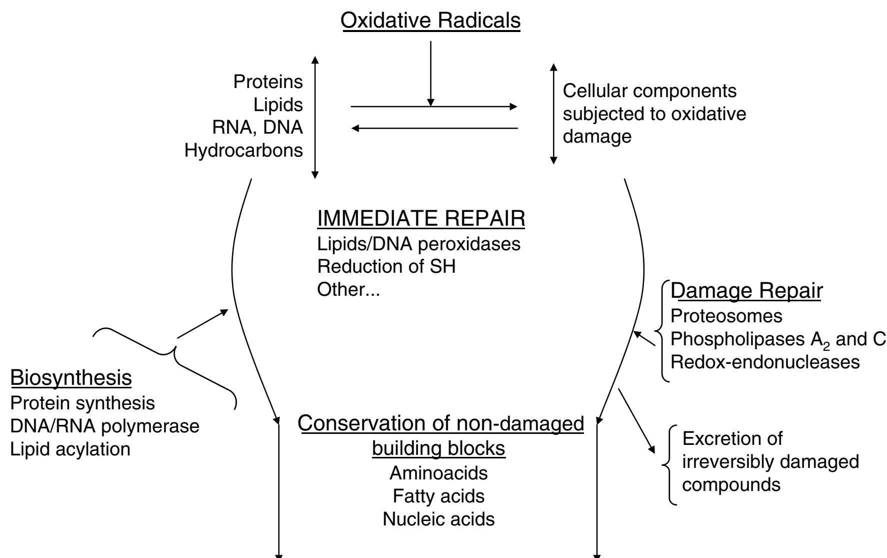


Fig. 1.4 Oxidative free radicals can damage cellular lipids, proteins and DNA

effect of free radicals is due to the instability of their electron structure; they detach an electron from other sources and make a pair with their single electron in order to stabilize it [33].

Lipid Peroxidation

Peroxidation of Polyunsaturated Fatty Acids

The group of polyunsaturated fatty acids is especially prone to free radical reactions. Peroxidation of lipids to fatty acids leads to free radical chain reactions. Due to these chain reactions, a radical substrate ($R^{\cdot}$) leads to the formation of equivalent lipid peroxides (LOOH) (Fig. 1.5). Peroxides are important intermediate products of lipid oxidative damage [34]. On lipid membranes such pathological reactions are usually accompanied by the formation of a great variety of products, including alkanes. Some of these products, especially hydroxyalkanes, are toxic, and they are useful secondary messengers for the free radicals which cause damage [35, 36]. In lipid peroxidation, products of primary peroxidation are formed, such as conjugated dienes as well as secondary products, such as malondealdehyde (MDA), 4-hydroxynoneal, hexanal and gaseous hydrocarbons [37, 38]. The lipid peroxidation procedure consists of a series of chain reactions which are initiated by the detachment of a hydrogen atom from the chain of an unsaturated fatty acid [39, 40]. In atmospheric air, oxygen is added to the carbon-centric lipid radical of the fatty acid (L) and a lipid peroxy-radical is formed ($LOO^{\cdot}$). As soon as $LOO^{\cdot}$ is formed it

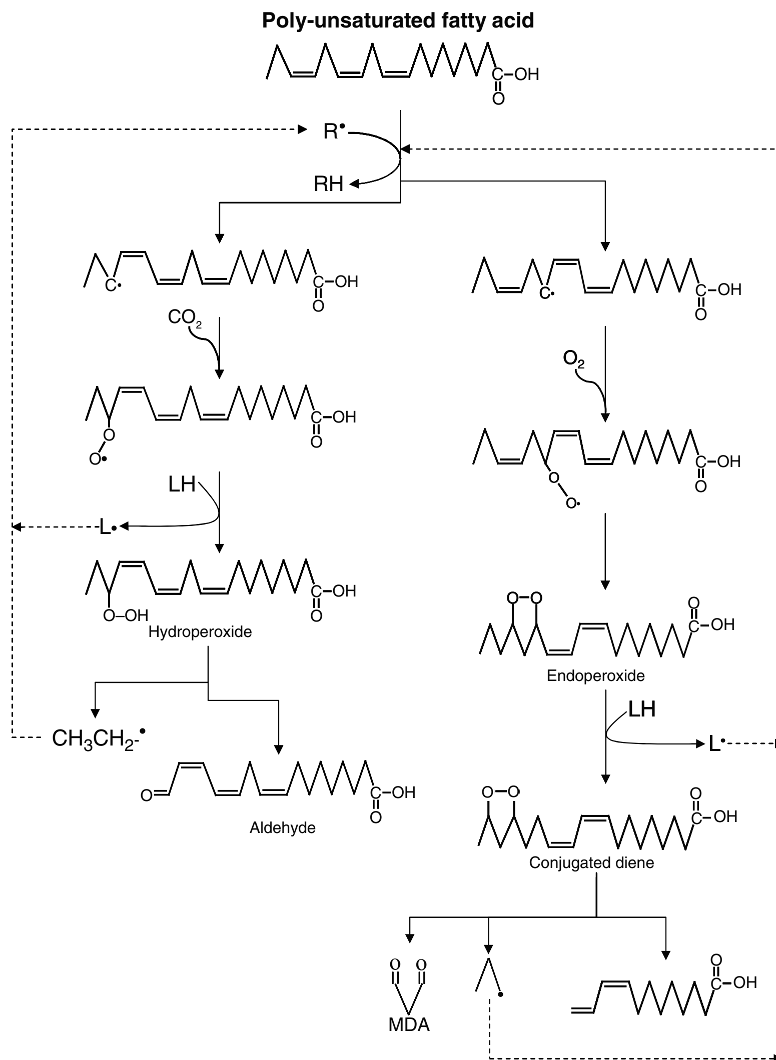


Fig. 1.5 Poly-unsaturated fatty acids peroxidation pathways

extends the peroxidation reaction chain (endo-peroxide route) by detaching a hydrogen atom from adjacent unsaturated fatty acids [39, 40]. The resulting hydro-peroxide (LOOH) (hydroperoxide route) is easily disintegrated to a more active species, among which are lipid alkoxy-radicals (LO·), aldehydes (e.g. malondealdehyde), alkanes, lipid epoxides and alcohol [39, 40]. Cholesterol is also oxidized to produce epoxides and alcohol. Consequently, the hydroperoxide route creates two radicals from one attack, whereas the endoperoxide route results in three radicals from one attack. Each one of the radicals produced, initiates by itself further lipid peroxidation following

the classic chain reaction. The result is extended peroxidation of more polyunsaturated fatty acid molecules from only a few initial radicals (Fig. 1.5). The lipid peroxidation caused, results in breaking and severe structural and functional lesions of the cellular membrane, which leads to increased sodium, chloride and calcium import into the cellular protoplasm and subsequent changes in the electrochemical density gradient of the membrane. This event causes cellular edema and a sarcoplasmic and mitochondrial Ca^{2+} concentration increase. The aldehydes produced are diffused through these membranes to other subcellular compartments [40–42]. This way, autolytic, destructive procedures are activated, relative to cell respiration.

Formation of Carbonyl Compounds

Biological reactions that involve lipid peroxidation are usually characterized by the formation of different products, including a large variety of carbonyl compounds [43, 44]. After peroxidation of ω-6 and ω-3 polyunsaturated fatty acids, the comparatively unstable fatty acid hydroperoxides are converted to more stable carbonyls after breaking and oxidation.

Some of these products are cytotoxic or genotoxic [45] and they react with biomolecules such as proteins and nuclear acids [46]. In addition, they act on receptors and on information transmission [47].

In contrast to free radicals, aldehydes are comparatively stable and therefore able to diffuse inside or outside the cell and act on targets distant from the site where they were originally produced.

Malondealdehyde (MDA) production is most widely used as a marker of lipid peroxidation.

Peroxidation of Arachidonic Acid

Production of Isoprostans

In the cell membrane there is a high potential of free radicals. The metabolism of arachidonic acid by cyclooxygenase to form prostaglandins and by lipoxygenase to produce leukotrienes, consists of a series of lipid peroxidation reactions that take place via free radicals. Byproducts of this metabolism are superoxide and hydroxy radicals [48, 49]. The intermediate products of lipid peroxidation are hydroperoxides and endoperoxides which decompose, resulting in the formation of radicals that trigger new chain reactions (Fig. 1.5). For example, peroxidation of prostaglandin G_2 to prostaglandin H_2 results in $\cdot OH$ production.

A sub-group of substances that belong to the prostaglandin group PFG_2 are known as F_2-isoprostans; they are produced in the human organism from a non cyclooxygenase mechanism which is catalyzed by free radicals and includes arachidonic acid peroxidation [50]. Formation of F_2-isoprostans is accompanied by the production of peroxy-radicals which are isomers of arachidonic acid. F_2-isoprostans are converted to prostaglandin of the PGG_2 type, which consequently is reduced to

prostaglandin, type PGF_2. F_2-isoprostans are converted in situ to phospholipids after the peroxidation of esterified arachidonic acid, which is catalyzed by free radicals. F_2-isoprostans are released in free form, probably from the phospholipases. Isoprostan formation is an early phenomenon that precedes cell death. In the urine of smokers, 8-epiPGF_{2a} levels were found to be extremely increased [51, 52].

Protein Peroxidation

Oxidation products and carbonyl protein derivatives can be the result of (a) oxidative alterations of amino acid side chains, (b) drastic cleavage of peptides by oxygen and (c) reactions that yield lipid and hydrocarbon oxidation products. The presence of carbonyl compounds in proteins shows that they have been subjected to oxidative damage by free radicals.

The loss of the sulfhydryl groups and the binding of the carbonyl groups to the side chains of amino acids like histidine, arginine, proline and lysine are two unfavorable effects of protein peroxidation. Oxidized proteins are more sensitive in proteolysis, compared to the non-oxidized. When the rate with which a protein is proteolyzed is higher than the rate it is synthesized, a cellular malfunction starts to appear and subsequently, if peroxidation continues, permanent cellular damage and cell death are observed.

This alteration of proteins is catalyzed by redoxo-cycline cations such as Fe^{2+}/ Fe^{3+} and Cu^{+}/Cu^{2+} [53]. The oxidative change in proteins results in loss of their catalytic activity, a change in their surface hydrophobicity and a change in their behavior in UV spectroscopy [54]. The determination of the carbonyls formed is the simplest method for studying protein alteration by oxidation.

Protein Oxidation Products

In contrast to the instability that most carbonyl compounds show, some products of phenylalanine and tyrosine oxidation are stable even after acid hydrolysis. Phenylalanine oxidation results in ortho-tyrosine (*o*-Tyr) formation [55]. One electron oxidation of L-tyrosine produces long-living tyrosine radicals which react with each other to form dityrosine (DT) [56]. During protein oxidation, both *o*-Tyr and DT levels are significantly increased. Since DT is a stable final product, it can be considered an excellent (cellular or urinary) marker of oxidative stress [57]. Oxidized amino acids in blood proteins are used as bio-markers for oxidative damage caused by free radicals [58].

DNA Peroxidation

DNA is a main target of ROS [59]. Nucleotide 8-hydroxy-2-deoxyguanosine (8-OHdG) was first mentioned in 1984 as a major product of DNA oxidative damage [60]. It was proven that hydroxyl radicals and single oxygen are responsible for

8-OHdG formation in biological systems [61]. DNA is constantly repaired during oxidation [62]. Until today, 8-OHdG, one of the 20 well known primary oxidative products of DNA damage, has been the bio-marker mostly used for estimation of oxidative mutations of DNA in humans and mice. 8-OHdG mutation and coupling to adenine and cytosine produce G:C to T:A transitions during DNA replication [64].

Thymine-glycol and thymidine-glycol are also two products of DNA hydroxylation that are found in urine. When they are detached from DNA, both glycols are excreted from blood through urine within 24 hours, without being further metabolized. Thymidine glycol is not dietary originated, whereas thymine glycol is, albeit at a very low percentage. Free thymine or mono-nucleotide thymine levels are very low in the cell. Consequently, high glycol levels in the urine can indicate DNA damage [65].

The ROS can generate bonds with DNA and damage the main body of phospho-deoxyribose, as well as cause specific chemical changes to purines and pyrimidines [66, 67]. Oxidative changes of the bases lead to mutations, whereas oxidation of half of deoxyribose causes detachment of the bases or breakage of DNA helices [68, 69], (Fig. 2.2). In vivo, when DNA is damaged, it is repaired by endonucleases. Damaged bases are excreted at once through urine and deoxy-nucleotides are further metabolized to mono-nucleotides, before they are excreted through urine [64, 70].

The key enzyme for DNA peroxidation is poly-(ADP-ribose)-polymerase which is activated by peroxidation during DNA damage [71, 72]. As soon as the enzyme is activated, it starts using large amounts of NAD in order to repair the damage caused to DNA by peroxidation. This disturbs electron flow into the mitochondria and consequently disrupts the ability of cell to produce ATP. The cell is led to energy deficiency, changes in its calcium levels are caused and subsequently it dies.

Protection of DNA by Metallothionein

The protection of cytoplasmic proteins from the effect of free radicals, especially ROS, is accomplished via the presence of protective enzymes, such as superoxide dismutase, catalases and peroxidases. Hydrogen peroxide (H_2O_2) is formed by the dismutation of $O_2^{-\cdot}$ in the presence of superoxide dismutase (SOD) and it is diffused towards the inner nucleus where it oxidizes the iron that is bound to chromatin and the Fenton reaction develops. This reaction produces the extremely damaging hydroxyl radicals, which because of their short life span (10^{-9} sec) can act in situ at an extremely short distance from DNA (2–3 Å).

Recently, it has been shown that a protective mechanism exists in the nucleus against the action of hydroxy free radicals; it is based in the non-enzymic protein metallothionein. This protein is of a low molecular weight, can be bound to metals and contains cysteine. One-third of the total amino acid composition of metallothionein is cysteine [73, 74]. It has been shown to be a neutralizer of oxygen radicals such as superoxide and hydroxyl radicals [75, 76]. Metallothionein production is achieved by some stress factors such as paraquat (PQ), *t*-butyl-hydroperoxide and menadione [77, 78].

Nitrogen Free Radicals

Nitric Oxide as a Free Radical

Nitric oxide (NO) is produced from NO-synthase (NOS) during the transformation reaction of arginine and oxygen to citrulline. It originates from NO-synthase expression when it is exposed to cytokines. It is released in a quantitatively defined way from two different NO-synthases, the constitutive and the inducible. NO-synthase functions on two biologically different routes, intracellular communication and cytotoxicity. The constitutive form is cytoplasmic, depends on Ca^{2+}-calmodulin and releases a pico-molar amount of NO molecules in response to receptor stimulation. The main activity of this synthase is increased rapidly during activation of specific receptors which are bound to a G-protein. The inducible form is cytoplasmic, does not depend on Ca^{2+}-calmodulin and releases a nano-molar amount of NO over a long time period. Both enzymes depend on NADPH and tetrahydrobiopterin (Fig. 1.6).

Studies have shown that a NO-synthase can regulate tumor development and metastasis in cancer cells. NO is a signal-molecule in brain cells and a potent activator of soluble guanyl cyclase, which acts as the main receptor of NO in the brain. It is freely diffused from the site it is synthesized towards intracellular site-targets in adjacent cells and acts independently from membrane receptors or lipids of cellular membranes.

In endothelia, NO regulates vessel tone, whereas in blood platelets it regulates the aggregation function. These procedures are induced from NO which stimulates guanyl cyclase and in turn produces a second messenger, cyclic 3:5-monophosphoguanosine (cGMP) (Fig. 1.6).

The NO radical also targets enzymes of the electron transport chain. The enzymes contain iron and inhibit DNA synthesis. Cigarette smoke contains high concentrations of NO (400–1000 ppm/cigarette).

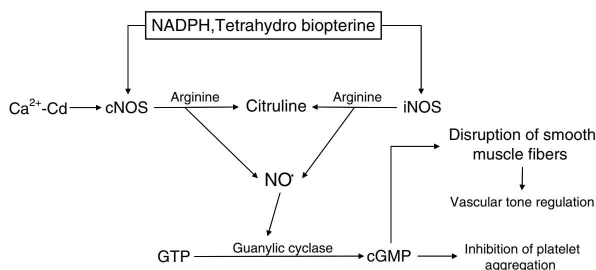


Fig. 1.6 Vascular tone and platelet aggregation mechanisms by NO. Cd = Calmodulin, GTP = Guanosine triphosphate, cGMP = cyclic guanosine monophosphate, cNOS = constitutive NO-synthase, iNOS = inducible NO-synthase

Nitric Oxide (NO) Toxicity

Nitric oxide (NO) in humans is produced by three isoforms of NO-synthase: endothelial (eNOS), neuronic (nNOS) and inducible (iNOS). NO reacts with superoxide ($O_2^{-\cdot}$) and produces peroxynitrite ($ONOO^-$), whereas with oxygen it produces NO_2 and N_2O_3. The latter or metric oxide react with thiols (RSH) and produce D-nitrosothiols : $RSH + N_2O_3 \rightarrow RS\text{–}NO + NO_2$ (or $NO + R\text{–}SH \rightarrow R\text{–}S\text{–}N{=}O$) (Fig. 1.7) [79]. NO plays two discrete roles in the human organism: in low concentrations, produced from eNOS and nNOS, it regulates physiological functions. In high concentrations, produced from iNOS, it acts as a cytotoxic molecule and is used by our immune system to kill the pathogenic cells of the host [80]. Cell death caused by NO is especially important for the killing of pathogenic microorganisms, cancer cells and non pathogenic host cells. A death of this kind is involved in a wide variety of inflammatory, pestiferous, ischemic and neuro-degenerative diseases.

NO can kill cells by depleting adenosine triphosphate (ATP) reservoirs, an effect that damages sodium and calcium pumps. Consequently, necrosis caused by NO is due to depletion of energy reservoirs, which is in turn caused by (i) inhibition of mitochondrial respiration, (ii) inhibition or uncoupling of glycolysis, (iii) activation of poly-(ADP-ribose)-polymerase (PARP) and (iv) activation of mitochondrial permeability transition (MPT) [81] (Fig. 1.8).

In the mitochondrial permeability transition (MPT) there is a significant increase of permeability of the inner mitochondrial membrane in all small molecules, caused by calcium and oxidative substances; this leads to lack of coupling, depletion of ATP, mitochondria swelling, outer membrane failure and cytochrome C release [82–84], resulting in apoptosis. The reasons that lead to NO-induced apoptosis are less well known and may vary (page 110); they include: (i) stress of the endoplasmic reticulum that causes CHOP transcription, (ii) MPT caused by NO derivatives, such as peroxynitrite and S-nitrosothiols, (iii) inhibition of mitochondrial respiration, (iv) activation of MAP kinases (which are activated by stress and mitogens) and, (v) oxidation of mitochondrial phospholipids [85–93]. A key in the apoptotic procedure is caspase activation (caspases are proteinases with cysteine on their active site), which causes breakdown events such as chromatin breaking and externalization of phosphatidyl-serine (which is originally located on the inner surface of the cellular membrane). One of the major ways of causing caspase activation is the release of cytochrome C from the mitochondria into the cytoplasm, where it binds the APAF-1, component of the apoptosome and activates pro-caspases [94, 95] (page 96). The release of cytochrome C takes place either from MPT or from pores that are created from the proteins Bax/Bak on the outer mitochondrial membrane [96, 97]. H_2O_2 and other oxidative substances cause apoptosis via activation of MAP kinases

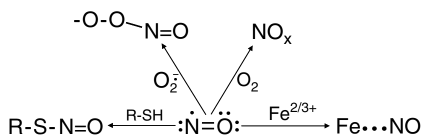


Fig. 1.7 Reactive pathways of nitric oxide

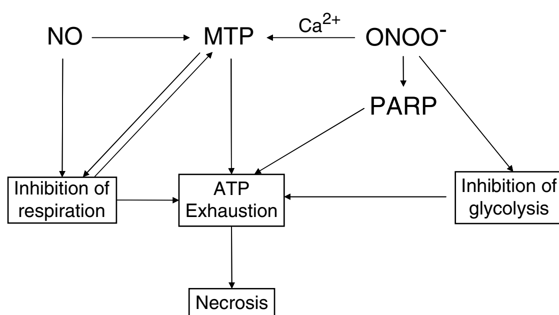


Fig. 1.8 NO causes cell necrosis due to exhaustion of ATP stores. ONOO– causes cell necrosis due to inhibition of glycolysis

by stress, acting positively on the activation of caspase [98–100]. Subsequently, NO has three different effects on the mitochondria in response to cell death: (i) inhibition of mitochondrial respiration, (ii) stimulation in the production of oxidative substances and (iii) causes MPT [93, 101, 102]. The inhibition of respiration due to NO is caused by the reversible inhibition of cytochrome oxidase from NO and by irreversible inhibition of different mitochondrial components from peroxynitrite and S-nitrosothiols [103–107]. The inhibition of respiration caused by NO results in cellular death and converts apoptosis to necrosis [108]. NO can stimulate the production of superoxide, hydrogen peroxide, peroxynitrite, NO_2 and N_2O_3 from the mitochondria. The above oxidative substances can either inhibit respiration or produce permeability transition events [81]. The production of oxidative substances from NO can cause depletion of the glutathione reservoirs, which in turn can cause necrosis [109–111]. As mentioned above, NO triggers apoptosis via induction of mitochondrial permeability transition. This fact shows that NO triggers the same chain of molecular events as that triggered by other activators (ROS) and by p53-dependent genotoxic stress [112–115].

The concept that NO causes apoptosis via permeability transition is according to the fact that Bcl-2 overexpression protects from the apoptosis caused by NO [116, 117]. It is well known that Bcl-2 acts as an endogenous inhibitor of mitochondrial membrane changes which are accompanied by premature apoptosis [94, 95,118–121]. These observations do not mean that NO is a global initiator of apoptosis and this is probably due to the differences that are noticed in the expression level of members of the Bcl-2 family or in the differences in the ability to counteract NO-caused toxicity in different cell lines [122]. The addition of NO to lung epithelial cell cultures does not cause damage to the cells. The cells are protected from oxidative stress by a variety of factors, such as the antioxidative ability of cells, their ability to withstand metabolic requirements by taking energy from other sources, their ability to repair molecules that have been modified by oxidation reactions and also the presence of suitable nutritious sufficiency [123]. On the other hand, the protection of cells from $ONOO^-$ is insufficient, in contrast to NO. Reduced thiols can possibly protect the cells from the direct toxic effects of $ONOO^-$, but not from the mechanism of slow cellular damage. It is known that depolarization of the mitochondria and the loss of reducing ability are premature events implicated

in slow cellular death from $ONOO^-$; these are noticed after exposure of the cells to oxidative substances in pathologic conditions [124]. The resulting need of energy in the cells is caused either by the modification of mitochondrial function and the inhibition of the glycolytic route [123] or indirectly from activation of poly-(ADP)-ribose-synthase. (page 248)

Toxicity of Nitric Oxide Present in the Gas Phase of Cigarette Smoke

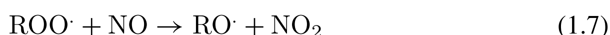
According to Pryor, [126, 127], in the gas phase of cigarette smoke there is a mechanism of a steady-state condition which is based on NO chemistry. According to this mechanism, the first step is slow oxidation of NO, from which the more active nitrogen dioxide is produced:

$$NO + 1/2\ O_2 \rightarrow NO_2 \qquad (1.5)$$

Nitrogen dioxide reacts rapidly with unsaturated substances which exist in smoke, such as isoprene and produces carbon-centre radicals ($R^{\cdot}$), which react with O_2 to produce peroxy-radicals:

$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot} \qquad (1.6)$$

Peroxy-radicals are converted to alkoxy-radicals after reacting with NO, and produce additional nitrogen dioxide (NO_2):



$$ROO^{\cdot} + NO \rightarrow RO^{\cdot} + NO_2 \qquad (1.7)$$

The reactions presented above show that isoprene plays an important role in the production of toxic radicals in the gas phase of cigarette smoke. When both isoprene and NO exist in large amounts in the cigarette smoke gas phase, an extremely toxic environment is created in the lungs during smoke inhalation. This toxicity is mostly due to the continuous production of NO_2 and results in the constant production of toxic free radicals, on a stable recyclable basis (Fig. 1.9). During the depletion of cigarette smoke from isoprene, reactions of NO recycling are stopped, the production of toxic free radicals pauses and NO is proven to be atoxic in vitro [125].

Cellular Protection from Nitric Oxide (NO)

When a vascular endothelium is damaged in any way, its ability to dilate in response to acetylcholine (ACH) is reduced. This incapacity of the vessels to dilate in response to ACH shows the inability of endothelial cells to produce nitric oxide. In this case, the ability of the blood platelets to aggregate on the site of the damage

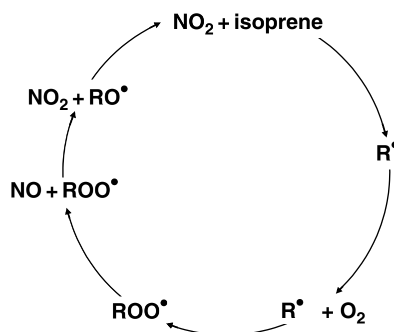


Fig. 1.9 Production of alcoxy-radicals in cigarette smoke, based on the mechanism of NO_2 recycling

is inhibited. These events reveal the ability of NO to act in different cytoprotective ways, which are very important for human protection.

NO is an endogenous inhibitor of the ability of polymorphonuclear granulocytes to adhere. Under physiologic conditions, this characteristic of NO limits the interaction between the endothelium and the polymorphonuclear granulocytes. In addition, it has been shown that the inhibition of NO production from the liver cells favors tissue damage. NO production from the liver cells is believed to regulate the cellular homeostasis of GSH and this leads to protection of the liver from oxidation reactions.

In addition, NO is a regulator of immune function and it probably participates in immune response to severe injuries and surgery. NO production by the macrophages seems to contribute to changes in immune response that follow serious surgical injuries.

Peroxynitrite ($ONOO^-$)

Nitric oxide (NO) is an important physiological regulator with many biological functions. It reacts with different cellular targets, such as heme and heme-associated iron, thiols, oxygen and superoxide anion. These reactions cause physiological changes such as the activation of guanyl cyclase, or pathological changes such as the production of peroxynitrite which is extremely toxic. Peroxynitrite is a powerful oxidative substance that is produced from the reaction of superoxide ($O_2^{-\cdot}$) with nitric oxide (NO) [128] (Fig. 1.10).

It plays an important role in the pathogenesis of tissue damage, in states of shock and different forms of inflammation [129–134]. Some peroxynitrite functions are induced through the activation of poly-(ADP-ribose)-synthase (PARS), an enzyme of the cell nucleus [135–137]. Peroxynitrite causes the breaking of DNA helices, induces PARS activation, which subsequently causes the dissociation of NAD from nicotinamide and ADP-ribose and catalyzes the addition of ADP-ribose polymers in proteins. This procedure exhausts intracellular ATP reservoirs and signals cellular death. It is known that NAD is important for the electron flow system in the

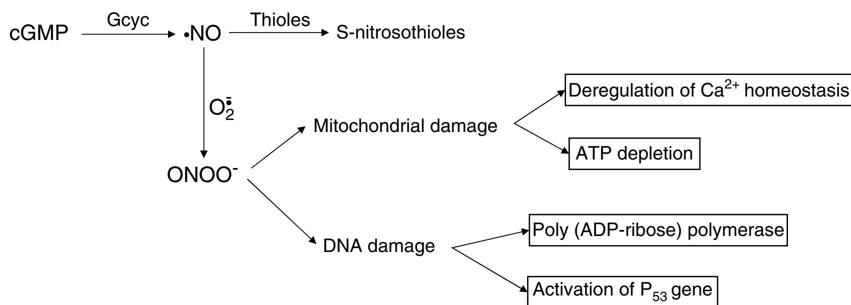


Fig. 1.10 Cell damages induced by ·NO. cGMP = Cyclic monophosphoric guanosine; Gcyc = Guanilic cyclase

mitochondria where ATP is mainly produced. The cytotoxic action of peroxynitrite, similar to other oxidative substances, (for example H_2O_2), depends on its concentration. Small amounts of peroxynitrite cause programmed cell death [138–140], whereas large amounts result in necrosis [140, 141].

The existing data on programmed cell death (apoptosis) mechanisms due to peroxynitrite is limited to studies that show that apoptosis is accompanied by the production of other oxidative substances in cells that have been treated with peroxynitrite [142] and that other substances like Bcl-2 can protect the cells from apoptosis caused by peroxynitrite [143]. Cellular intermediates that are involved in the regulation of programmed cell death caused by peroxynitrite, are unknown. The members of the protease family, caspases (cysteinic, aspartate-specific proteases) which are all related to the death enzyme ceanorhabditis elegans are of significant interest [144–147]. The substantial role of these enzymes in the programmed cell death procedure is presented via different apoptosis motives [148–152]. The exposure of cells to peroxynitrite results in the inhibition of their mitochondrial respiration and the activation of caspase-3 [153]. There is evidence that caspase-3 and not caspase-1 is the key regulator of DNA breakage caused by peroxynitrite [154].

Reactions of $ONOO^-$ with Components of the Mitochondrial Electron Chain

Concerning the components of the electron transfer chain [105], $ONOO^-$ action is different from that of NO. NO inhibits cytochrome C oxidase [103, 105,155–158], whereas $ONOO^-$ inactivates complexes I, II, and V [159–164] following mechanisms that need to be defined. There are indications that the inactivation of complex I by $ONOO^-$ may, at least partly, depend on the oxidation of thiols and the formation of the S-nitrosothiol complex [163, 165]. The mechanism of complex II inactivation includes oxidation of the thiol of succinic dehydrogenase [166] which exists at the dicarboxyl attachment site (Cys252 of subunit A) [167]. The simultaneous

inactivation of complexes I and II by $ONOO^-$ and the subsequent inhibition of NADH and of mitochondrial respiration, dependent on succinic acid, are similar to those that are observed during the attack on target cells by NO [105]. Since NO is not able to directly attack complexes I and II, $ONOO^-$ is considered to be the most direct oxidative factor in the irreversible inhibition of mitochondrial electron transport which depends on NO [168]. In addition, peroxynitrite inactivates and nitrates mitochondrial ATPase (complex V). ATPase is also nitrated by NO when it is produced in excess in the cell [105, 169]. Possibly, ATPase that contains tyrosine in its molecule is of critical importance and is responsible for activity loss [170]. In contrast to complexes I, II and V, respiration that is dependent on cytochrome C oxidase (complex IV) is not affected by peroxynitrite [105, 164]. In mammals, cytochrome C contains 4 tyrosines, two of which Tyr67 and Tyr48 are significantly protected due to their position near heme, contributing to the stereochemical structure and redox activity of cytochrome C.

Toxicity Mechanisms of $ONOO^-$ via Endogenous CO_2 in the Mitochondria

The mitochondria are a primary site where the production of peroxynitrite ($ONOO^-$) by the reaction between NO and the free radicals $HO^\cdot$ and $O_2^{-\cdot}$ takes place. $ONOO^-$ is diffused from the outer mitochondrial compartments to the mitochondrial interior where it reacts with CO_2 and produces secondary free radicals. The reaction of peroxynitrite with CO_2 is related to the mitochondria since they are a site where CO_2 is produced by the Krebs cycle and decarboxylation reactions are catalyzed by pyruvate dehydrogenase. Peroxynitrite anion and peroxynitrous acid (ONOOH) are in equilibrium. Both substances, acting like oxidants, react with various molecules such as thiols and transition metal centers, or they associate to free radicals, mainly carbonate ($CO_3^{-\cdot}$), nitrogen dioxide radicals ($NO_2^\cdot$) and to a lesser degree, hydroxyl radicals ($^\cdot OH$) (Fig. 1.11). An alternative secondary route for $ONOO^-$ production in the mitochondria is the reaction of nitroxyl anion (NO^-) with molecular oxygen [171]. Nitroxyl in the mitochondria is produced from NO reduction by an electron provided from mitochondrial electron donors, such as ubiquinol [172] or reduced cytochrome C [173]. The free radical route begins with the reaction of peroxynitrite with carbon dioxide and the formation of radicals [174–176]. Nitration reactions in the mitochondria (probably nitration of tyrosines in proteins) are related to the routes of the transition metals (Mn-SOD) as well as with the formation of $CO_3^\cdot$ and $NO_2^\cdot$ radicals.

The Role of Mitochondrial Creatine Kinase (MCK) in the Protection Against Oxidative Stress

Mitochondrial Creatine Kinase (MCK) is located in the area between the mitochondrial membranes. It regulates energy production from the mitochondria and connects

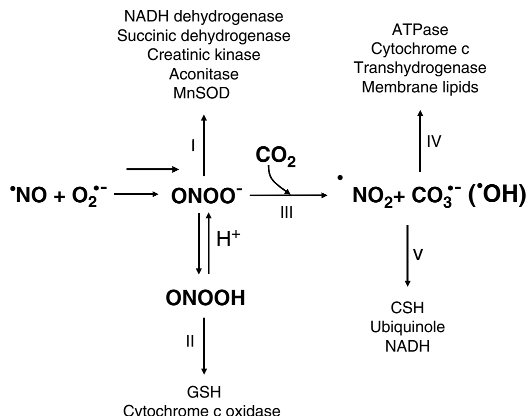


Fig. 1.11 Peroxynitrite reaction pathways in the mitochondria. The controlled diffusion reaction between ·NO and $O_2^{\cdot-}$ results in the formation of the peroxynitrite anion, which is in equilibrium with peroxynitrite acid. Peroxynitrite either undergoes spontaneous bi-molecular reactions with target-compounds (I, II) or reacts with CO_2 (III) towards the formation of $CO_3^{\cdot}$ and $\cdot NO_2$ radicals, that initiate the free radicals chemistry (IV, V). The oxidative damage from peroxynitrite (I, IV), is partially inhibited from decomposition or binding systems (II, V). [From: Radi, R., Cassina, A., et al. Free Radic. Biol. Med. 33(11): 1451–64 (2002), with modifications]

them with the sites in the cytoplasm where this energy is used. In fact, the ATP that is produced in mitochondrial complex V is transported to the area surrounding the membranes where it is exchanged with ADP. At these sites, mitochondrial creatine kinase catalyzes the formation of phosphocreatine from creatine and ATP, which is then transferred to the cytoplasm [177]. Creatine kinase plays an important role in the regulation of the opening of transition permeability pores [82, 178, 179]. Peroxynitrite inactivates creatine kinase via oxidation of an important thiol [180]. It also inactivates nicotinamide-nucleotide-transhydrogenase, which catalyzes the following reaction in the mitochondrial matrix, using the transmembrane proton density gradient:

$$\mathrm{NADH + NADP^+ + H \rightarrow NAD^+NADPH + H^+} \tag{1.8}$$

It is known that NADPH is required for the action of mitochondrial glutathione-reductase. A possible collapse of the mitochondrial proton density gradient and inactivation of transhydrogenase by $ONOO^-$ will lead to exhaustion of NADPH reservoirs, reduction of reduced glutathione and inactivation of glutathione-reductase, contributing to the development of oxidative stress in the mitochondria.

Signaling of Programmed Cell Death by Peroxynitrite

The biological half life of peroxynitrite in extracellular compartments is at a range of 10ms. $ONOO^-$ and ONOOH can be formed inside the mitochondria on both

sides of the inner mitochondrial membrane. Peroxynitrite anion ($ONOO^-$) as well as peroxynitrous acid (ONOOH) can be diffused through the mitochondrial membranes [174, 181, 182]. Small amounts of $ONOO^-$ are also diffused towards the outer mitochondrial space. During diffusion, $ONOO^-$ reacts with proteins that exist in the four compartments and it modifies their structure and biological behavior. More specifically, components of all four mitochondrial compartments react with peroxynitrite. Voltage Dependent Anion Channel (VDAC) (pages 87,88) proteins, creatine kinase, ATPase and aconitase which are present in the area outside of the membranes at the space in between the inside membranes and the mitochondrial matrix are oxidized by $ONOO^-$. Under physiological conditions, when short duration flows of $ONOO^-$ are noticed, peroxynitrite is dissociated after reacting with cytochrome-c oxidase, glutathione, ubiquinol and probably NADH (Fig. 1.11) and subsequently oxidative damage is limited and can be repaired quickly. Under extended flows of $ONOO^-$, the oxidative procedure towards targets like Mn-SOD, aconitase, electron transport chain components, ATPase and creatine kinase, thiols, results in the disturbance of calcium homeostasis and the opening of MPT pores, which together induce mitochondrial signaling of cell death. It is interesting that changes in the composition of mitochondrial ATP which is necessary for promoting the apoptosis cascade, are counterbalanced initially by increased glycolysis. After the collapse of mitochondrial energy metabolism, glycolysis is inhibited (for example triphospho-glyceraldehyde dehydrogenase is inactivated by $ONOO^-$) [183, 184]. Consequently, ATP reservoirs are exhausted and the above events lead to cell death.

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Chapter 2
Oxidative Stress

Oxidative stress is a condition of increased biomolecular transport caused by non-enzymic oxidation.

The Role of Oxygen in Oxidative Stress

Every oxygen atom has one uncoupled electron in its outer valency orbital, whereas molecular oxygen has two uncoupled electrons. Therefore, atomic oxygen is a free radical, whereas molecular oxygen is a free double radical. The tetravalent reduction of oxygen by the electron transport chain in the mitochondria for water production, is considered a safe process, whereas its monovalent reduction creates intermediate, extremely potent substances of oxygen, such as the superoxide anion radical, hydrogen peroxide and hydroxyl radicals, which all together are considered to be responsible for oxygen toxicity. Therefore oxygen in its atomic form exists as a free radical (O), whereas in its molecular form it exists as a double radical (O_2). In the non-enzymic form, the reduction pathway of oxygen sustains 4 successive reductions of one electron (Fig. 2.1)

At a percentage larger than 95%, inhaled oxygen sustains tetravalent reduction to form water with reactions catalysed by cytochrome oxidase (cytochrome-C: oxygen oxidoreductase) of the IV complex of the mitochondrial electron transport chain ($O_2 + 4e^- + 4H \rightarrow 2H_2O$). Cytochrome oxidase is the terminal electron recipient of the chain. If the flow of electrons along the chain stops, the power from proton transport is lost and ATP production cannot continue (Fig. 4.1). Therefore, the major role of oxygen for all aerobic organisms is to simply act as a tank or the cleaning base of electrons. Although the mitochondrial electron transport chain is a very effective and safe system, redox reactions predispose each electron carrier to react with the adjacent molecular oxygen. For instance, inside the circle of the electron transport chain of quinone (fully oxidized), semiquinone (one electron reduction product) and quinol (two electron reduction products), a tendency is observed for an electron to pass readily to oxygen ($O_2^{-\cdot}$ production) instead of passing to the next electron carrier of the chain. Therefore, mitochondrial $O_2^{-\cdot}$ production reflects the largest intercellular free radical source under physiological conditions [1]. Proteins, lipids,

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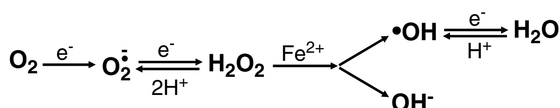
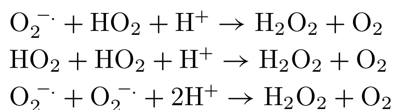


Fig. 2.1 The monovalent reduction pathway of oxygen

hydrocarbons and nucleic acids are sensitive to oxidative modifications from a large variety of free radicals and from the ROS in general. Superoxide is not particularly potent with lipids, hydrocarbons and nucleic acids, but it has limited activity on certain proteins: it reacts with proteins which contain prosthetic transitional metal groups [2, 3]. $O_2^{-\cdot}$ reactions through transitional metals conclude in amino acid damage, especially those that are connected to the metal catalyst and thus, proteins lose their protein or enzyme functionality.

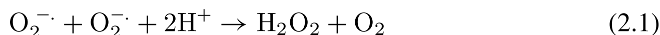
Reactive Oxygen Species in Oxidative Stress

One of the most significant reactions of superoxide is that in which it reacts with another superoxide molecule. All of the following reactions are dismutation reactions in which one $O_2^{-\cdot}$ or HO_2 molecule reacts as a reduction molecule, whereas the other acts as an oxidation molecule. H_2O_2 and O_2 are substances derived from superoxide dismutation, beginning either from $O_2^{-\cdot}$ or from HO_2:



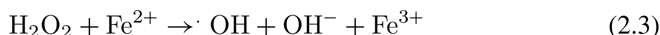
$$O_2^{-\cdot} + HO_2 + H^+ \rightarrow H_2O_2 + O_2$$
$$HO_2 + HO_2 + H^+ \rightarrow H_2O_2 + O_2$$
$$O_2^{-\cdot} + O_2^{-\cdot} + 2H^+ \rightarrow H_2O_2 + O_2$$

Hydrogen peroxide is an oxidative substance for many biological molecules, especially for those that contain the sulfhydril groups, ferrous-sulfur aggregates and copper additive groups [1,4–7]. Hydrogen peroxide readily reacts with transition metals-catalysts producing hydroxyl radicals (OH):



$$O_2^{-\cdot} + O_2^{-\cdot} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (2.1)$$

$$O_2^{-\cdot} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \qquad (2.2)$$



$$H_2O_2 + Fe^{2+} \rightarrow {}^{\cdot}OH + OH^- + Fe^{3+} \qquad (2.3)$$

[Fenton HJH, (1894) J Chem Soc (London) 65:899–910]

In reaction (2) $O_2^{-\cdot}$ acts as a reducing agent and reduces Fe^{3+} to Fe^{2+} [8]. Hydroxyl radicals are the most reactive of all oxygen radicals and readily oxidate proteins, lipids, hydrocarbons, DNA and RNA. Proteins that contain iron or copper additive groups react with H_2O_2 or with $O_2^{-\cdot}$ and produce high class oxidation with the attached metal. Other compounds have a role in oxidative stress as well, such as

hypochlorous acid which can be found in a 50/50 relationship as $HOCl/OCl^-$ in physiological pH. $HOCl/OCl^-$ is produced in the presence of the enzyme myeloperoxidase in some phagocytes, such as the polymorphonuclear neutrophils. Phagocytes produce $O_2^{-\cdot}$ in the presence of the membrane attached NADPH oxidase [9]. The $O_2^{-\cdot}$ produced in this manner is subjected to dismutation to H_2O_2. Neutrophil granulocytes utilize myeloperoxidase to oxidise the abundant halogen chlorine for the production of hypochlorous acid. These cells use $HOCl/OCl^-$ to kill microbes. NO and NO_2 are usual constituents of the photochemical cloud [10]. Today it is believed that apart from the ROS, NO is capable of inducing oxidative stress to the cells, either directly or through the production of relevant substances such as peroxinitrite ($ONOO^-$) [11–13].

Enzyme Role in Oxidative Stress

The human organism synthesizes a large number of antioxidative enzymes in order to minimize oxidative damage (Table 2.1). Perhaps the most known of these enzymes is superoxide-dismutase (SOD) which is considered to be the greatest finding in the free radical field [14]. SOD catalyses the reaction $O_2^{-\cdot} + O_2^{-\cdot} + 2H^+ \rightarrow H_2O_2 + O_2$. All SOD members use transitional metals at their reactive sides [15]. All mammals and certainly humans, utilize distinctive cytoplasmic and extracellular forms of SOD, such as Cu-SOD, Zn-SOD and mitochondrial Mn-SOD. The genetic deletion of SOD results in a lethal mutation in lower organisms; this fact shows the great significance of the physiological presence of these enzymes. H_2O_2 is a reaction product of SOD, which is certainly toxic and must be readily removed from the cells. This removal in mammals is achieved with the help of two enzyme families: the glutathione peroxidases and the catalases. Glutathione peroxidases [16] as well as the catalases [17] render H_2O_2 non toxic, via reduction, converting the molecule to water and oxygen. Glutathione peroxidases utilize the reduction capacity of glutathione (GSH). The sulfhydrilic half of its cysteine offers the reduction equivalents required for glutathione peroxidase activity. When two GSH molecules are oxidized, a complex substance is formed which is connected via a disulfide bond and which causes the reduction of a H_2O_2 molecule to water. The accompanying glutathione reductase enzyme utilizes NADPH in order to restore the GS-SG molecule to its reduced form; in other words, it is reduced to two GSH molecules, thus allowing the glutathione peroxidases to continue their activity. The role played by enzymes utilizing glutathione in the physiological functions of the cell is so important that

Table 2.1 Antioxidative enzymes

- Glutathione peroxidase
- Glutathione reductases
- Catalase
- DT-diaphorase

most cells contain an abundance of glutathione in excess of 5mM [16]. Another important antioxidative enzyme is DT diaphorase, also known as quinone reductase [18]. DT diaphorase catalyses the immediate divalent reduction of many (dihydro) quinones to (dihydro) quinoles. By catalysing the immediate reduction of electrons in the quinone-substrates, DT obviates the production of intermediates of the reactive semiquinone radical.

Oxidation of Proteins in Oxidative Stress

The loss of the sulfhydryl groups and the linking of the carbonyl groups with the side chains of the amino acids histidine, arginine, proline and lysine, constitute the adverse sequential of protein peroxidation. Oxidised proteins are more sensitive to proteolysis as compared to the non oxidised proteins. When proteins are damaged by oxidative stress, their amino acids are re-used in a de novo protein synthesis. However, during strong oxidative stress, the cell's proteolytic capacity is not able to respond to a large number of oxidized molecules [19]. Under these circumstances, oxidized proteins, which do not undergo proteolysis, join up together and form long hydrophobic bonds. These accumulations are catastrophic for normal cell function.

The amino acid, cysteine, is very prone to auto-oxidation or to oxidation catalysed by metals. When two adjoining cysteines in a protein molecule are oxidised, a bisulfide bond is formed, resulting in the formation of a stiff protein. Bisulfide bonds are also formed between two proteins, thus causing large supermolecular totals which consist of inactivated enzymes and some other proteins. Another important oxidative process is the oxidation of methionine to methionine sulfoxide. This oxidation process induces loss of the enzymic or protein properties [20]. This protein transformation is catalysed by cations of redox-cycline, like Fe^{2+}/Fe^{3+} and Cu^{+}/Cu^{2+} [14]. Protein oxidative transformation alters their surface hydrophobic condition and this changes their behavior in the ultraviolet spectrum [21].

Degradation and Substitution of Oxidised Proteins in the Mitochondria, Cytoplasm and Nucleus

Most amino acids of an oxidised or degraded protein are re-utilised by the de novo protein synthesis. During oxidative stress, the cell's proteolytic activity is not efficient enough to cope with the large number of oxidised protein molecules that are continuously formed. Under these circumstances oxidised proteins may not be degraded efficiently and instead, they are inter-coupled, or they form extensive hydrophobic bonds. These accumulated proteins can induce functional problems to the cells [22–25]. In animal cell mitochondria a series of proteolytic enzymes seems to guide degradation of the soluble proteins which are altered by oxidation [26]. In the cytoplasm and nucleus of the eukaryotic cells the oxidised soluble proteins are degraded by the proteosomic complex (Fig. 2.2) [24].

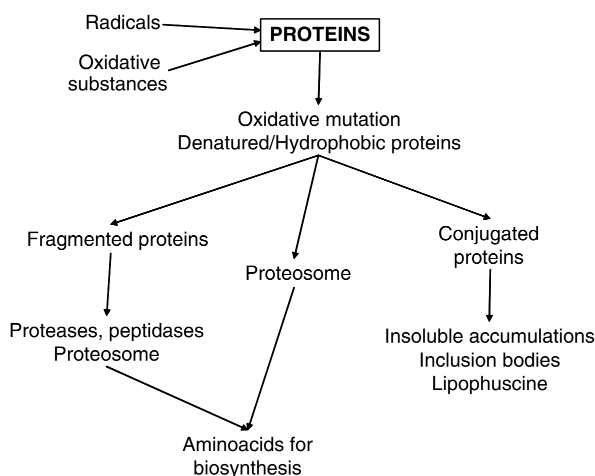


Fig. 2.2 The oxidized soluble proteins are degraded by the proteosomic complex

A proteosome is a poly-enzymic complex, (670 KDa), which is expressed in the cytoplasm and nucleus of all eukaryotic cells. More than 15 polypeptides, each in multiple copies, and with a molecular mass of 20.000 to 35.000 Da, constitute the proteosome complex, which is a non-classical protein complex. The nucleus of this complex recognises the transformed proteins and induces their selective degradation. Recognition and degradation of the oxidised proteins can take place during the lifespan of the cell [27]. Recognition of oxidised soluble proteins in the cell nucleus and cytoplasm leads to the association of the proteosome with the hydrophobic spots in the damaged proteins. The oxidation of proteins may cause alterations in their amino acids, rendering them more hydrophilic. Modifications in the electrical charge of proteins may cause unfolding and partial transformation of their molecules. The unfolding of the protein molecules exposes the protected primary amino acid sequences which are, by nature, hydrophobic. The exposed hydrophobic spots on the surface of the oxidised proteins play a role in connecting and recognising the amino acid series for the nuclear proteosome [27].

Direct Repair Systems of Oxidative Damage

The elimination of the oxidised cell constituents and the cell repair mechanisms are classified as direct and indirect processes [27–29]. Direct repairing systems have only been evident in a few cell constituents. A very important process in direct repair mechanisms is the de novo reduction of the protein sulfydryl groups. Cysteines in the protein molecules are very prone to auto-oxidation as well as to oxidation catalysed by metals. When two adjacent cysteines are oxidised in a protein molecule, a disulfide bond is formed which leads to a very stiff protein formation (intramolecular bisulfide bonding). These bisulfide bonds are formed between two proteins, further promoting the creation of a large supramolecular assembly

of activated enzymes and proteins (transmolecular bisulfide bonding). Both processes, the intramolecular bisulfide bonding and the transmolecular bisulfide bonding can be partially inverted by the bisulfide reductases [27]. Another important process of sulfydrylic oxidation is the oxidation of the amino acid methionine to methionine-sulfoxide, leading to a loss of enzyme/protein function. The reductase of methionine-sulfoxide is able to regenerate methionine and reconstitute its function [20]. Direct repair of the DNA hydroperoxides by glutathione peroxidases has been reported in vitro studies [30]. It is not yet known to which degree DNA superoxides are able to directly repair the DNA hydroperoxides by the glutathione peroxidases, *in vivo.*

Indirect Repair Systems: Elimination of Damage

Indirect repair includes two distinguished stages [27, 28]. One, the damaged molecule (or the damaged part of the molecule) must be recognised, cut off and eliminated or degraded. Two, the whole damaged molecule must be replaced by the synthesis of a new molecule or part of the molecule must be constructed and incorporated at the damaged site.

Lipid Peroxidation in Oxidative Stress

An interesting approach to the study of oxidative stress is the estimation of the lipid peroxidation rate in the case of membrane or in the lipid acids, since superoxides constitute a very important intermediate of lipid peroxidation [31]. During lipid peroxidation, different primary and secondary peroxidation products are created. Primary peroxidation products are coupled dienes, while secondary peroxidation products are malondealdehyde (MDA), 4-hydroxynoneale, exanale and hydrocarbon gases [32]. Phospholipids of the cell membrane can always be found during the oxidative challenge. The lipid peroxidation process includes a whole series of chain reactions which start with the detachment of a hydrogen atom from the chain of an unsaturated lipid acid and the creation of a carbon-centered radical (L) [33, 34]. In the atmospheric air, oxygen is added to the lipid acid (to the carbon-centered lipid radical), thus creating a lipid peroxy-radical (LOO). Soon as LOO is formed the chain reaction of peroxidation is further promoted (endoperoxide pathway) by eliminating a hydrogen atom from the adjacent unsaturated lipid acids [33, 34] (Fig. 1.5). The resulting hydroperoxide (LOOH) (hydroperoxide route) is easily decomposed further, producing a number of active products, among which are lipid alkoxy-radicals (LO), aldehydes (i.e. malonaldehyde), alkanes, lipid epoxides and alcohol [33, 34].

In conclusion, oxidative stress in humans and animals is due to a disturbance in the balance between pro-oxidants and antioxidants in the intracellular compartments. When the equilibrium is disturbed in favor of the pro-oxidants, a potential of free radicals develops on the cell membrane. The peroxidised cell membrane becomes stiff, loses its selective permeability and under extreme conditions, loses its integrity.

Aldehydes, which are produced under these conditions, diffuse through the cell membranes to other subcellular compartments [34–36]. Dialdehydes function as a joining reagent, contributing to the protein accumulation to form lipofuscin [37].

When lipids of the cell membrane are oxidised, it becomes a suitable substrate for phospholipases. Phospholipase A_2 exerts its action at the position Su-2 of the phospholipid glycerol backbone, creating free lipid acids and phospholipids. Changes in the microfluidity of the cell membrane and the increased hydrophilic nature of the oxidised lipids are responsible for the increased activity of phospholipase A_2 [38].

The hydroperoxides of the lipid acids flowing into the cell cytoplasm become substrates for glutathione-peroxidase. Glutathione-peroxidase renders superoxides of the lipid acids, atoxic, by reducing their corresponding hydroxy-lipid acids [38]. Lysophospholipids serve on the substrates for recycling reactions (addition of lipid acids into the position Su-2) in the regeneration of normal phospholipids [39, 40].

DNA Peroxidation in Oxidative Stress

DNA oxidative damage normally takes place in vivo [41–47]. The quantity of oxidative damage is extensive, covering one mutated base for every 130,000 bases in nuclear DNA. Different forms of DNA damage are classified as follows:

i. single or double strand breaks
ii. interchange of sister chromatids
iii. coupling of DNA-DNA and DNA-protein and
iv. base modifications.

All four DNA bases can be modified by oxidation (Fig. 2.3). However, pyrimidines (cytosine and thymidine in particular) are the most prone to oxidation [48].

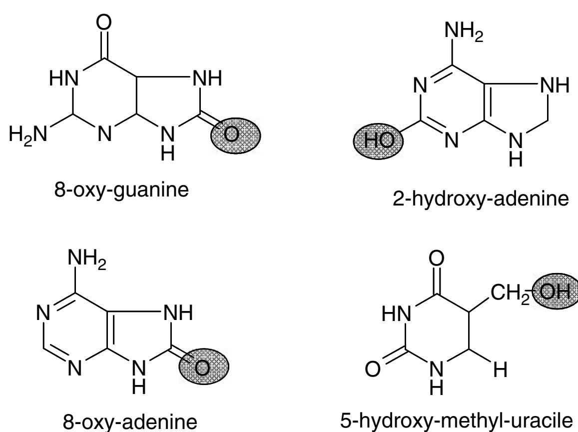


Fig. 2.3 Modifications of the nuclear-DNA bases due to free radicals

In 1984, hydroxylated nucleotide 8-hydroxy–deoxyguanine (8-OHdG) was for the first time referred to as a major product of oxidative DNA damage [49]. It has been shown that hydroxy-radicals, singlet oxygen or direct photodynamic action are responsible for the formation of 8-OHdG in biological systems [50]. Until now, 8-OHdG has been one of the 20 well known primary oxidative products of DNA damage [51]. The mutation of 8-OHdG and its coupling with adenine and cytosine, create the G:C $\rightarrow$ T:A transversions during DNA replication [52]. Oxidised DNA is continually repaired [53].

These conversions lead to the loss of aromatic character and of the space arrangement of the DNA bases and they cause local deformities in the DNA double helix [48]. Altered oxidised bases are found, either bound to, or detached from, the DNA molecules, thus creating an apurinic/apyrimidinic gap (AP) [44, 54, 55]. There are more than 20 forms of DNA base damage which can be produced during the cell's exposure to oxidative stress. The prevalent damage of purines is 7,8-dihydro-8-oxoguanine (7-oxo-guanine), while the most common damage of pyrimidines is thymidine glycol (Tg). The creation of 8-oxo-G is due to hydroxyl-radicals ($OH^{\cdot}$) which react with guanine to produce a radical-adduct, the C8-OH. Subsequently, the loss of one electron (e^-) and one proton (H^+) creates 8-oxoG. The radical adduct, C8-OH, can also be reduced by uptaking one electron and one proton, thus creating 7-hydro-8-hydroxylguanine which is converted to 2,6-diamino–hydroxy-5-formamido-pyridine (FaPy) (which is also a major oxidation product of guanine) [56]. A central enzyme in DNA peroxidation is poly-(ADP-ribose)-polymerase which is activated by peroxidation [57, 58]. As soon as the enzyme is activated, a large quantity of NAD is consumed; this impairs the electron flux in the mitochondria and thus, impairs ATP production. Under these circumstances, the cell suffers from energy depletion, its calcium concentration changes and this leads to cell death. Endonuclease III, a N-glycosylase activity, splits off DNA at the side $3'$ of gap AP [59] and it is able to react with glycolthymine and urea, two well known products of DNA oxidative damage. The phosphodiesteric backbone may also undergo oxidative damage; phosphate and sugar damage, which constitutes the structure of the DNA backbone, leads to breakage of the DNA helix (Fig. 2.2) [47, 54].

Terminals of non-$3'$-OH and non-5-PO_4 are formed due to DNA damage induced by free radicals. These abnormal terminals are not substrates for the DNA polymerases and they must be eliminated before any DNA damage repair [55]. Exonuclease III with its $3'$-nucleolytic activity is able to eliminate these fragmented sugars which are created from breakage of the DNA helixes during oxidation [59]. Exonuclease III expresses 85% of the endonucleolytic activity.

Oxidative damage of the nucleic acid polymers intercepts the transcription, translation and replication of DNA, leading to mutations and finally to death [48,60–64]. The term "redoxo-endonucleases" has been proposed for nucleases which participate in DNA oxidative damage [65]. Oxidation may induce DNA oxidative demethylation [48, 54]. DNA methylases restore the damaged patterns of methylation.

Endogenous DNA Damage

Most endogenous cell damage originates from products of oxygen metabolism (ROS), from internal instability of chemical DNA bonds, from endogenous methylating agents and from errors during DNA replication.

Endogenous damage only affects the primary structure of double strand DNA. Modifications during the course of endogenous damage are due to DNA oxidation and methylation.

DNA Oxidation

DNA oxidation is mainly due to by-products of oxygen metabolism (ROS) and concerns the oxidized DNA bases produced in large quantities under normal conditions and may cause mis-coding (8-oxo-7,8-dihydroguanine [8-oxo-G], thymin-glycol and other oxidized bases). Probably, the more frequent and the most dangerous of this damage is 8-oxoG, which very often mis-pairs with adenine [66, 67]. It has been estimated that about 7500 damages due to 8-oxoG are formed daily in a mammalian cell (about 5% of all oxidative damage) [67].

The best studied oxidised pyrimidines are thymine-glycols, which can, *in vivo*, induce cytotoxicity [68–70] and mutations [71].

Further to base oxidation, the ROS are able to cut off the DNA helix. It is unknown as yet to what extent this damage in single DNA strands contributes to self-mutation. An immediate response to the breakages of single strand DNA is poly-(ADP)-ribose polymerase (PARP) activation [72], the biological significance of which has not yet been determined. Position $3'$ in the broken strands induced by oxygen free radicals is related to the breaks of the sugar-phosphate framework (Fig. 2.4). Reconstituting diesterases $3'$ cut off and eliminate these breaks leaving $3'$ OH behind, in order for the DNA polymerases to intervene [73]. Another issue is the superoxidation of polyunsaturated lipid acids that generates substances which can damage DNA. These substances include the lipid hydroperoxides and different substances with uncoupled electrons (such an alcoxy- and peroxy-radicals) [74]. Also, lipid peroxidation generates substances which contain carbonyls (such an malondealdehyde), which produce some exocyclic DNA adducts [75].

In normal biological systems, part of these endogenously-produced substances by lipid peroxidation maintain their toxicity in order to induce DNA damage.

DNA Methylation

The DNA bases are endogenously methylated by small molecules, such as 5-adenosyl- methionine. From a quantitative point of view, guanine-methylation products such as 7-methyl-guanine (7-meG) is the most important (4000 molecules are

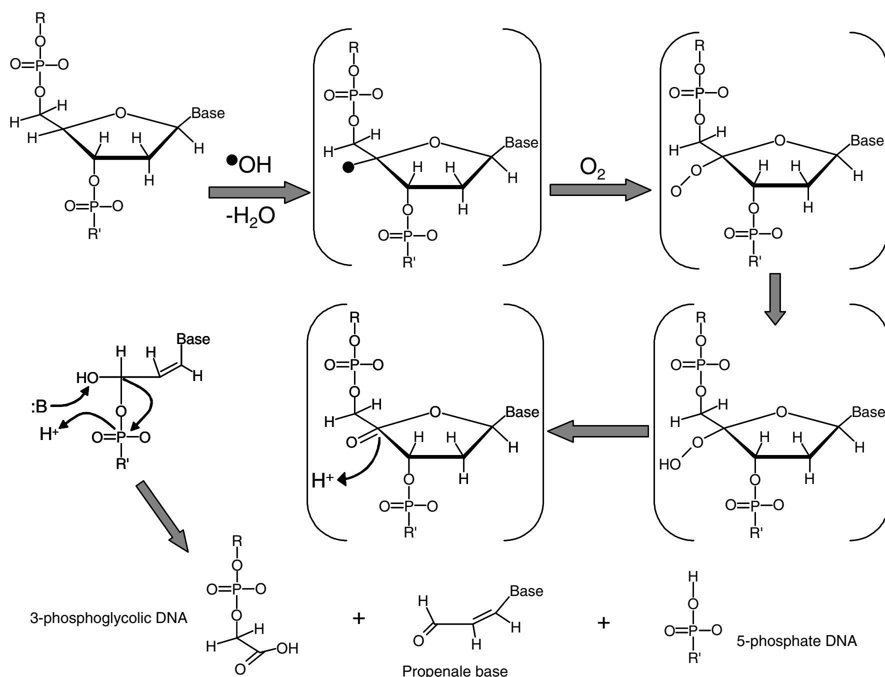


Fig. 2.4 Fragmentation of the nuclear-DNA helixes

created daily in each cell), and according to recent evaluation [76] remains particularly constant (steady state level), at about 3,000 molecules/cell. With respect to toxicity, it has been shown that this base is relatively harmless, since it does not induce mistranscription or cytotoxicity [77]. On the contrary, methyl-adenine (3-meA) produced endogenously at a rate of one hundred molecules daily, participates as a structural material in DNA transcription, and for this reason it must be reconstituted promptly. Furthermore, small quantities of O^6-methyl-guanine (O^6meG) are produced endogenously, possibly as a consequence of the endogenous production of nitrosamines which make an important contribution in self (automatic) mutagenesis [78, 79].

Mechanisms of DNA Oxidative Damage

Data from the literature show that DNA damage due to oxidation is attributed to hydroxyl radicals ($^{\cdot}OH$), but not to H_2O_2 nor to superoxide radicals ($O_2^{-\cdot}$). Hydroxyl radicals are created by the copper or iron ions present in the cell nucleus which are associated with nuclear chromatin [80–84]. A second interpretation of the capacity of oxidative stress to induce damage to DNA is that it can begin with a series of metabolic processes in the cell [85, 86], which lead to the activation of nucleasic enzymes that disrupt the DNA backbone. The two mechanisms of DNA damage by hydroxyl ions or by activating nucleasic enzymes do not rule out each other, but it is possible for them

to take place simultaneously. Intracellular calcium can play an important role in the process of DNA damage. Quinones, for example, induce DNA breakages following activation of the endonuclease, the action on which the calcium ions (Ca^{2+}) depend. [87–89]. During oxidative stress, high concentrations of calcium ions are caused by the restriction of the Ca^{2+} capacity to bind to the endoplasmic reticulum, and also to the inhibition of its exocytosis through the cytoplasmic membrane and at the same time, to its release through the mitochondria, resulting in an increase of endoplasmic Ca^{2+}, activating endonuclease and inducing DNA breakage.

Inducible Systems of Defense and Repairing Against Oxidative Stress: The Role of the OxyR and OxoRS Proteins

These systems are a very important cellular defense mechanism against oxidative stress. Both prokaryotic and eukaryotic cells are able to up-regulate the arsenal intended for their protection from oxidative substances, as a response to oxidative stress. These phenomena are regulated by an adaptation protocol. This can be ascertained by an experiment in which cell cultures are exposed to low concentrations of the same oxidative substances at different time limits, before the cells are exposed again to a lethal concentration (challenging dose) of oxidative substances. The re-treatment of the cells with oxidative substances render them resistant to the challenging dose. These adaptation responses to oxidative stress create extensive changes in gene expression [29, 90–93]. For example, the adaptation of bacteria to hydrogen peroxide includes the OxyR regulon protein [94–103]. OxyR gene codons the OxyR protein which can be associated with nine gene targets, by regulating their expression. During oxidative stress, as for example during exposure to H_2O_2, the OxyR protein constructively regulates in a dramatic manner, the gene-target transcription, in which a catalase is included, as well as an alkyl-hydroperoxidase-reductase, a glutathione-reductase, a non-specific protein bound to DNA and the heat-shock protein DNAK. Each of these enzymes/proteins offers a clear protection advantage to the cells, which have been undergoing oxidative stress. Recent studies have shown the existence of a second protein which is induced by H_2O_2, the so-called OxoR-regulon, which seems to offer inducible protection to *E. Coli* [104, 105]. When *E Coli* is exposed to the superoxide ($O_2^{-\cdot}$) instead of H_2O_2, the OxoR-regulon offers inducible protection [106–113]. Inducible genes, in the case of OxoR-regulon, are the genes of Mn-SOD, exonuclease IV, glucose-6-phosphate-dehydrogenase, fumarase and the antisense regulator RNA.

Gene Expression as a Response to the Oxidative Stress

Following cell exposure to oxidative stress, increased expression of some “antioxidative genes” can take place. Instead, inductions take place in glutathione peroxidase and at the catalase mRNA level, but some smaller inductions occur in the

superoxide-dismutases of Cu, Zn and Mn [114]. The mechanisms regulating gene expression from ROS have been well established in the prokaryotic systems [115], while parallel mechanisms have been ascertained. The ROS stimulate the kinase cascades to the signal transduction pathways [116–118] and activate or inhibit specific transcription factors [119]. Among the transcription factors present in the cell redox conditions, those more researched are NF-kB and AP-1 [120, 121]. In this respect, it is important to note that redox regulatory regions have been found in all genes for anti-oxidative enzymes and also in the NF-kB and AP-1 factors. To the extent that NF-kB could be a messenger of major importance for the redox-sensitive transcription regulation, it is logical that there is activation and an increased binding activity to the cell DNA as a response to the pro-oxidative action [122]. The response of the transcription factor NF-kB during irregularities of the redox cell homeostasis, plays an important role in immune function regulation [121].

Oxidative Damage of DNA in Cancer: Role of Lipid Peroxidation and 8-oxoGUa

There is a constant rate of DNA damage in the cells. Much of this damage is created by endogenous factors which affect DNA. These factors include ROS, such as superoxide anion ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\cdot OH$), which originate from oxidative respiration. The DNA damage due to free radicals can induce a whole series of such damage, including altered bases (Fig. 2.4). Hydroxyl radicals cause a large amount of DNA damage, derived from pyrimidines and purines [123]. Some of these altered bases can yield an important potential that damages the integrity of the genes [124, 125]. DNA 8-oxo-7,8-dihydroguanine (8-OxoGua) leads to GC $\rightarrow$ TA transversions unless repaired before DNA replication [126]. Consequently, 8-Oxo-Gua can lead to mutations. In oxidative stress, there is a direct relation between 8-OxoGua formation and carcinogenesis, in vivo [127]. Oxygen free radicals can induce mutagenesis in the codon hot spots of the human genes p53 and K-Ras [128, 129]. Therefore, the substrate of the DNA oxidative damage is imperative for carcinogenesis due to oxidative stress, with the participation of OxoGua in the p53 and K-Ras gene mutations at the start and development of cancer. 8-Oxo-Gua is a very strong mutagenic substrate for DNA synthesis, as it can pair off with adenine and cytosine, with the same frequency [130]. In this case, it is possible for both AC and GT transversions to occur [126]. Furthermore, in oxidative stress, the ROS start with lipid peroxidation resulting in oxidative degradation of the cholesterol phospholipids and the other unsaturated lipids, as well as the formation of lipid hydroperoxides (LOOHs). Cytotoxic lipid peroxidation is involved in the development of carcinogenesis via changes that take place in the cell function, such as permeability to ions in cell membrane fluidity, coupling of amino lipids with polypeptides and inactivation of the cell membrane enzymes and receptors. Lipid peroxidation leads to the formation of aldehydes, superoxides and epoxides. Aldehyde MDA produced during superoxidation is a well known mutagenic substance and a possible carcinogen [131].

ROS Activity in Mitochondria

Superoxide and the other reactive oxygen species (ROS) are produced in the mitochondria as by-products of normal aerobic metabolism. Since the ROS play an important role in normal cell functions, such as signal transduction, high concentrations of ROS during an oxidative challenge by tobacco smoke can induce cell death [132], while low concentrations can induce apoptosis (Fig. 2.5). The increased production of mitochondrial ROS is extremely harmful. The ROS can damage the surrounding biomolecules inducing neurodegenerative diseases related to aging and cancer [133]. For example, the ROS can increase mitochondrial lipid peroxidation [134, 135], induce oxidative damage to mitochondrial DNA [136, 137] and carbonylate proteins [138–140].

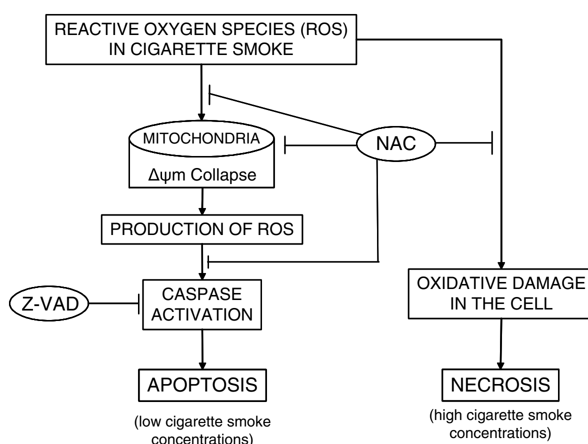


Fig. 2.5 The reactive oxygen species in cigarette smoke can cause necrosis (high cs toxicity) or apoptosis (low cs toxicity)

Antioxidative Enzymes in Saliva

Among the damaging components in cigarette smoke, there are some other substances which are directly toxic to the epithelial cells of the aerogastric tube.

The antioxidative protection offered to smokers by enzymes in the saliva are very important: saliva contains a significant antioxidative protection system. The most important constituent of the antioxidative system is saliva-peroxidase which along with myeloperoxidase, constitute the mouth-peroxidase activity. Saliva peroxidase is excreted from the large saliva glands [141] whilst myeloperoxidase is excreted from the white blood cells [142]. Saliva peroxidase degrades the H_2O_2 produced by the bacteria in the mouth, while an active product of saliva peroxidase, ion thiocyanate (OSCN), plays a decisive role in the killing of the mouth bacteria by inactivating bacterial glycolytic enzymes, such as hexokinase, aldolase and pyruvate kinase [143–146].

It has been shown that plasma exposure to tobacco smoke can induce protein modification. This is evidenced by the increase in protein carbonyls [147] and the oxidation of the plasma lipids [148]. The volatile aldehydes in cigarette smoke are the source of increased protein carbonyls [149]. The function of the saliva enzymes, such as amylase, lactic dehydrogenase (LDH) and acid phosphatase, are very much impaired by tobacco smoke. Aldehydes contained in cigarette smoke, such as acrolein, crotonaldehyde as well as the oxygen free radicals, are involved as causative factors in the damage to these enzymes [150, 151].

Klein and colleagues [152] have shown that a small unchangeable molecule in the saliva is responsible for the inactivation of the peroxidase of the mouth. This concerns the cyanide ions (which are very strong peroxidase inhibitors), present also in the gas phase of tobacco smoke. In addition to cyanide ions, there are some other harmful factors which can affect the activity of the mouth peroxidase. Preincubation of the saliva with OH-CO gives protection to the mouth peroxidases from tobacco smoke KCN. This shows that the presence of cyanate salt in the saliva is the most probable cause of peroxidase inactivation. It is well known that 70–350 μmol of hydrogen cyanide present in the gas phase of tobacco smoke is a causative factor in the pathogenetic mechanisms involved in damage to the mouth.

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Chapter 3
Oxidative Damage of Genome DNA: Repair Mechanisms

In all forms of life, the restoration of the integrity and genetic constancy of DNA repair mechanisms is of essential importance. To achieve this goal, nature has developed a series of effective and highly selective systems, in order to be able to detect DNA damage, locate it and activate its repair mechanisms. The importance of repairing the pathways which target DNA damage can be evidenced by the fact that some defects present in the constituents of these pathways result in the inability of the cell to maintain its viability. On other occasions, such damage, even if well tolerated, can lead to abnormalities, such as the development of cancer [1–3]. The cell responds to DNA damage with a high frequency of responses, ranging from DNA damage repair to the delay of the evolution of the cell cycle. Ingenious transmitters transduce the signal of DNA damage to all systems, as soon as the damage ensues. The transmitters must be able to sense the damage and create an immediate contact with a large number of signal transduction networks. The transformed protein of the teleangiectasic ataxia is a good example of such a transmission mechanism. The latter responds quickly when significant DNA damage exists (i.e. double strand breaks) by phosphorylating key proteins to a large number of signaling networks. For detailed descriptions concerning cell response to genetic damage see references [3–5].

The elimination of the damage by cell repair systems is both direct and indirect [6–8].

A). Direct damage repair: An important process of direct repairing of oxidative damage is, for example, the reduction of the sulfhydryl groups. The enzyme methionine-sulfoxyl-reductase regenerates methionine on the oxidized proteins and restores their function [9].

B). Indirect damage repair: This involves two concrete stages; the first is the recognition and elimination of the damaged molecule and then a new molecule is placed at the site of the damaged one, by neo-synthesis (for example, this could happen to the DNA bases).

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Reconstitution of DNA Damage

Daily DNA damage in mammalian cells due to auto-destruction is estimated to be approximately 10^4 DNA [10]. Everyday, people are exposed to a large number of harmful environmental molecules such as cigarette smoke oxidants which can induce damage to the different cell tissues and particularly to the broncho-epithelial cells. However, more than 130 repair genes which incessantly try to reconstitute the aforementioned damage, have been identified [11]. The presence of such a large number of genes is justified by the fact that different kinds of damage are repaired by different mechanisms, even if some contributions to the different pathways which serve as reconstitution mechanisms do overlap [12–15]. The reconstitution of DNA damage is also associated with the regulation of the cell cycle and the transcription and replication of the DNA, whereas irregularities in the damage reconstitution mechanisms are related to the induction of cancer [16].

The Role of DNA-polymerase ß in the Repair Mechanism of DNA Damage

DNA polymerase ß is non-activated in mammalian cells and it exists in a constitutive form. Its transcription follows DNA damage [17]. In this case DNA polymerase ß reconstitutes the damaged DNA by repairing a nucleotide and filling the gap with a suitable nucleotide [18, 19]. The elimination of the modified DNA bases has been considered to be a protective mechanism which works against the accumulation of altered DNA which is responsible for the mutational and carcinogenic process. Most oxidized DNA products are mutagenic, inducing GC $\rightarrow$ TA transversions and resulting in the miscoding and misreading of the DNA polymerases [20, 21] which sometimes override abnormal nucleotides; the latter then accumulate on the DNA, inducing mutations and leading to carcinogenesis.

DNA Damage-Repairing Pathways — Reconstitution of Genomic DNA

Introduction

DNA reconstruction enzymes continuously supervise the chromosomes in order to be able to repair damaged nucleotides when they are exposed to genotoxic substances. DNA damage can be the consequence of environmental factors such as tobacco smoke. A large proportion of these DNA alterations are due to endogenous mutagenic compounds such as ROS and alkylating-like metabolites. The inconstancy of the genome caused by these compounds can be a huge problem for the cells, when reparative mechanisms are absent. Four directly overlapping pathways operate in the reconstitution of DNA damage: nucleotide excision repair (NER),

base excision repair (BER), mismatch repair (MMR) and reconstitution and rejoining pathways for the repair of DNA strand breaks [22, 23]. Recombination mechanisms can repair any DNA lesion, provided that an intact copy of the affected region resides in the same cells [West SC, Annu Rev Biochem 61:603–640, 1992]. Recombination machinery can be recruited when strand breaks remain open at the site of a lesion or particularly when unrepaired damage blocks the progress of DNA replication. NER reconstitution involves a broad class of damage which results in the distortion of the DNA backbone which affects base pairing and normal transcription.

Reconstitution by BER targets small chemical base alterations. These alterations are sometimes able to prevent transcription and they very often miscode. BER is able to avoid mutagenesis. The proteins of the BER mechanisms cut off and substitute the bases of the damaged DNA (mainly those bases which result from endogenous oxidative or hydrolytic damage) [23].

The Role of Glycosylases in BER Reconstruction of DNA Damage

DNA glycosylases start the BER process by eliminating the modified bases. Fragmentation of the phosphoglycolate chain, excision of the abasic constituent and in situ DNA synthesis take place as follows.

The cell nuclei and mitochondria contain a large number of different glycosylases. Three different glycosylases excise and isolate the oxidative damage of DNA, while a fourth glycosylase specifically excises the alkylating purines. Four of the eight identified DNA glycosylases, which are able to eliminate uracil from the DNA are nNG, SMUG1, mBD4, and IDG. Each of these glycosylases has a specific function. nNG glycosylase is related to the transcription of forked DNA and it repairs mismatched uracil against adenine. SMUG1 glycosylase which is unique in higher eukaryotics, can possibly eliminate the uracil which originates with the deamination of the DNA cytokine. mBD4 cuts uracil and thymine in the deaminated sites of CpG and 5-methyl-CpG series, while IDG eliminates ethino-C, a product of lipid peroxidation, and it also slowly removes uracil and thymine from the GU and GT DNA pairs. Most NER damage comes from external sources, with the exception of some oxidative damage; BER is mostly, but not exclusively for internal damage. Damage in both of these reconstitution classes is related to only one DNA strand. In a model of “cut and sew,” the abnormal base is eliminated and the gap created at the lesion is restored, using the template of the complementary undamaged strand.

Double Strand DNA Damage

Disruption of double-strand DNA causes increased disarray to the repair process since both DNA strand breaks require that the cell be aware of which part of the cell the edges of the DNA strands belong to. Sometimes this is extremely difficult, when taking into consideration the enormous size of the mammalian genome. Under these

circumstances, reconstitution follows two pathways: homologous recombination repair and non-homologous end-joining (NHEJ) mechanisms. These pathways exist in order to solve the problem of double strand break (DSB). During DNA transcription, the homologous recombination mechanism prevails in the S and G2 phases of the cell cycle. This offers a primitive second copy of the base series so that the alignment of the breaks is easily done. Otherwise, the less accurate non-homologous end-joining is placed in the G1 phase of the cell cycle [24].

The Role of Methyl-Transferase in Reconstitution of DNA Damage

A few unique reconstitution proteins restore some DNA damage. For example, O6-methyl-guanine-methyl-transferase eliminates O^6-methyl-guanine. This extremely mutagenic damage allows pairing with both the C and T bases, and it is able to deceive the reconstitution system by incorporating a wrong base on the damaged site, aiming at the apparent reconsitutive transcription. Otherwise, regular methyl-transferase is able to detach the methyl group from O^6-meG and restore the guanine to normal in different mammalian cells [25, 26]. This suicide enzyme uses one of its own cysteines as a methyl group receptor, thus inactivating itself [27]. This shows how a whole protein must be sacrificed in the context of the reconstitution of a single DNA base.

Reconstitution of DNA Damage by Base Excision Repair (BER)

Base excision is the essential mechanism which eliminates the endogenous damage which can induce small distortions to the DNA strands [10]. The endogenous sources of DNA damage include hydrolytic processes such as hydrolytic deamination of the cytokine, adenine and guanine, or the hydrolytic loss of bases (mainly purines), oxidation by the ROS (hydroxyl radicals) and also the endogenously-produced alkylating agents such as S-adenosyl-methionine (SAM) [13–15]

BER reconstitution is the most flexible among the DNA repair mechanisms because of its capacity to eliminate a large amount of damage.

The common denominator of the different forms of endogenous damage induced by the different chemical substances is the production of a large quantity of adducts which can induce distortion of the DNA strands.

The first step in BER consists of the recognition and elimination of the altered base; this can be done by the intervention of DNA glycosylases. The intervention of each of the DNA glycosylases can split a specific part of the bases [23, 24, 26]. Glycosylases are classified as mono or bifunctional according to their AP-lyase activity which dissociates DNA at its abasic (AP) site. Mono-functional glycosylases delete only the base, leaving the AP site untouched. Twelve different human-type DNA-glycosylases have been identified up to now.

In bifunctional DNA glycosylases, the AP site created by glycosylase activity is cut off further at position 3, with b-elimination, thus giving rise to the

obstructive 3′-terminal. The conventional monofunctional glycosylases are uracil-DNA-glycosylases (UDG) and 3-alkyl-N-purino-glycosylases (ANPG), while human 8-oxo-DNA glycosylases (hOGG1) and endonuclease III (hNTH1) are authentic bifunctional glycosylases. Each glycosylase is specific to a limited number of damaged DNA bases [28]. In human cells, the normal AP sites left behind the monofunctional glycosylase activity are severed at terminal point 5′ by the hydrolytic AP endonuclease called APE, HAP1 or Ref-1 [29], leaving the 5′-phosphate-2-deoxyribose 5′ terminal and a substrate 3′ OH terminal at this site. This protein (APE) can afford an important reducing activity, that regulates the joining of the transcription factors to DNA [29]. The APE is inducible by the oxidative stress [30].

Apart from the elimination of the altered base, reconstitution is completed with the repair of microspots (gaps of two to eight bases) [31, 32]. These two mechanisms use different enzymes and complementary proteins. The restoration steps include the formation of a single strand gap, covering this gap with DNA synthesis and binding. The choice of the pathway is determined at least partially, by the nature of the DNA glycosylase [33], the nature of the AP site, and also the right moment in the cell cycle [34]. The macro-spot pathway demands different factors which have also been used during DNA replication. The choice of the pathway can be extremely important for the reconstitution of the oxidized bases since such bases can be found in a cumulative form and combined with DNA strand breaks.

Interestingly, with regard to the oxidative bases, the well known glycosylases are all bi-functional. Thus, the DNA backbone is ruptured at side 3′ of the abasic site, while the abasic sugar remains at the 3′ terminal, leading to a lapse in APE1. This mechanism avoids polymerism with the neighbouring strand break; otherwise, this could lead to double strand breaks. The high frequency of a base auto-destruction and the high degree of maintenance of the restoration mechanism of the excised bases demonstrate the importance of the restoration mechanism in the maintenance of DNA integrity.

Reconstitution of DNA Damage with Excision of the Oxidized DNA Bases by the Specific Glycosylases NEIL 1 and NEIL 2

Recently, two glycosylases which are specific to oxidized DNA bases have been discovered: these are NEIL1 and NEIL2 which constitute the background for a new restoration pathway. This pathway is based on the principles of base excision repair (BER). The restoration process based on BER includes participation of the AP endonucleases (APE) by excision of the damaged base. APE produces the substitute 3-OH at the DNA-strand-break site and synthesizes the restorative material. The glycosylases NEIL1 and NEIL2 accomplish deletion in the “abasic site” following base excision, by inducing a terminal 3′-phosphate completion. Polynucleotide kinase (PNK) bearing endo-3′ phosphatase activity permits the restoration step to proceed further. Thus, NEIL1 forms a constant protein complex (PNK and APE1) which is essential to the next restoration step. APE1 is a necessary protein in the

production of the 3′-OH terminal, following the activity of some other DNA glycosylases, such as OGG1 and NTH1. NEILs excise the damaged DNA base substrates with a bubbling structure. All of the other mammalian glycosylases are inactive in the context of bubbling structures, since the latter are formed temporarily during the transcription and replication processes. NEILs seem to participate in restoring the DNA bases which are oxidized and connected to the replication and transcription processes.

Reconstitution of DNA Damage by Nucleotide Excision Repair (NER)

Most NER damage comes from external sources with the exception of some oxidative damage. Restoration by NER eliminates different kinds of damage, the most important of which are chemical adducts and some form of oxidative damage. This is the most flexible DNA restoration mechanism because of its capacity to eliminate a large amount of damage unrelated to normal DNA. The creation of a large volume of base-adducts which can induce significant distortion of the DNA strands, apart from the many alterations which occur in DNA chemistry, is the common denominator of the different forms of DNA damage produced by chemical substances, to which the cells are very sensitive [35, 36]. There are 20–30 proteins which contribute to the NER processes. These processes include the recognition of the damage, the opening of the DNA double helix around the damage and the excision of the damaged strand at both sides. Following the excision of the damage-bearing nucleotide, the ensuing gap is filled with newly-synthesized DNA, and the strand is tied.

Recognition of the Damage

During NER, the initial stage of damage recognition is the binding of the XPC^{-}/HR23B complex to DNA [37]. The binding of the XPC^{-}/HR23B complex recruits a full device of restoration proteins at the site of the DNA damage. XPC^{-}/HR23B complex indicates xeroderma pigmentosum group protein complex, and it is the initiator of global genome nucleotide excision repair [37].

Base Excision at Both Sides

Following local unwinding and dissociation of the damaged DNA, an oligonucleotide which contains 24–32 nucleotides is cut away. In this process, the activity of the specific endonuclease XPC is necessary, in order to take action on the side of the opened complex XPC^{-}/HR23B [38].

Filling the Gap and Binding

The last NER phase involves filling the gap which has been formed as a result of the oligonucleotide excision, with a reconstitutive synthesis of new DNA. In order to

accomplish this goal, the transcription factors replication protein A (RPA), replication factor C (RFC) and proliferating cell nuclear antigen (PCNA) are necessary as are the polymerases δ and ε [39]. NER processes are completed by tying the newly synthesized DNA. This reaction proceeds with the aid of ligase I.

Reconstitution of the DNA Lesions Caused by Mismatch Repair (MMR)

Post-transcriptional reconstitution of mismatch repair utilises the MutSLHomologue family genes, such as MSH2, MSH6, MSH3, MLH1 and PMS2 genes and possibly the MLH3 gene. Until recently it was believed that the correction of errors in human cells was based on the "specific" of MMR proteins and also on the members of the replication mechanism. Today, we know that there is one more member of the MMR family, the MLH3, which is involved in the reconstitution process [40].

The primary role of the post-transcriptional reconstitution of MMR lesions is the elimination of polymerase errors (such as the mismatch repair of the DNA bases [base/base] and the insertion/deletion of loops on the template strand), from the newly synthesized helix. These processes consist of three principal steps: The recognition of the mistake at the mismatched bases which is accomplished by the Muts eterodimer (MSH2 and MSH6). Another eterodimer (MSH2 and MSH3) is able to initiate the reconstitution process. The next step in this process is directed by the ATPase which is present and functions in all of the carboxy-terminal half of the Muts homologues [41]. The completion of the process is described in detail in references [42–45]. The recognition of the mismatched bases (base/base) and the insertion/deletion loops are accomplished in the dimeric of the MutS homologues (MSH) and in the eterodimer MSH2 and MSH3 proteins, which can start the reconstitution processes.

The degradation of the error-bearing helix and the reparative synthesis process are further promoted in the presence of a protein complex which participates in the replication process [46, 47].

Reconstitution of DNA damage by Nucleotide Pool Sanitation

The most stable mutagenic damage due to DNA oxidation, 8-oxoG, can be coupled with the nucleotides, cytosine and adenine, resulting in the formation of GC → TA transversions [48]. The harmful behaviour of 8-oxoG is faced by complicated systems, such as the "GO system", which includes the MutT, MutY and MutM proteins [49, 50]. In human cells this system has additional levels of complexity containing the BER, MMR and NER processes. The first line of defense is the elimination of 8-oxodG from the pool of nucleotides, caused by the effect of the nucleotide hydrolase hMTH1 (human MutT homologue 1) which completely degrades 8-oxodGTP (and also 8-oxo-dATP and 2-hydroxy-dATP) and their equivalent monophosphoric

derivatives [51–53]. MTH1 is localised in the nucleus, the cytoplasm and mitochondria [54]. The important contribution of MTH1 to the avoidance of the mutation is shown by the increased incidence of automatic oncogenesis in mice mth-/- [55]. If oxodGTP could escape sanitation in the nucleotide pool, it could possibly be incorporated into the DNA by the polymerases. The level of 8-oxodGTP in the mitochondria is relatively high because its levels are related to the oxidative phosphorylation machinery; in this case, 8-oxodGTP escapes sanitation, carried out by the nucleotide hydrolase hMTH1, and is incorporated into the DNA opposite the TA or TC. This fact can justify the development of a second line of defense by removing the 8-oxodGTP from the opposite of the two bases. This defense system must be able to eliminate oxodGTP which is formed by oxidation of DNA guanine. At present, there is a large number of glycosylases which remove 8-oxodGTP from the DNA.

In this case, in which the reconstitution of the 8-oxo:C lesion is not completed before the second round of replication, it is possible that ATP instead of dGTP can be incorporated into the DNA [56] leading to a CG to TA transversion. Mammalian cells are also supplied with a third line of defense in cases of glycolytic removal of the adenine which has been mis-incorporated opposite the 8-oxoG. This is a function of the hMYH (human MutY homology), a glycosylase adenylate of the double helix which targets adenine or 2-hydroxyadenine (2-OHA), both of which are placed by mistake in the position of 8-oxoG [57, 58]. Thus, MYH along with the mitochondrial isoforms MTH1 and OGG1 form a complete "GO system" which protects the mitochondrial 8-oxoG from the mitogenic activity of adenine and 2-OHA.

Role of the Mut Proteins in the DNA Repair Processes

The cell faces two different problems related to GO adducts. One problem is the miscoding of GO damage into chromosomal DNA and a second problem is the incorporation of 8-oxo-dGTP into the DNA. The mutation rate is minimized when cells have an increased number of lines of defense against GO damage. In the case of chromosomal damage due to the GO system, nuclear DNA normality depends on the MutT and MutY proteins in order to avoid GC $\rightarrow$ TA transversion [59, 60].

The MutM protein eliminates GO damage from chromosomal DNA [59]. This protein has a high affinity for A/GO miscoupling and thus removes the misincorporated adenine [60]. The MutY protein remains joint at its site, preventing action against GO damage at the opposite AP site [59]. Unless MutM acts on the AP/GO site, it could lead to a base loss and to a double stand break. This joining of MutY serves as a signal to specific proteins which promote the DNA repair process [60].

Another problem facing cells is the mis-incorporation of 8-oxo-dGTP into the DNA. Additionally, the cell has two systems involved in avoiding errors due to this mutagenic substrate. First, the MutT protein hydrolyses 8-oxo-dGTP to 8-oxodGMP [61]. This eliminates triphosphate from the nucleotide pool, contributing to the avoidance of its incorporation into the opposite adenine template.

The second defense system takes place during DNA polymerism in which the choice of the nucleotide and its disposal by the DNA-polymerase, establishes a two-fold more potentiality than the incorporation of O6-oxo-dGTP into the opposite frame of adenine [62]. The defense of the GO system starts off with the enzymic and non-enzymic systems which try to avoid DNA damage by the reactive oxygen species (ROS). If the ROS are able to overcome the primary defense system, they can damage the nucleic acids [7, 63]. The second line of the defense system attempts to overcome damage. The MutY protein and the DNA-polymerases offer a second level of defense against the mutagenic potential of the GO adduct to the DNA or the nucleotide pool.

Finally, GO (8-hydroxyguanine) represents one of the most stable products of DNA damage [63, 64].

Restoration of Oxidized Pyrimidine

The restoration of oxidized pyrimidine, the thymine glycol of BER (base excision repair) is very complicated. Bifunctional glycosylase MTH1 is able to detach thymine glycol and some other related bases, but not the oxidized purines from the damaged DNA [65]. The main substrate for three proteins with enzymic activity (NEIL-1, NEIL-2 and NEIL-3) is the oxidised pyrimidine [66, 67]. NEIL1 can detach 8-oxoG, its specific substrate; it can also detach thymine glycole, 5-hydroxyuracile, 5, 6-dihydroxy-uracil (5,6 DHU) and 5-hydroxy-cytosine (5-OHC). NEIL-1 is accumulated during phase S of the cell cycle, playing a distinguished role in restoration during replication.

Direct Restoration of DNA Damage

The direct reconstitution of damaged DNA bases can occur in human cells. However, this mechanism does not participate in the reconstitution of damage due to oxidation. Repairing O6-methyl-guanine by O6-methyl-guanine-DNA-methyltransferase (MGMT), restores the DNA guanine to normal (a methyl group is transferred to one of its cysteines by MGMT). The enzymic activity of MGMT then becomes inert [68].

Reconstitution of Double-Strand Breaks by Homologous Recombination (HR) and Nonhomologous End Joining (NHEJ)

The generation of double strand breaks (DSB) is due to different genotoxic factors. The solitary non-coupled DSB site may lead to cell death, and for this reason reconstitution is extremely important. Mammalian cells respond to DSB damage using different systems: the arrest of the cell cycle and the apoptosis and response

to survival adaptation. The reconstitution of DSB in mammalian cells is accomplished by homologous and non-homologous mechanisms. These mechanisms are however, inefficient in correcting different diseases [69, 70]. According to the literature, NHEJ happens more frequently as compared to HR. However, the reconstitution mechanism depends on the condition of the cell cycle. Recent research has shown that HR is the prevailing mechanism in the completion of the S-phase of the cell cycle, provided that there is the same copy of the DNA helix (sister chromatid) available. In the G1 phase of the cell cycle, NHEJ is the preferable mechanism for the reconstitution of the cell cycle [71, 72]. Exactly the same copy of the DNA helix, the sister chromatid, is mostly preferred (twice out of three times) in the homologous or heterologous chromosome [73]. Even if HR is exempted from a mistake, NHEJ may lose or win some nucleotides. HR and NHEJ are different mechanisms which demand completely different protein factors. Both, however, need the ataxia telangiectasia mutated (ATM) protein which codes the ATM gene for damage recognition and possibly for the recruitment of reconstitution factors. ATM is a kinase which regulates key proteins [69, 74]. ATM mutations can induce telangiectasic ataxia, a rare syndrome characterized by extremely increased susceptibility to ionizing radiation, cerebellum degeneration, telangiectasia, increased chance of cancer development and immunodeficiency.

Oxidation Damage of DNA: A Causal Factor of Cancer Development

In living cells, there is increased diversity of DNA oxidation damage. A large amount of such damage is found in endogenous factors which damage the DNA at a continuous and stable rate. The ROS are included among these factors, which are derived from oxidative respiration.

Free radicals which damage the DNA can induce a whole series of damage, including base modification. Hydroxy-radicals ($^{\cdot}$OH) can induce a large amount of DNA damage caused by pyrimidines and purines [75]. Some modified DNA bases have an increased potential for damaging the integrity of the genome [76, 77]. 8-oxo-7,8-dihydroxyguanine (8-oxoGua) damage has been very well researched. When 8-oxoGua contributes to the DNA structure, it leads to GC $\rightarrow$ TA transversions, unless these abnormalities are repaired before DNA replication [78]. Consequently, the presence of 8-oxoGua in these cells can lead to mutations. There is good evidence showing the relationship between 8-oxoGua and cancer [79], and also between oxygen-free radicals and mutagenesis in the hot spots of human p53 and K-Ras genes [80, 81]. This shows that oxidised DNA can be proven to be imperative in the development of cancer.

The basic level of the 8-oxo-Gua in the DNA of the cell represents a potential equilibrium between oxidative damage and the rate of damage repair in these cells. The presence of repair enzymes which identify and eliminate 8-oxo-Gua from the DNA indicates the biological significance of the damage.

Overexpression of Enzymes Which Participate in the Reconstitution of Endogenous Damage

Sufficient reconstitution of endogenous damage in mammalian cells is presumed from the steady presence of certain damage in human cells, as compared to the number of cells produced daily [82]. For example, while in human cell DNA many thousands of abasic sites (AP) are produced every day, momentary control shows less than 10 AP sites in each cell genome. The highly efficient reconstitution of the AP sites is ascertained in in vitro [83] and in vivo [84] experiments where it can be shown that these sites are eliminated quickly, in a very short period of time. Unfortunately, all endogenous damage is not reconstituted sufficiently, as happens in the case of the AP sites. For example, at uracil sites (the most easily reconstituted base in mammalian cells), in normal cell extracts, reconstitution is three to ten times slower, as compared to AP sites in vitro. 8-oxo-Gua is repaired at a still slower rate as compared to uracil [85]. Furthermore, the reconstitution of purine damage is slower, as compared to pyrimidine. The above indicate that with some exceptions, enzymic DNA reconstitution mechanisms are kept at a high level in nature. However, the superexpression of reconstitution enzymes is not able to lead to increased resistance during endogenous or exogenous mutagenesis.

1. According to existing evidence, in BER, there are two phases which can limit the excision rate: the elimination of the 5-phosphate-2-deoxy-ribose (dRP) when the AP site is excised by intervention of the APE/HAP1 protein (human abasic endonuclease) and the elimination of the 3′ part when the AP site is excised by AP lyase, which is connected to bifunctional glycosylase. By strengthening these phases, the total adequacy of NER in mammalian cells is drastically improved. This can be accomplished by both the overrexpression of the dRPase (5-deoxyribose-phosphodiesterase) region, of polymerase-ß and also by the overexpression of the AP1 protein (fungal abasic endonuclease) which has sufficient reconstitution activity 3′.
2. The overexpression of O^6-alkyl-guanine-DNA-alkyl-transferase is useful since it can decrease the mutagenic activity of O^6-meG (O^6-methyl guanine). By suppressing this protein, mammalian cells are able to succeed in achieving an increased resistance to the toxic and mutagenic activities of the alkylating agents.
3. However, the overexpression of certain BER enzymes may be proven to be more dangerous than useful, as compared to the protection offered by automatic mutagenesis. This is obvious, particularly in the enzymic activity of the 3-alkyl-N-purine-glycosylase (ANPG) and DNA polymerase-ß, whereby the non-equilibrated activities (while keeping the other important BER activities) may lead to cell sensitisation against DNA damaging factors, and to a phenotype of genome instability. Thus, the increased restoration of endogenous damage demands a concerted expression of at least four enzymes which are graded proportionally to the contributing glycosylase, as follows:

a) mono-functional DNA glycosylase [86]
b) bifunctional DNA glycosylase and APN1 protein for reconstitution activity
c) DNA polymerase ß
d) DNA ligase III.

Even if these proteins are at a ratio of 1:1:1:1, an imbalance in the normal ratio can possibly be created, leading to a phenotype of genetic instability.

4. In somatic eucaryotic cells, DNA polymerase ß (Pol ß) is considered to be a reconstitution polymerase which excises the DNA bases (BER). It is expressed steadily and at a low level, during the cell cycle [87] and it is prone to making errors as it has a low DNA replication accuracy in vitro [88], as well as a small ability to distinguish the linking capacity of nucleotides [89]. Dysregulation of polymerase ß expression is considered to be an important event and it predisposes the cell to genetic instability. The increased expression of DNA polymerase ß following oxidative stress may cause a transformed phenotype, enforcing oxidative damage mutagenesis as well as increased apoptosis.

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Chapter 4
Mitochondria: Structure, Function and Relationship with Carcinogenesis

Mitochondrial Contribution to Oxidative Processes

It is in the mitochondria where energy is produced – the energy needed by the cell in order to fulfill its various biological needs. Energy production is a matter of life and death for the cell. It is based on the following: the ionization of hydrogen, electron transport and the production of water. The first step towards the production of energy inside the mitochondria is the ionization of hydrogen atoms (provided by food), in pairs. One of the two hydrogen atoms becomes the hydrogen ion (H^+) while the other binds with NAD^+ and becomes NADH. The first reaction is the release of one hydrogen atom from NADH and the production of one more H^+ (Fig. 4.1). This cycling reaction generates NAD^+ which can be used again and again. The electrons that are removed from the hydrogen atoms during ionization immediately enter the electron transport chain which is an inseparable part of the inner mitochondrial membrane. The electron transport chain consists of a series of electron acceptors which get reduced or oxidized in a reversible manner, accepting or releasing electrons. The major proteins of the electron transport chain are the flavoproteins (FMNs), various sulphuric iron proteins, ubiquitin and cytochromes b, c_1, α and α_3. Every electron travels from one acceptor to the other until it reaches cytochrome α_3 also called cytochrome oxidase, because it is capable of provoking the release of two electrons from atomic reduced oxygen, which in turn binds with the hydrogen ions to produce water.

Pumping of H^+ to the Outer Part of the Mitochondrion, Induced by the Electron Transport Chain

As electrons pass through the electron transport chain, a large amount of energy is released. This energy is used for the pumping of hydrogen ions from the mitochondrial inner matrix to the inter-membrane space. This procedure creates high concentrations of H^+ in the inter-membrane space and simultaneously a powerful electric potential in the inner matrix.

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ATP Production

The next step is the formation of ATP from ADP. This happens in conjunction with a large protein molecule which protrudes from the inner mitochondrial membrane with a drum head shape in the inner matrix (Fig. 4.1). This molecule is the ATP-ase or ATP-synthase. The high concentration of positively charged hydrogen ions in the outer section and the difference in potential at either side of the inner membrane, force hydrogen ions to move towards the mitochondrial matrix through the ATP-ase. The energy produced from this movement of electrons is used by the ATP-ase to transform ADP to ATP by attaching inorganic phosphorus (Pi) to ADP and in this way, adding a high energy bond to the molecule. The final step of the procedure is the transport of ATP outside the mitochondrion to the cytosol. This happens by simple diffusion through the inner and outer membranes. ADP is transported in the opposite direction, to the inner matrix for the continuation of energy production, (i.e. its transformation from ADP to ATP). Under normal conditions, the energy in the cell is provided mainly from this mitochondrial phosphorylation [1–3] (Fig. 4.2).

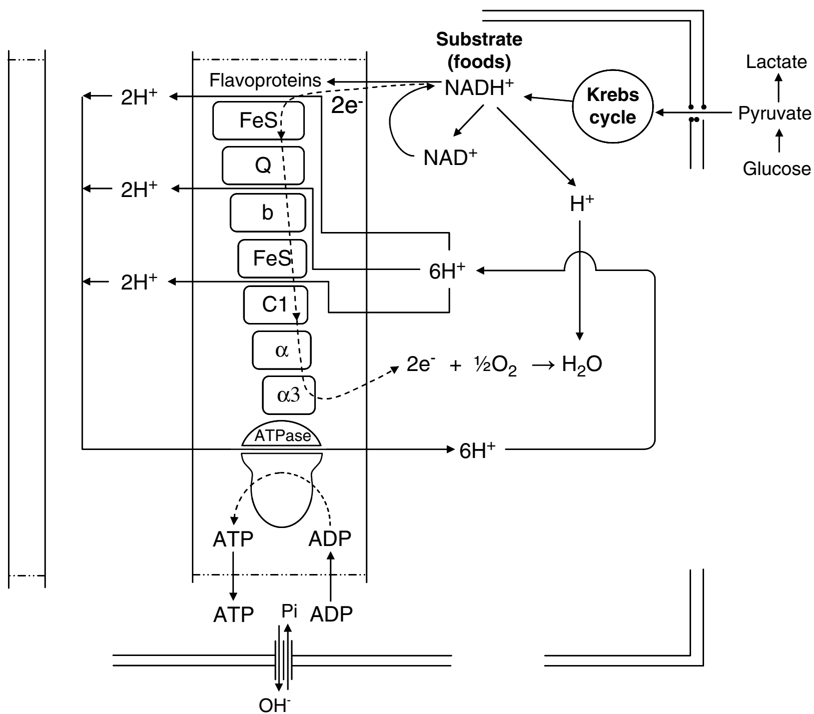

Fig. 4.1 Mitochondrial chemosmotic mechanism for the production of large amount of ATP (energy)

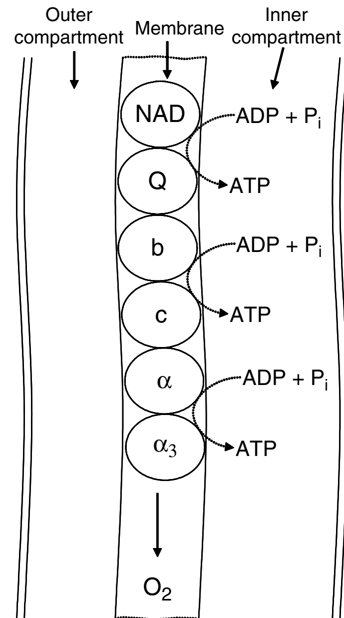

Fig. 4.2 Under normal conditions, the energy in the cell is provided from mitochondrial oxidative phosphorylation

Respiration-dependent Calcium Transport to the Mitochondria

The mitochondria accumulate certain cations, against a concentration gradient, by a procedure which couples with electron transport. Calcium (Ca^{2+}) accumulation is accompanied by the uptake of equivalent amounts of phosphorus. For each pair of electrons that pass through NAD to oxygen, roughly six calcium ions accumulate, (two for each side sustaining energy) (Fig. 4.3). When Ca^{2+} is accumulated in this way inside the mitochondria, oxidative phosphorylation cannot proceed. Therefore, the released energy from electron transport is used either for Ca^{2+} accumulation or ATP formation, but not for both at the same time.

Intramitochondrial Ca^{2+} Cycling

Under normal conditions, Ca^{2+} enters the mitochondria electrophoretically via the Ca^{2+} uniporter [4] and exits by exchange with Na^{+} using the Na^{+}/Ca^{2+} carrier [5, 6]. Both of these transport systems, together with the Na^{+}/H^{+} antiporter, constitute a transport cycle which creates a slow continuous cycling of Ca^{2+} across the inner mitochondrial membrane, driven by the expulsion of hydrogen ions from the respiratory chain [7, 8]. The continuous cycling of Ca^{2+} across the inner membrane implies that mitochondrial Ca^{2+} is regulated by the kinetic properties of the uniporter and efflux systems rather than by the forces driving the fluxes. The rate of Ca^{2+} recycling cannot be greater than that provided by the Ca^{2+} uniporter.

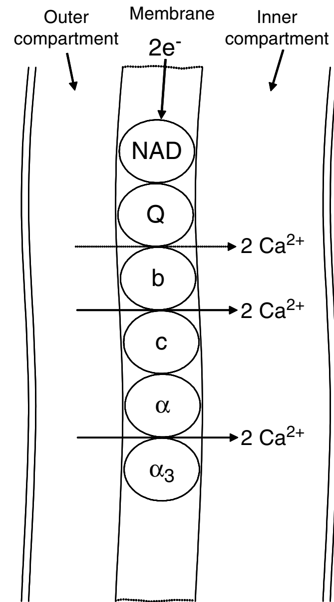

Fig. 4.3 Ca^{2+} oxidative accumulation in the mitochondrion. Ca^{2+} enters through a specific calcium transporter

Mitochondrial Permeability Transition

Mitochondrial permeability transition (MPT) is the dramatic increase in the permeability of the pores of the inner mitochondrial membrane in small molecules (<1.5 kDa) [9]. The mitochondrial membrane potential and the mitochondrial matrix Ca^{2+} determine the potential of other substances to cause MPT.

Components of the MPT Pore

The mitochondrial permeability transition pore is formed by a complex of the voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT) and cyclophylin D (Cyp-D) at the contact sites of the inner and outer mitochondrial membrane. There are serious indications that the VDAC-ANT-Cyp-D complex can recruit other proteins, including Bax (page 94), in order to participate in the apoptotic process.

The basic component of the mitochondrial pore is adenine nucleotide translocase (ANT). The opening of the pore is extremely susceptible to ANT. The substrates of ANT are ADP, dADP and ATP and these are the only nucleotides found to react with the pore [10]. ANT operates as a gated pore (Fig. 4.4). When occupied by a transportable substrate it alternates between two conformations in which the ADP/ATP-binding site is either on the matrix side of the inner membrane (m-state) or on the cytoplasmic side (c-state). ANT ligands that bind to the m-state inhibit the PT pore, whereas c-state ligands activate it. This suggests that the c-state conformation is required for PT-pore opening.

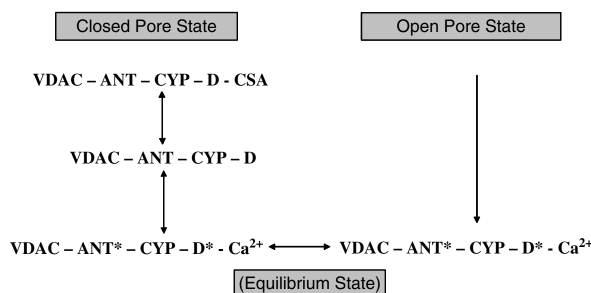

Fig. 4.4 Possible activation mechanism of the metaptotic permeability pore in pathological conditions. Under oxidative stress conditions, for example, the VDAC-ANT-CYP-D complex binds to mitochondrial Ca^{2+} with spatial transition (*), capable to evoke PT pore opening activity

Role of Cyclophilin-D

It is clear that ANT by itself does not create the PT pore, and other components are needed. A number of studies have shown that CyP-D binds to ANT on the VDAC-ANT complex. It appears that the VDAC-ANT-Cyp-D complex determines the components of the PT pore. Both calcium and Pi are necessary for the function of the pore. When the complex is incorporated into liposomes, the addition of Ca^{2+} and Pi evoke PT pore activity which is blocked by cyclosporine A (CSA). CSA is a cyclic endecapeptide with largely unmodified alkyl side chains which sit in a hydrophobic pocket in CyP-D, corresponding to the active site. It is assumed that Cyp-D associates with ANT via the active site and that CSA blocks the pore by preventing this association. CyP-D binding to ANT is enhanced on the modification of the ANT thiol group during oxidative stress. The binding of Cyp-D with ANT promotes its ability for adaptive transformation triggered by Ca^{2+}. The binding of ADP and ATP at the lumen of the mitochondria, where ANT is localized, competes with the action of Ca^{2+}. The modification of different thiol groups of ANT inhibits its binding to ADP and sensitizes mitochondrial permeability towards Ca^{2+}.

General Remarks

A striking aspect of the PT pore is that it is assembled from components that have other well established roles in the life of a cell. The general function of VDAC is to allow low molecular weight substance-solute access to the solute-specific transport systems of the inner membrane. ANT mediates the ADP to ATP exchange, essential for the basic bioenergetic function of the organelle. The function of CyP-D has not yet been established, but a likely role is the catalysis of protein folding. The function of the VDAC-ANT-Cyp-D complex is the establishment of contact sites between the inner and outer membranes. The role of contact sites in energy transduction is very important. There are indications that the contact sites are involved in the transfer of phosphatidylserine between the endoplasmic reticulum and the

mitochondrial inner membrane [11, 12]. In order to carry out these tasks, the sites recruit other proteins (e.g. kinases for efficient phospholipid transfer) [13]. There is evidence that the sites also recruit Bax and possibly, other Bcl-2 family proteins. Thus, the VDAC-ANT complex operates as a multifunctional recruitment center for other proteins, assembling them into the appropriate complexes depending on the job to be done. In the case of apoptosis, it appears that the task involves lysis of the outer membrane. Potentially, this activity could include phospholipases for outer membrane destabilization.

Role of Calcium in the Function of the PT Pore

During Ca^{2+} accumulation in the mitochondria, an increased inner membrane permeability to solutes with molecular mass below 1.5 kDa is observed [9]. This leads to the collapse of the electrochemical H^+ gradient and therefore puts ATP synthesis to a halt [14]. The consequent release of Ca^{2+}, the osmotic swelling and the hydrolysis of the remaining ATP by the ATPase are responsible for mitochondrial loss of function [15]. In vitro, the PT pore opening is fully reversible [16]. After PT pore closure, the mitochondria re-polarize, their functions associated with energy production are restored and under appropriate conditions, volume recovery is possible by solvent extrusion [17]. The reversibility of the the PT pore is fully controlled by the mitochondrial membrane potential and the mitochondrial matrix pH suggesting a physiological procedure associated with Ca^{2+} homeostasis [18]. Mitochondrial permeability transition is induced in isolated mitochondria with a Ca^{2+} overload under conditions promoting mitochondrial depolarization [19–22]. The mitochondrial PT pores function as:

1. voltage sensors
2. thiol sensors
3. sensors of the redox function of the pyridine nucleotide pool
4. mitochondrial matrix pH sensors
5. adenine nucleotide sensors.

Role of Adenine Nucleotides and Pi

Both ADP and ATP, but not AMP, inhibit the PT pore. In general, ADP is the most inhibitory [23, 24]. When adenine nucleotides are used to prevent pore opening, then only ATP is effective [25]. This ATP prevention of pore opening involves its acting on the outside of the mitochondrial inner membrane, whereas inhibition by ADP (after pore opening) could occur by binding to the translocase at the inner face of the inner membrane. A concentration of ATP, similar to that found normally in the cytosol, blocks pore opening completely when bound to any of the pore components. Such a component may be the kinases that bind to the VDAC-ANT complex.

Evidence of Increased Mitochondrial Permeability (MP)

When isolated mitochondria are re-suspended in a buffer solution, the increase in MP causes colloidosmotic swelling of the mitochondria. The large extent of the swelling leads to changes in optical density at 540nm. MP can also be measured with the use of radioactive markers (sucrose, Ca^{2+}). Patch-clamp techniques recognize the so-called mitochondrial mega-channels, which are possibly similar to the pores of mitochondrial transitional permeability.

The periodical reversible opening of the pores under normal conditions allows the release of Ca^{2+} from the mitochondrial matrix for cellular Ca^{2+} homeostasis. Moreover, the transitional permeability of the pores facilitates the transport of proteins to the mitochondrial matrix through the membrane potential. In addition, it is involved in the rapid response to the energy fluctuations of the cell. Mass opening of the mega-channels leads to cell death.

Rupture of the Outer Mitochondrial Membrane Due to Swelling: The Influence of the Irreversible Transitional Permeability

Mitochondrial permeability transition (MPT) takes place during apoptotic cell death [26, 27] which is programmed to remove unwanted, old and damaged cells [28]. During apoptosis, two different proteins are released in the mitochondrial intermembrane space: cytochrome c [29,30–33] and the apoptosis-inducing factor (AIF). Cytochrome c binds to APAF-1 and recruits procaspase 9 which is then processed to its active form [34] and it activates other caspases and recruits a factor that activates nucleases [35]. On the contrary, AIF acts as a direct activator of nuclear DNases, resulting in the characteristic oligo-nucleosomic DNA fragmentation which is observed during apoptosis [35, 36]. Finally, the irreversible mitochondrial membrane permeability transition that leads to mitochondrial swelling, results in further release of cytochrome c and AIF due to the mechanical rupture of the outer mitochondrial membrane.

Mitochondrial Membrane Potential

The channels (pores) of the mitochondrial membrane remain closed under normal conditions. It is believed that a sensor capable of decoding potential membrane changes during the opening of the pores, exists [37]. Today, we know that a more negative potential favors pore opening. Oxidative factors move the potential towards negative values and therefore towards values that favor the opening of the pore, while reducing factors behave in the opposite manner and favor the closure of the pore [37]. Thus, the opening of the pores is accomplished either by depolarization or by changes of the threshold potential.

Mitochondrial Matrix pH

The pores are exceptionally sensitive to mitochondrial matrix pH changes. Mitochondrial membrane transition permeability is inhibited by H^+ [38]. The inhibitory action of H^+ is linked to the reversible protonation of histidine [39]. Thus, a number of active or inhibitory substances control MTP.

Mitochondria and Accidental Programmed Cell Death (PCD)

Necrosis-apoptosis

When a cell is seriously damaged, there is an irreversible loss of vital functions, forcing the cell to enter a random cell death process or necrosis. However, most cells possess mechanisms that lead to cell death (or facilitate cell suicide) even under circumstances when their components have not suffered irreversible damage. This type of PCD is called apoptosis [40]. Apoptosis is a tightly regulated process during which the cells show characteristic morphological and biochemical features. These features include chromatin aggregation, nuclear and cytoplasmic condensation, and the partition of the cytoplasm and the nucleus into membrane-bound vesicles. This is a physiological process, necessary for multicellular organisms to sustain life, since it provides the means by which unwanted or useless cells are eliminated. It is known, for example, that ATP production is crucial for cell integrity. If the inner mitochondrial membrane loses proton impermeability, which is a result in changes in Ca^{2+} homeostasis, ADP phosphorylation forming ATP is disrupted, leading to cell death.

Elaboration on Apoptosis and Necrosis

Apoptosis or programmed cell death (PCD) is a response to physiologic or pathologic stress, in which cell death is completed in an orderly manner, via activation and synthesis of gene products, aiming at cell destruction. There is disarray in the balance between the rate of cell division and elimination. Apoptosis provides the means by which organisms remove unwanted or useless cells [41]. If the process of apoptosis is disrupted, various pathologic conditions such as cancer, acquired immunodeficiency syndrome and autoimmune diseases may arise. The biochemical basis of the apoptotic machinery exists by constitution in all mammalian cells and is activated by various extra- and intra-cellular signals [27]. Apoptosis and necrosis are two forms of cell death with distinct morphological and biochemical characteristics [42]. However these two types of cell death may occur simultaneously in the cells [43–46]. The intensity of the stimulus determines the type of cell death [47]. The intracellular energy levels fall during necrosis but not in apoptosis [44]. ATP is necessary for apoptosis to occur and threshold ATP concentration is needed in order for the cell to carry out the apoptotic process; this is crucial since

the intracellular ATP levels will determine the type of cell death. During the last phase of the apoptotic process, ATP is produced by glycolysis in the mitochondria.

Today, it is believed that the execution of programmed cell death is centrally controlled and is enhanced by the presence of the proto-oncogene Bcl-2 (corresponding to the ced-9 protein in *C. elegans*). Some members of the Bcl-2 family inhibit or delay apoptosis while others initiate death by an unknown mechanism. Bcl-2 failure to protect the cells from apoptosis is explained by the notion that there are other parallel pathways to death, different from that of Bcl-2.

Mitochondrial Potential (ΔΨm) and Programmed Cell Death (PCD)

Zamzami et al. [48] suggest that a reduction in ΔΨm constitutes an irreversible step of ongoing cell death, preceding other alterations of cellular physiology, thus allowing for the ex vivo assessment of PCD. In addition, it has been shown that overexpression of Bcl-2 prevents apoptosis by regulating an antioxidant pathway [49] at sites of free radical generation and inhibits the ΔΨm decrease [50]. The overexpression of Bcl-2 is associated with increased ATP production from the mitochondria [51]. Long-term exposure of the cells to H_2O_2 causes an immediate reduction of ΔΨm, suggesting that the ROS may be implicated in this reduction. Exogenous antioxidants and Bcl-2 [52] disturb the mitochondria balance, possibly via a regulated pathway [53] or as a consequence of mega-channel opening, accompanied by a partial, non-reversible reduction in ΔΨm. As an alternative process ΔΨm reduction might be secondary to changes in cellular glucose concentration. The reduction of ΔΨm signifies an initial non-reversible process in the cascade of metabolic changes accompanying PCD.

Release of Cytochrome c in the Cytosol During Apoptosis

Cytochrome c is a central protein of the mitochondrial respiratory chain. Following its synthesis, the apo-cytochrome c molecule is transported into the mitochondria where it is located (loosely attached to the inner mitochondrial membrane) [54]. Oxidative stress causes cytochrome c release to the cytosol prior to mitochondrial membrane potential loss [55]. This implies that the MPT does not open prior to the release of cytochrome c. The release of cytochrome c in the cytosol activates caspase-dependent apoptotic signals. Recent reports suggest that cytochrome c released during apoptosis is progressively accumulated in the nucleus, while in parallel, acetylated histone H_2A is released in the cytosol from the nucleus. Cytochrome c has been shown to cause nuclear condensation and chromatin reorganization [56].

Synthesis and the Role of Cytochrome c

Similar to all other mitochondrial proteins, cytochrome c is coded by a nuclear gene. Apo-cytochrome c is synthesized in the cytosol and is transported, unfolded into

the mitochondrial intra-membrane space where further modifications take place, including the addition of a heme group. Cytochrome c is the only water soluble cytochrome and it acts as a portable electron transporter between the Bcl-2 complex and cytochrome c oxidase. It is bound to the negatively charged Bcl-2 complex and the outer surface of the inner membrane [57]. Because electrons flow along the electron chain, cytochrome c can bind and unbind very quickly with both complexes (vibrational movement).

Roles of Bid, Bax and Bcl-2

The Bid protein belongs to the Bcl-2 family of proteins. Some members of this family together with Bax, promote apoptosis, while others such as Bcl-2 block the process. The Bcl-2 protein is localized on the outer mitochondrial membrane and in the endoplasmic reticulum as well as in the nucleus [58]. Bax is a cytosolic protein which translocates to the mitochondria after an apoptotic signal [58, 59]. This translocation is mediated via caspase in order to penetrate the outer membrane where it heterodimerises with Bid via BH3 domains [58, 60, 61]. It seems that the pro-apoptotic proteins, Bid and Bax, are recruited to the outer mitochondrial membrane where they react with the anti-apoptotic protein Bcl-2 during apoptosis. The release of apoptogenic proteins from the intra-membrane space is determined by the relative amounts of Bid, Bax and Bcl-2 on the outer membrane. How these proteins control the release of cytochrome c and AIF protein is currently under investigation. According to Kroemer and co-workers [62], the opening of the PT pore that leads to the rupture of the outer membrane is the mechanism by which intra-membrane apoptotic proteins are released. The same group of researchers suggest the existence of a close functional and physical connection between the PT pore and Bcl-2 and Bax on the outer mitochondrial membrane (where these proteins control the activity of the PT pore). Therefore, both pro- and anti-apoptotic proteins of the Bcl-2 family react with components of the PT pore (probably with VDAC on the outer membrane). It seems that the VDAC-ANT-Cyp-D complex reacts with the apoptotic proteins and consequently plays an apoptotic role in the physiological function of the cell.

Caspases as Mediators of Apoptosis

Cellular proteases appear to play an important role at the initiation of apoptosis:

a. specific proteolytic activity has been demonstrated in almost all apoptotic processes [63–68]
b. various protease inhibitors block the apoptotic process [69, 70]
c. some viral proteins which have the ability to suppress the apoptotic process are protease inhibitors [71, 72].

Cellular damage during apoptosis is due to caspases, a family of 14 cysteine-dependent aspartyl proteases. Caspases are constitutively present in cells as inactive proenzymes that must be cleaved and processed for activation. Caspases can be classified as initiator caspases (caspase-2, caspase-8, caspace-9 and caspase-10) that subsequently activate effector caspases (caspase-3, caspase-6, and caspase-7), which are responsible for most of the proteolytic cleavages associated with cellular self-destruction (Fig. 4.5).

Activation of initiator caspases can occur through two general mechanisms. The first mechanism involves death receptor activation and apoptotic signaling. Death receptors represent a subgroup in the tumor necrosis factor (TNF) superfamily of receptors that includes TNF-R1, Fas, and TNF-related apoptosis-inducing ligand (TRAIL) receptors, TRAIL-R1 and TRAIL-R2. Specific ligands to these death receptors include TNF-a that activates TNF-R1, Fas ligand (FasL) that activates Fas, and TRAIL that activates TRAIL-R1 and TRAIL-R2.

Following activation, death receptors complex with adaptor proteins such as Fas-associated via death domain (FADD) and initiator procaspases such as procaspase-8, form the death-inducing signaling complex (DISC) at the membrane. Within the DISC, procaspase-8 is cleaved to form the active p20 subunit of caspase-8 that subsequently cleaves and activates effector caspases, such as caspase-3, caspase-6, or caspase-7. The second mechanism mediating caspase activation is the "mitochondrial stress" apoptotic signaling pathway. Various stresses such as chemicals and radiation destabilize the outer mitochondrial membrane and cause the release of apoptotic proteins such as cytochrome-c and apoptosis-inducing factor

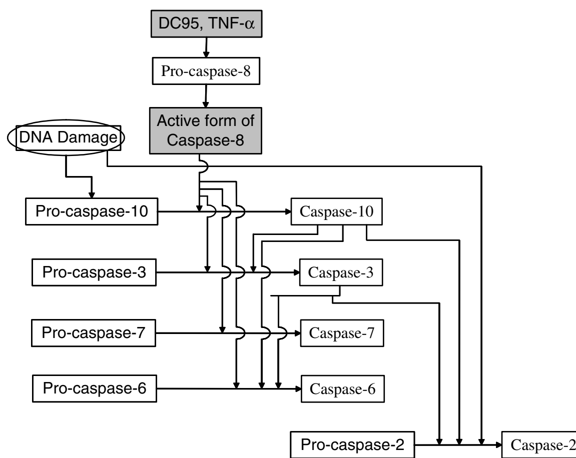

Fig. 4.5 Apoptosis is triggered by the DNA fragments or by TNF-α and CD95 (FAS/APO-1) factors, which react with their own receptor. Both receptors transport their apoptotic signal inside the cell through the intracellular C-terminal death region, which participates in protein-protein interactions. Both receptors result in cleavage and activation of caspase-8 which cleaves all the rest known caspases. Caspase-10 which also cleaves all other known caspases, is activated by different stimuli, probably following DNA damage

(AIF) into the cytoplasm. Cytochrome-c then interacts with apoptotic protease activation factor-1 (APAF-1) [73] and initiator procaspases, such as procaspase-9, to form the apoptosome. Following apoptosome formation, procaspase-9 is cleaved to form active caspase-9 subunit that is then released into the cytoplasm and activates downstream effector caspases. The requirement for cytochrome-c involvement by an apoptotic route is the most important indication that mitochondria participate in apoptosis.

Alterations of Mitochondrial Functions that Constitute Initiators of Apoptosis

Until recently, it was believed that the mitochondria retain their morphology during apoptosis whereas they swell during necrosis. More recent studies have shown: the initial dissipation of the mitochondrial membrane potential difference ($\Delta\Psi$m) which is correlated to the loss of electron transport and ATP production, as well as to the reduction of the rate of mitochondrial loss of precursor cytoplasmic mitochondrial protein maturation. When it was shown that in cells lacking a functional respiratory chain, Bcl-2 was capable of blocking apoptosis, it was proven that the anti-apoptotic activity of Bcl-2 was not linked to mitochondrial respiration. It is possible that in mitochondrial function which is not directly linked to respiration but to mitochondrial membrane potential, $\Delta\Psi$m is involved in the apoptotic process. It has been proven that increased Bcl-2 expression raises $\Delta\Psi$m and that this action is linked to the anti-apoptotic activity of Bcl-2. This process is also linked to the opening of mitochondrial mega-channels and the MPT pore – the opening and closure of which is controlled by various substances such as divalent cations, pro-oxidants, thiol-linked factors and especially connective proteins that are believed to take part in the formation of MPT (adenine nucleotide translocator and benzodiazepine receptor). These substances cause one of the major forms of MPT, such as loss of $\Delta\Psi$m and superoxide production ($O_2^{-\cdot}$). When a cell loses its $\Delta\Psi$m, it enters apoptosis, and conversely, when a cell is protected from apoptosis, it maintains its $\Delta\Psi$m. Bcl-2 offers protection after the dissipation of $\Delta\Psi$m due to MPT. In addition, when there is a loss of $\Delta\Psi$m, the mitochondria produce a protein > 10 KDa, which in isolated nuclei causes chromatin condensation and nuclear destruction [36, 62].

Gene Regulation of Apoptosis

Apoptosis is a distinct form of cell death, controlled by an internally coded suicidal program [74, 75]. The morphological changes which accompany apoptosis include nuclear and cytoplasmic condensation, blebbing of the cell membrane, partition of the cytoplasm and nucleus into membrane bound-vesicles (apoptotic bodies) which are phagocytosed by the neighbouring cells [42, 76]. The biochemical markers of apoptosis include DNA fragmentation-laddering [77], caspase activation [78, 79] and the degradation of various substrates of the protease family

ICE, in which poly(ADP-ribose) polymerase (PARP) is included [80, 81]. The cell suicidal program is depicted better in genetic studies of the nematode Caenorhabditis elegans [82]. Two genes which are involved in the regulation of programmed cell death of C. elegans have been extensively studied: one gene, ced-9, which codes for a protein that protects cells from apoptosis [83] and the other ced-3, which codes for a protease - the activity which is necessary for apoptosis to occur [84]. The Bcl-2 gene family is the ced-9 homolog [82]. Bcl-2 overexpression protects mammalian cells from apoptosis, by blocking cytochrome c release from the mitochondrial intermembrane space [85]. The Bcl-2 protein is localized mainly in the outer membrane of the mitochondria [86, 87]. In mammals, the closest homolog to ced-3 is CPP32 (Caspase-3) [88], which breaks down PARP and sterol regulatory element binding proteins (SREBPs) in cells undergoing apoptosis [89, 90]. CPP32 exists normally in the cytosol of a cell in an inactive state which is proteolytically activated in apoptotic cells [78, 89]. Because CPP32 is activated by proteolysis in an aspartic residue, it seems that the whole ICE (Interleukin-1β Converting Enzyme) proteolytic cascade drives cells to apoptosis [80, 90]. Activated CPP32 is capable of cleaving its inactive precursor [89], indicating that CPP32 is activated by autocatalysis.

Oxidative Stress and the Mitochondria

Respiratory Chain Function During Oxidative Stress

Oxidative stress is capable of causing oxidation of any biomolecule through the reactive oxygen species (ROS). The mitochondria are extremely sensitive to the harmful action of ROS, which are continuously produced from the mitochondrial respiratory chain ($O_2^{-\cdot}$, H_2O_2, $\cdot OH$) [91, 92]. In addition, the mitochondria are an important place for the low molecular weight complexes of Fe^{2+}. These complexes mediate oxidation of the membrane lipids [93–95]. The reduction of O_2 is accomplished in four consecutive stages via univalent reduction. During this process no oxygen components are released in a partial reduction state due to the high chemical resemblance of cytochrome c. Thus, no production of superoxide radicals takes place through the univalent O_2 reduction via cytochrome c. However, 1–2% of the reduced O_2 from the mitochondria is converted to $O_2^{-\cdot}$ at intermediate stages of the mitochondrial respiratory chain function [91, 92, 96]. This production takes place at the level of NADH dehydrogenase [97] or at the level of coenzyme Q [98]. During normal function of the respiratory chain, electrons are transported from NADH to the oxidized form of coenzyme Q (UQ) for the production of the reduced form of coenzyme Q (UQH_2). Then, UQH_2 transfers electrons to cytochrome c oxidase and it is again converted to UQ after it has passed through a quinone radical ($UQ^{-\cdot}$). This process takes place initially at the cytoplasmic side of the inner mitochondrial membrane and is then repeated on the matrix side of the membrane. $O_2^{-\cdot}$ production is a continuous physiological process which can take place because the mitochondria possess an adequate anti-oxidant system. This system is comprised

of hydrogen peroxide dismutase, glutathione peroxidase, glutathione reductase, glutathione, NAD(P) transhydrogenase, NADPH, vitamins E and C [99, 100], thiol peroxidases such as SP-22 [101] and mitochondrial respiration itself [102]. Superoxide radicals which are produced by the respiratory chain are dismutated by MnSOD [103] to produce H_2O_2. Under normal conditions, H_2O_2 is converted to a non-toxic substance by glutathione peroxidase or by the inner or outer mitochondrial catalase [104, 105]. Mitochondrial glutathione peroxidase remains in a reduced state by glutathione, which is consequently reduced by NADH and NADPH via NAD(P) trans-hydrogenase [105]. Thus, oxidants of the mitochondrial pyrimidine nucleotide inhibit the lack of H_2O_2 toxicity produced by the mitochondria [106, 107].

Conditions of Elevated $O_2^{-\cdot}$ Production or Shortage of Antioxidants

Under conditions of increased production of mitochondrial $O_2^{-\cdot}$ or when antioxidant systems of the cell are depleted, an accumulation of H_2O_2 is observed and this leads to a state of mitochondrial oxidative stress. Under these conditions, H_2O_2 reacts with mitochondrial Fe^{2+}, forming highly reactive hydroxyl radicals via the Fenton reaction [99, 100]. Calcium ions represent an interesting case of mitochondrial ROS accumulation stimulus [108] which mediates structural changes in the inner mitochondrial membrane such as an increased accumulation of lipids [107]. Accumulated lipids increase ROS production due to the non-functional mitochondrial respiratory chain, since most of its active components are integral parts of the proteins of the inner mitochondrial membrane. Thiol groups of the mitochondrial membrane proteins suffer extensive oxidation under conditions of mitochondrial oxidative stress caused by calcium ions [106]. Hydroxyl radicals, and to a lesser extent the other components of the ROS, are capable of promoting cysteine and methionine oxidation and the production of disulphide bonds and sulphoxymethionine, respectively. The loss of mitochondrial function is possibly linked to the oxidation of the cysteine residues of proteins of the inner mitochondrial membrane. The presence of Ca^{2+} and oxidative alterations of the thiol groups of the inner mitochondrial membranes induce a non-specific permeability of the inner membrane which is reported to be mitochondrial permeability transition [109]. The membrane becomes permeable to protons and even to small proteins [107].

Function of the Pores During Oxidative Stress

The opening of the pores during oxidative stress is reversible. The in vitro opening of the pores from H_2O_2 in the presence of Ca^{2+} is fully reversed when the oxidative status is removed [110]. Under conditions of oxidative stress, $O_2^{-\cdot}$ produced by the respiratory chain presents a linear increase [111], whereas H_2O_2 is formed by superoxide dismutase. The mitochondria lack catalase and for this reason, H_2O_2 is reduced by GSH (glutathione peroxidase). As a result, it appears that

pore activation is probably mediated via oxidation of the GSH, NADPH or NADH pools. There is substantial evidence that oxidative stress triggers PT pore opening by oxidizing vic-thiols in the pore proteins. Arsenite attacks vic-thiols as a co-activator with Ca^{2+} [112, 113]. This has led to a model in which the linkages –S–S– and –S–As(OH)–S– in the pore structure yield pore opening, whereas $(–SH)_2$ and –SH produce pore closure. According to this model, the pore is maintained in the closed form by the normally highly reduced state of the intramitochondrial glutathione. Oxidation of the GSH pool during excess production of peroxides allows dithiol formation and pore opening. The capacity of internal adenine nucleotides to inhibit the PT pore has been decreased by thiol oxidants, suggesting that thiol oxidants activate by preventing the binding of inhibitory adenine nucleotides at the m-state of the inner membrane (page 87). This agrees broadly with the properties of the PT pore, reconstituted from the VDAC-ANT-CyP-D complex, which yields Ca^{2+}-induced PT pores in the absence of thiol oxidants [114]. Thus, oxidative stress may activate the PT pore by overriding inhibition by the intramitochondrial adenine nucleotides.

Opening of the Mitochondrial Membrane Pores During Oxidative Stress

The mitochondria are the most important intracellular source of the ROS. Almost 90% of the oxygen consumed by mammals is offered to the mitochondria, where the reduction of one electron from O_2 in the respiratory chain causes a proton electrochemical gradient in the inner membrane used for ATP production. Four percent of this oxygen is partially reduced, creating the ROS, which under normal conditions, are consumed by the antioxidant defense of the organelle [115]. In pathological situations in which there is an accumulation of radicals, the mitochondrial membrane undergoes lipid peroxidation and an increase in permeability, known as mitochondrial permeability transition (PT). This phenomenon depends on the opening of non-selective pores by Ca^{2+} and other factors, such as pro-oxidants and oxidative phosphorylation factors. Permeability Transition is a key phenomenon in the toxic and oxidative form of cellular damage as well as in apoptosis [22, 26, 37, 116].

It is already known that oxidative stress increases the possibility of MPT pore opening [117] and that this pore opening is the key to various oxidative forms of cellular damage [118–121]. This fact suggests that pore dysfunction is the common pathway via which many harmful factors, such as cigarette smoke constituents, mediate their effects. The important observation that the pores participate in the apoptotic process [27] and the decisive role of oxygen radicals as death mediators in a number of pathological conditions [122, 123], explains our interest in the loss of mitochondrial function due to pore opening.

Role of Ca^{2+} in Pore Opening During Oxidative Stress

According to Crompton and co-workers [17, 124], changes in Ca^{2+} and Pi as well as in adenine nucleotides [24] during oxidative stress, trigger PT pore opening. Because

opening of the pores leads to hydrolysis of mitochondrial ATP, with subsequent loss of energy, the metabolism suffers additional damage and further increases in Ca^{2+}, causing a vicious cycle, leading to cell death. According to the above authors, the PT pore opening is of critical importance for the normal function of the cell [17, 125].

Lipid Peroxidation of the Mitochondrial Membrane

Lipid peroxidation, results in major oxidative damage to the biological membranes and it can be caused by various oxidative agents [126]. This process begins with free radicals which attack and remove one hydrogen atom from the unsaturated lipids of the membrane and create lipid radicals, which in turn can bind to molecular oxygen ($L^{\cdot} \rightarrow LOO^{\cdot}$). The radicals formed recycle the cascade of lipid peroxidation which comes to a halt with the reaction between two lipid radicals or with antioxidants [127]. Lipid peroxidation of the mitochondrial membrane results in an irreversible loss of mitochondrial functions such as mitochondrial respiration, oxidative phosphorylation and ion transport [128, 129]. Under normal conditions, the mitochondrial membranes contain iron capable of initiating lipid peroxidation by a yet unknown mechanism. However, it depends on the micromolar concentrations of Ca^{2+} [130]. Trivalent substances, such as acetone [131], are capable of initiating lipid peroxidation of the mitochondrial membrane. These substances in the presence of Ca^{2+} and high concentrations of non organic phosphorous, stimulate lipid peroxidation of the mitochondrial membrane [132]. Under these conditions, the generation of mitochondrial ROS is stimulated by the presence of Ca^{2+} and Pi, resulting in the peroxidation of the poly-unsaturated membrane lipids. The aldehydes produced during peroxidation lead to the formation of enoles by a reaction catalysed by Pi. Enoles are capable of reproducing the chain reaction of lipid peroxidation. Peroxynitrite anion, a product of the reaction of $O_2^{-\cdot}$ with NO, is believed to be responsible for a number of actions previously attributed to NO [133]. Recently, it has been shown that peroxynitrite is in a position to initiate lipid peroxidation of the mitochondrial membrane in combination with oxidation of the thiols of the membrane proteins, independent of the presence of Ca^{2+} [134].

Oxidative Stress and the Mechanisms of Apoptosis

The mitochondria are considered to be the initiators of the apoptotic process. Loss of the mitochondrial membrane potential, permeability transition and depletion of ATP have been incriminated in the triggering of apoptosis [62, 116,135–137]. Mitochondrial permeability transition occurs because of the opening of the mega-channels of the mitochondrial membrane. This alteration of the membrane occurs under oxidative stress. The mega-channel opening allows the passage of substances up to 1.5 KDa which is accompanied by a loss of membrane potential and the release of a number of proteins including cytochrome c [30]. Cytochrome c binds to

APAF-1 and activates caspase-3, initiating apoptosis [136]. Thus, at the beginning of apoptosis, cytochrome c is released to the cytosol prior to caspase-3 activation [138]. Anti-apoptotic proteins such as those of the Bcl-2 family, stabilize the pores and inhibit apoptosis [139, 140]. Adenine nucleotides play a role in apoptosis, in the living cells and tissues [141, 142]. Today, it is believed that the complexes of mitochondrial proteins that participate in important mitochondrial/cellular processes (such as opening of the MPT pore), are proteins of the inner mitochondrial membrane containing very important thiols that can easily be oxidized by oxidants and peroxynitrite [9, 143]. In MPT pore, ANT is a significant protein which is affected by oxidants and peroxynitrite [23, 144, 145]. During oxidative stimulus, the complex VDAC-ANT-CyP-D forms and triggers the opening of the mitochondrial permeability transition pore leading to a subsequent release of pro-apoptotic proteins (e.g. cytochrome c, AIF, pro-caspase-9) to the cytoplasm. Peroxynitrite can cause PTP opening [144, 146, 147] partly via oxidation of the critical thiols of ANT [144] and possibly via the reaction with VDAC which has been found to be nitrated under high concentrations of NO [148]. In a similar manner, oxidation of critical thiols of the pyridine nucleotide, promotes the release of mitochondrial Ca^{2+} [143]. Low ATP levels lead to cellular death due to lack of energy, via a non-apoptotic pathway. Adequate cellular ATP levels during the initiation of apoptosis are of critical importance [149–152]. Changes in cellular energy balance (e.g. ATP/ADP ratio) determine if cells will die via necrosis or apoptosis. In the case of a large drop in ATP levels, necrosis will prevail. Accordingly, apoptosis requires adequate ATP levels in order to reach cellular death – for example, enzymic hydrolysis of proteins and DNA degradation. DNA fragmentation does not occur in cells depleted of ATP. These cells appear to die via necrosis, but if ATP levels are restored partially from glycolysis [152], cellular death is considered apoptotic. The depletion of cellular ATP levels inhibits apoptosis induced by corticosteroids in the thymus cells [142], while both ATP and adenosine induce apoptosis [142]. Various toxic and anoxic conditions known to cause oxidative stress and disturbances in energy metabolism are linked to MPT and can lead either to apoptosis or necrosis [153–155]. Although depletion of cellular adenine levels can cause apoptosis, the exact molecular mechanism of how adenine nucleotides control pore opening at various levels of oxidative stress is not fully understood [155–158]. Polynucleotide degradation during oxidative stress is unique to the mitochondria [159].

ROS, Bcl-2 and Programmed Cell Death

Initially it was believed that Bcl-2 is localized only at the mitochondrial membrane [160]. Immunocytochemical methods however, revealed that it is localized at the outer mitochondrial membrane, the endoplasmic reticulum membrane and the nuclear membrane. Recent studies suggest that Bcl-2 has antioxidant properties due to its function to inhibit programmed cell death through repression of the formation and action of the ROS [49, 161]. Thus Bcl-2 functions to protect the cells from oxidants or from the ROS produced as by-products of physiological

mitochondrial reactions during ATP synthesis. Aerobic cells are supplied with extensive anti-oxidant defenses in order to cope with the harmful action of the ROS. Under constant oxidative "siege" such as smoking, their survival depends on the balance between ROS production and anti-oxidant activity [73]. Because the ROS are a highly reactive species and generally non-selective, it is unlikely that they participate in the highly coordinated and regulated processes which take place during programmed cell death. However, the ROS seem to participate in some cases of programmed cell death (PCD) [162] because (a) the addition of the ROS or depletion of the endogenous antioxidant pool is capable of initiating PCD, (b) PCD can be inhibited by endogenous or exogenous anti-oxidants and (c) PCD is associated with increases in ROS levels in the cells, especially in cases where apoptosis is caused by TNF-α or by mitochondrial ROS production [163]. What is not clear is if ROS production is essential for the progress of PCD [164, 165]. Hypoxia can induce PCD, while Bcl-2 can inhibit hypoxia-induced PCD [166]. These findings suggest that Bcl-2 can inhibit PCD in the absence of ROS. Although the ROS do not seem to be necessary for the execution of PCD, they participate at the beginning stage as intracellular signaling molecules [167].

Bcl-2 belongs to a family of proteins that either inhibit (Bcl-2, Bcl-xl) or promote (Bax, Bad, Bak) apoptosis. Proteins associated with Bcl-2 penetrate the outer mitochondrial membrane and nuclear and endoplasmic reticulum membrane [29, 168]. Bcl-2 overexpression prevents cells from entering apoptosis induced by a variety of stimuli including NO [49, 169, 170]. Thus, Bcl-2 inhibits MPT, prevents the release of caspase activators (especially cytochrome c) from the mitochondria and prevents $\Delta\Psi$m and DNA damage from oxidants and ceramide [110]. According to other investigators, high Bcl-2 levels are capable of delaying cell death, even when cytochrome c has already been released to the cytosol [171]

Oxidative Modifications of the Mitochondrial DNA Bases

Today, the biological properties of some oxidative modifications of mitochondrial DNA bases are well known. For example 8-OH-guanine is a promutated base when it is present in the DNA during its replication and thus, the incorporation of dAMP which leads to the mutation (GC $\rightarrow$ TA) occurs. Oxidative damage of the mitochondrial DNA bases is considered to be an important factor in cancer development [172–176]. Mitochondrial DNA (mtDNA) is especially exposed to the ROS produced by the mitochondrial electron transfer system. However, it is possible for oxidized monophosphoric deoxynucleotides to be incorporated into mtDNA. The levels of 8-OH-Gua in mtDNA have been found to be 4.4 times more elevated compared to nuclear DNA. Similar values are observed for 5-OH-Cyt. This ratio is higher in the case of the other three bases: 5-hydroxy hydantoin (5-OH-Hyd), 5-hydroxymethyl hydantoin (5-OHMeHyd) and 5-hydroxymethyl uracil (5-OHMeUra). The highest level of modified by oxidation bases in a resting state in mtDNA compared to nuclear DNA depends on many factors:

1. The large quantity of the ROS produced inside the mitochondria.
2. The lack of histones associated with mtDNA.
3. Inadequate DNA repair mechanisms present in the mitochondria.

Recently, it was demonstrated that the mitochondria possess a fine number of repair enzymes. However the exact mechanisms involved in the restoration of the oxidized bases remain unclear [177].

Oxidative Damage of Mitochondrial RNAs

Oxidative stress can be the outcome of an increased activity of the enzyme Cu/Zn-SOD (SOD-1). If the relative activity of SOD-1 to catalase and glutathione peroxidase (GPx) is increased in favor of SOD-1, more active hydrogen peroxide is produced by SOD-1 than that which can be consumed by catalase and GPx. This fact leads to a serious disruption of the oxidative balance [178–180].

The mitochondria are exceptionally sensitive to damage caused by H_2O_2 and other oxidants. Up until now a variety of mitochondrial oxidative damage has been reported, including the depletion of the ATP produced, loss of electron transport ability, the release of Ca^{2+}, lipid peroxidation, protein oxidation and DNA damage [181–184]. It is important to note that in cultures of hamster HA-1 fibroblasts exposed to low concentrations of H_2O_2 (160μm), down-regulation of mitochondrial RNAs was observed [185]. This down-regulation involves the decrease and degradation of many mitochondrial RNAs, while in higher concentrations apoptosis is observed [186, 187]. This preferential down-regulation is specific to the mitochondria. Altered mitochondrial gene expression upon oxidative stress, includes total transcriptional down-regulation in response to xanthine oxidase activity [188] and peroxyl radicals [189]. In addition, oxidative stress has been shown to cause generalized protein damage and degradation which is associated with decreased maximal rates of electron transport and diminished respiratory control [190].

Nitric Oxide (NO) Toxicity in the Mitochondria

Nitric oxide can harm the mitochondria by three main means: reversible inhibition of respiration, non-reversible inactivation of the mitochondrial enzymes and induction of permeability transition of the mitochondrial membrane pores. In addition, NO nitrosylates thiol groups of creatine kinase, and affect ATP production from the mitochondria [191, 192].

Nitric Oxide (NO) Action in Mitochondrial Respiration

Nitric oxide and its products inhibit mitochondrial respiration via oxidative stress, DNA damage, activation of PARP and deregulation of cytoplasmic calcium. While NO inhibits cytochrome c oxidase in a reversible manner [139,193–195], its products

inhibit complexes I, IV, V via S-nitration in an irreversible manner [196]. The inhibitory action of NO on cytochrome oxidase is accomplished even at nanomolar concentrations [197] and for this reason it is considered to be a potential regulator of respiration. Inhibition is exerted in the position where oxygen binds to the oxidase. Thus, NO prevents the oxidase from binding to the oxygen. NO binds either to reduced cytochrome a_3 and produces cytochrome a_3^{2+}-NO, or it binds to and reduces oxidized Cu_B^{2+} and produces Cu_B^{+}-NO^{+}. The binding of oxygen to cytochrome oxidase occurs at a position where two different metal ions exist: the iron of heme-a_3 and the copper of the active centre Cu_B. Bound oxygen is placed in between the two metals and is therefore reduced rapidly, while the two metals are reduced to a_3^{2+} and Cu^{+}. Nitric oxide therefore, binds to the reduced form of cytochrome c oxidase at the position where oxygen is bound [198]. The inhibitory action of NO to cytochrome oxidase is considered to be important for a number of physiological processes and it is possibly the only known physiologic regulator that acts directly with the respiratory chain. In addition, an important mechanism by which NO mediates its cytotoxic activity is the reversible inhibition of cellular respiration as a consequence of inactivation of the mitochondrial aconitase and components of the electron transport chain [199–201]. The inhibition observed in response to NO is competitive to oxygen [202–205], while the reversible binding to heme-a_3^{2+} seems to be more important to the cells. Very small (on a nanomolar scale) NO concentrations are capable of inhibiting electron transport at normal oxygen levels [202]. In vivo, NO harms mitochondrial function in a reversible manner, by inhibiting respiration and ATP synthesis and by causing mitochondrial Ca^{2+} release [191, 206, 207].

Long-term Activity of NO Causes Production of ONOO⁻

Cells exposed to NO show a direct and reversible inhibition of respiration due to the inhibition of cytochrome oxidase. However, after long exposure to NO, an irreversible inhibition is observed, probably due to the transformation of NO to the reactive nitrogen species (RNS) [196, 208–210]. It is known that NO produced in an aerobic environment is transformed to a powerful oxidant after reacting with $O_2^{-\cdot}$ and forming peroxynitrite ($ONOO^-$) [211–214]. Peroxynitrite is a highly reactive and toxic molecule which is produced during oxidative stress and which causes inhibition of the oxygen consumption dependent on complexes I and II; it mainly inhibits the activity of succinate dehydrogenase and ATPase without affecting respiration dependent on complex IV nor the activity of cytochrome c oxidase. $ONOO^-$ inactivates complex I via S-nitration [215]. Nitration inhibits aconitase and complex IV, possibly because of the removal of iron from the iron-sulfur centers [216–218] where $ONOO^-$ forming conditions are created. As soon as $ONOO^-$ is produced, it is capable of inhibiting the activity of complexes I, II, III, ATPase, aconitase, Mn-SOD, creatine kinase and many other proteins [219–221]. $ONOO^-$ inhibits complex I via tyrosine nitration [222]. This inhibition of complex I by $ONOO^-$ plays an important role in cellular dysfunction leading to death. The consumption of thiol deposits occurs preceding this

inhibition [208]. The $ONOO^-$ reaction with the mitochondrial respiratory chain causes further $O_2^{-\cdot}$ [223] and $ONOO^-$ production, inactivating Mn-SOD and increasing $O_2^{-\cdot}$ availability in the mitochondrial matrix [224]. Thus, NO is capable of initiating a destructive cascade via $ONOO^-$. The respiratory chain complexes I, II, III and enzymes of the citric acid cycle such as aconitase, contain iron-sulfur centers which are reversibly affected by $ONOO^-$ [196,225–228]. Peroxynitrite causes the translocation of calcium into the mitochondria, causing the opening of MPT pores due to the oxidation of thiols and NAD(P)H [147,229–231]. MPT is caused by oxidative stress in combination with elevated calcium concentration, which causes the formation of a protein pore, 2–3nm in diameter in the inner mitochondrial membrane. The formation of the pores causes, in turn, depolarization and swelling of the inner mitochondrial membrane leading to the release of solvents from the mitochondrial matrix [22, 108]. A depletion in ATP levels does not allow the cells to maintain their inter-membrane calcium density gradient by inhibiting the function of endoplasmic reticulum and cytoplasmic membrane Ca^{2+}-ATP-ases [232, 233].

Nitric Oxide in the Mitochondria Causes Formation of the ROS and RNS and Mediates MPT

Nitric oxide in relatively small amounts increases the production of $O_2^{-\cdot}$ and H_2O_2 in the mitochondria, thus inhibiting mitochondrial respiration, while in higher concentrations it inhibits H_2O_2 production, scavenging $O_2^{-\cdot}$ and producing $ONOO^-$ [223, 234]. NO reacts with ubiquinone (QH_2) and forms $NO^{-\cdot}$, which reacts with oxygen and produces $ONOO^-$. It also reacts with ubi-semiquinone (QH) which reacts with oxygen and produces $O_2^{-\cdot}$ [235]. Thus, NO is capable of producing $O_2^{-\cdot}$ and $ONOO^-$ and under different conditions, NO^-, NO_2 and N_2O_3. NO or RNS inhibit catalase, deplete intracellular glutathione levels and inhibit glutathione peroxidase, by increasing intracellular H_2O_2 levels [236, 237]. Indeed, NO and H_2O_2 contribute to cell death possibly via $ONOO^-$ production catalysed by hydrogen peroxide dismutase [238].

Nitric Oxide Activity on MPT

Mitochondrial permeability transition as mentioned before, is the dramatic increase in mitochondrial inner membrane permeability in small (up to 1,5KDa) molecules [9,239–241]. The mitochondrial membrane potential and mitochondrial matrix Ca^{2+} determine the ability of other substances to cause MPT. Thus, the inhibition of respiration by NO and the subsequent reduction of membrane potential, facilitate the opening of the MPT pore. Cyclic guanyl monophosphate (cGMP), formed by guanyl cyclase [GC] after stimulation by NO can inhibit MP in the cells via protein kinase C [242]. Nitrogen oxide (NO) in high concentrations possibly promotes MP via either (1) $ONOO^-$, nitrothiols and NO_2 or (2) depletion of glutathione

stores. NO and ROS can directly oxidize the thiol groups of various proteins and regulate the opening of the MPT pore [243, 244]. MPT plays an important role in both necrotic and apoptotic cell death. The dissipation of the proton motive force causing the uncoupling of oxidative phosphorylation and the reversal of ATP synthase hydrolyzing cellular ATP, leads to necrosis. MPT causes mitochondrial swelling with outer membrane rupture thus releasing intermembrane proteins, such as cytochrome c, to the cytosol. The release of cytochrome c and other matrix components such as NADH, inhibits respiration, causing necrosis. The release, however, of cytochrome c and other apoptogenic factors such as AIF and Smac/Diablo, triggers apoptosis. The transient opening of the MPT pore can be a physiological process and usually does not cause cellular damage while a long-lasting opening can cause either apoptosis or necrosis. Calcium is believed to cause cytochrome c release from the mitochondria and the consequent apoptosis, inducing the mitochondrial NO synthase (Mit NOS) to produce NO and subsequently $ONOO^-$ which in turn can cause MPT [245].

Pathophysiological Function of NO

The main physiological function of NO is the activation of GC, cGMP production and concomitant protein phosphorylation [246]. The normal function of NO may be dependent on or independent of GC and cGMP [247, 248].

Nitric oxide is capable of affecting various cellular functions directly (nitrosylation, nitration) or indirectly (methylation and ribosylation). The cytotoxicity of NO is associated with superoxide ($O_2^{-\cdot}$), thiols and transition metals. The reaction products of NO with these substances are NOx, $ONOO^-$ and metal adducts (Met-NO). NO toxicity leads to PCD or apoptosis. Under normal conditions, apoptosis begins with NO binding to specific (trimeric) receptors such as the TNF receptor (CD95/Fas/Apo-1, TNFR1 and TRAIL). The binding of the ligand with the trimeric receptor leads to the recruitment of adaptor molecules such as FADD in order to form Death Inducing Signaling Complex (DISC) and activate caspase-8 (page 96). Caspase-8 is responsible for initiating an apoptotic signaling cascade. High NO and $ONOO^-$ concentrations cause apoptotic cell death in various cell types [249]. Factors affecting cellular sensitivity to NO are the cellular redox state and transition metal complexes inside the cell [250]. Therefore, the threshold levels of NO action triggering apoptosis differ from one cell to another.

Activation of Apoptosis by NO

Nitric oxide is capable of inducing cytochrome c release from the mitochondria with a loss of mitochondrial membrane potential. Cytoplasmic cytochrome c activates the caspase-dependent apoptotic signaling cascade. Initially NO binds to cytochrome oxidase (complex IV) on the mitochondrial electron transport chain [223]. Mitochondrial-produced $O_2^{-\cdot}$ reacts with the NO bound to cytochrome oxidase and

generates $ONOO^-$ which causes mitochondrial dysfunction and cytochrome c release. The cytotoxic activity of NO and $ONOO^-$ results in DNA damage which causes p53 accumulation [251]. p53 accumulation leads to cell cycle arrest with the positive regulation of p21, increasing the ratio of Bax/Bcl-xl and cytochrome c release as well as caspase activation. However, the exact mechanism by which NO causes caspase activation is not yet fully understood. There are serious indications that these mechanisms cause the following conditions:

1. NO-induced DNA damage causing p53 accumulation [251]
2. activation of GC leading to apoptosis [252]
3. direct activation of MPT [159, 253], cytochrome c release and subsequent caspase activation through cytoplasmic APAF-1
4. peroxidation and degradation of mitochondrial phospholipids resulting in cytochrome c translocation to the cytosol [254]
5. inhibition of mitochondrial respiration which results in the generation of oxidants and the decrease in mitochondrial membrane potential, leading to the opening of MPT pores and the release of cytochrome c [168, 219, 255]

Nitric oxide is also capable of causing direct cytochrome c release and activating the MAP kinase pathway, independent of p53 pathway. According to Messmer and Brune [251], p53 accumulation precedes apoptotic DNA fragmentation in response to DNA damage [256]. Normally, cells express low levels of the p53 protein due to its short life. Although p53 is capable of causing apoptosis, there are other signaling pathways such as the lack of activation of certain transcription genes, negative regulation of Bcl-2 signalling cellular survival and positive regulation of Bax promoting cell death [257, 258]. NO activates cJun N-terminal kinase (JNK), SAPK and MAPKs, which are involved in apoptotic cell death caused by NO. There are indications that JNK/SAPK, MAPK and p53 play a critical role in NO-induced caspase-3 activation [259–261]. Another important property of NO is that it increases ceramide levels leading to the activation of sphingomyelinase and caspase-3 in the presence of Mg^{2+} [262]. Ceramide is capable of inducing various apoptotic pathways such as cytochrome c release, caspase-3 and -9 activation, activation of JNK/SAPK [263] and inhibition of Bcl-2 expression [264].

Anti-apoptotic Activity of NO

NO induces the expression of many cytoprotective genes such as HSP70 and HSP32 which protect the cells from TNF-induced apoptosis or oxidative stress [265, 266] as well as from glucose depletion [267]. Biochemical anti-apoptotic NO mechanisms are specific for each cell type and are either dependent or independent of cGMP. In some cells, such as hepatocytes, the anti-apoptotic function of NO depends on cGMP production which prevents cytochrome c release [266] from the mitochondria with the production of ceramide [268] and the activation of caspases [269]. Nitric oxide and cGMP protect some cells such as B lymphocytes from

PCD by inducing Bcl-2 expression [270] and the activation of Akt/PKB (Protein Kinase B) [271] which causes Bax phosphorylation as well as the increased expression of cytoprotective genes via activation of NF-kB. NO can inhibit caspase activation via S-nitrosylation. All caspases contain a critical cysteine residue in their catalytic centre. This thiol is susceptible to nitrosylation by NO. By inhibiting caspase activity via nitrosylation, NO is capable of inhibiting apoptosis in some cell types [272]. NO is an inert molecule with weak chemical activity, reacting with thiols at neutral pH in comparison to other highly reactive NO^+-type products. NO^+ can be produced with the loss of one electron from NO. The known electron acceptors are molecular oxygen and transition metal ions such as iron and copper which are directly activated with NO in vivo. Therefore, NO is able to nitrosylate caspase thiols as well as the availability of other thiol targets such as glutathione and free cysteines. The product of the reaction of NO with the complexes of iron-sulfur, causes S-nitrosylation of caspase via NO^+ formation [273].

Cellular Necrosis by NO

ATP depletion is capable of inducing necrosis mainly via four different mechanisms: (1) inhibiting mitochondrial respiration, (2) inducing MPT (3) inhibiting glycolysis from 3-phosphoglyceraldehyde dehydrogenase and, (4) activation of PARP or PARS. NO does not inhibit glycolysis directly, while $ONOO^-$ directly oxidizes the critical cysteine of the 3-phosphoglyceraldehyde dehydrogenase, causing irreversible inhibition [274]. Reduced thiols such as glutathione can prevent this sort of inhibition and thus effective inhibition can only take place in the case of glutathione depletion. When cells are exposed to NO, ATP production through glycolysis is considered to be important for cell survival, since moderate NO levels inhibit mitochondrial respiration and consequently mitochondrial ATP production. In the absence of glycolysis, moderate NO levels can cause necrosis due to the inhibition of respiration and therefore ATP depletion [275, 276]. In many cell types, necrosis induced by NO can be prevented by glucose [276, 277]. However, if MPT is triggered over a long time period by NO products, respiration can be inhibited, oxidative phosphorylation dissipated and glycolytic ATP hydrolysed, due to the reversal activity of ATP-ase. Necrosis can therefore proceed even if glycolysis occurs at a rapid pace.

DNA Damage by NO

An important cause of glycolysis inhibition is NAD^+ depletion caused by PARP or PARS. When PARP, localized in the nucleus, is activated by DNA fragments caused by $ONOO^-$, it catalyses the ADP-ribosylation of proteins using NAD^+ as a substrate [278]. In cases of extensive DNA damage, PARP activity is so great that intracellular NAD^+ stores (inhibiting glycolysis) and possibly adenine nucleotide levels are depleted (either because they serve as a substrate for NAD^+ production

or because glycolysis is inhibited). PARP activation contributes to the depletion of cellular energy supplies perhaps in synergy with the inhibition of respiration, the inhibition of glycolysis and the activation of MPT. Recently it was demonstrated that PARP activation can cause MPT and the release of factors such as AIF, inducing caspase-independent apoptosis [279].

NO-induced Apoptosis

NO-induced apoptosis is mediated via caspases and specially via caspase-3 while it is completely inhibited by caspase inhibitors [276, 280, 281]. The release of cytochrome c suggests that NO-induced apoptosis is mediated by the mitochondria. However, in some cell types, the premature activation of caspases-8 and -2 indicates that apoptosis caused by NO can be triggered by other pathways independent of the mitochondria [281–283]. NO induces apoptosis via many pathways such as:

1. induction of MPT [159, 280, 284]
2. oxidation of mitochondrial lipids [251, 254, 285, 286]
3. positive regulation of pro-apoptotic proteins such as p53 [251, 286] and Bax, and negative regulation of anti-apoptotic Bcl-2 [287]
4. activation of the MAPK pathway [288]
5. activation of the stress responsive endoplasmic reticulum-mediated pathway [289, 290].

Nitric oxide by itself does not cause MPT but via $ONOO^-$, S-nitrosothiols, NO_2 and N_2O_3 which are capable of inducing apoptosis, directly activating MPT, followed by the release of cytochrome c and caspase activation. All of these processes are completely inhibited by the addition of cyclosporine A, a MPT inhibitor [159, 280].

Lipid peroxidation of the mitochondria constitutes an important event in the apoptotic process [291]. NO is capable of positively regulating Bax and translocating it from the cytosol to the mitochondrial outer membrane. It seems that the activation and translocation of Bax occurs after the activation of p38 MAP kinase [288]. The reduction of the mitochondrial membrane potential is enough to trigger Bax translocation which can lead to apoptosis, assuming that ATP levels are maintained [292]. NO is capable of inhibiting apoptosis induced by various other factors. A number of different mechanisms have been suggested to explain this effect:

1. the inhibition of cytochrome c by cGMP leads to the inhibition of MP in the presence of PKC [242]
2. the inhibition of ceramide synthesis by cGMP
3. nitrosylation of caspases
4. depletion of energy stores
5. hyperpolarization of the mitochondria
6. activation of MAPK pathways
7. activation of NF-kB and AP-1 and
8. increase in heat shock and Bcl-2 protein expression [289].

In summary, NO inhibits mitochondrial respiration in two ways:

1. by reversible inhibition of cytochrome oxidase
2. by irreversible inhibition or inactivation of complexes I, II and III, of cytochrome oxidase, ATP synthase, creatine kinase and aconitase via $ONOO^-$ or via S-nitrosylthiols.

The reversible inhibition of cytochrome oxidase is accomplished either via NO binding to the reduced heme-a_3 in competition with oxygen or via NO binding to the oxidized Cu_B with the consequent formation of an inhibitory nitrate ion.

Mitochondria and Carcinogenesis

The mitochondrial genome consists of circular DNA encoding 37 genes on the L and H strands. These include 22tRNAs, 2rRNAs and 13 respiratory chain subunits [1, 2]. The mitochondrial genetic code varies slightly from the nuclear genetic code and the mutation rate of the mitochondrial genome is 10fold higher than that of the nuclear genome [3–5]. This is due to a lack of protective histones [6].

Mutations alter mitochondrial functions that may have some effects on tumor development. Although most cancer cells suffer somatic mutation, in mitochondrial DNA (mtDNA) the question of whether such mutations contribute to the promotion of carcinogenesis remains undefined [7]. There are sometimes confusing and conflicting data on seemingly disparate mechanisms that influence phenotype and carcinogenesis. Although it has been shown that a majority of cancer cells harbor mutant mtDNA, it has not yet been determined whether mtDNA mutations precede and lead to carcinogenesis.

Studies of the presence and mechanisms of mitochondrial DNA mutation focus attention on the role of the mitochondrial membrane potential ($\Delta\Psi m$). The mitochondrial membrane potential is maintained by electron transport coupled to the pumping of protons from the mitochondrial matrix to the intermembranous space. This creates a pH gradient and the $\Delta\Psi m$, which are of critical importance in both the translation and stability of proteins encoded by mitochondrial DNA and synthesized within the organelle [8], and also in the translocation across the mitochondrial membrane of nuclear encoded proteins destined for the mitochondria [9].

Anything that affects the $\Delta\psi m$ can induce damage to proteins encoded by mitochondrial DNA and also the nuclear encoded proteins destined for the mitochondria. Mitochondrial proteins and subunits of mitochondrial complexes involved in electron transport and oxidative phosphorylation are encoded by nuclear genes. Furthermore, mitochondrial oxidative phosphorylation is associated to many pathways of intermediary metabolism. Therefore, mutations in nuclear genes related with altered profiles of gene expression, will directly or indirectly interact with mitochondrial mutations.

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Chapter 5
Oxidative Activation of Transcription Factor Pathways by Cigarette Smoke Oxidants

Activation of the Transcription Factor NFkB

Structure and Function of NFkB

The NFkB transcription factor is a dimer (homo or hetero) belonging to the Rel family proteins [1–5]. Only five of these proteins have been identified and they are the p65(RelA), p50, c-Rel, p52 and RelB. They all share a common N′ terminal which consists of 300 amino acids (aa) named Rel Homology Domain (RHD) which is responsible for dimerization, DNA binding, nuclear localization and binding to its inhibitor IkB.

Under normal conditions the NFkB protein is found in the cytosol bound to its inhibitor IkB in an inactivated form. There are seven members of the IkB family of proteins: IkBα, IkBβ, IkBε, IkBζ, BCL-3, p100/IkBδ, p105/IkBγ and IkBR. The N-terminal of the IkB proteins includes a section consisting of ankyrin repeats, each consisting of 30–33 aa [1–6] with a functional role in Rel binding. The C-terminal of the IkBs is required for inhibition of the NFkB binding to the DNA [7]. The binding of IkB to NFkB results in the masking of the Nuclear Localisation Sequence (NLS) of the NFkB protein, which is responsible for the DNA binding ability of the transcription factor, thus sequestrating the NFkB protein in an inactive state in the cytosol.

Upon cell activation, IkB becomes phosphorylated at specific serines, e.g. in the case of IkBα the phosphorylations occur at serines 32 and 36. Serine phosphorylation leads to the release of IkB from the NFkB dimer and its immediate degradation from the 26S proteosome [5]. The release of the NFkB dimer from its inhibitor unmasks the NLS and allows for the transcription factor to enter the nucleus and bind to DNA specifically through its kB motifs (GGGRNNT(Y)CC). This NFkB binding to the DNA leads to sequestration of important mediators of the transcriptional machinery and induces the expression of the genes located near the kB motifs. Different dimers can bind specifically to approximately 128 different motifs. So far there have been a large number of genes identified that can be activated by the NFkB dimer and these control immune response, the generation of nitric oxide (NO), cell to cell interaction, apoptosis and cell proliferation [8]. Other genes controlled by NFkB also encode for enzymes, growth hormones, chemotactic factors, adhesion

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molecules, acute phase proteins and many other substances [9]. Finally, activation of NFkB is accompanied by induction of the anti-apoptotic pathway, thus inhibiting programmed cell death.

When the existent IkBα pool becomes degraded, the newly expressed IkBβ binds to NFkB with no previous phosphorylation [10]. IkBβ is a less effective substrate than IkBα for the IKKα enzyme [11, 12]. Upon binding of the NFkB to the non-phosphorylated IkBβ, the complex enters the nucleus, binds to the DNA and activates transcription. Through this mechanism, IkBβ is able to induce expression of IkBα and allow the prolonged DNA binding and transcriptional activity of NFkB.

Control of lkBa by Tyrosine Phosphorylation

During oxidative stress IkBα phosphorylation occurs at tyrosine 42 instead of at serine 32 and 36 [13]. This phosphorylation at tyrosine 42 results in the release of IkBα from NFkB. IkBα release is not followed by its degradation since in phosphorylated form IkBα cannot be recognized by the proteosome. Oxidative stress inhibits the transcription activity of NFkB, while its ability to control gene expression is dependent on cysteine 62 which is found on the p50 subunit and must remain in a reduced form. This preservation of cysteine 62 in a reduced form is dependent on thioredoxin, a small protein molecule which controls certain functions such as cell growth and programmed cell death [14]. Therefore, the inability of NFkB to bind to DNA is probably due to the absence of reduced thioredoxin. In a few hours following oxidation, there is cell response to the oxidative stress with an increased production of thioredoxin reductase mRNA, along with an increase in GSH and the recovery of the NFkB/thioredoxin complex. Therefore the redox state of the cells exposed to cigarette smoke directly influences the redox state of the NFkB p50 subunit. In turn, reduced GSH plays an important role in protecting the cell from oxidative stress. The high concentrations of GSH in the cytoplasm does not permit NFkB to translocate to the nucleus, while the GSH found inside the nucleus facilitates the DNA binding of the NFkB. Thus, oxidative stress which reduces the cytoplasmic GSH, promotes the nuclear translocation of NFkB, while a reduced state in the nuclear environment ensures its DNA binding ability. Besides the transcriptional activity, the cell redox state influences many other important cell functions (transmembrane substance transportation, concentrations of receptor molecules and attachment factors), through inactivation of many enzymic active sites bearing cysteines, which in turn are important electron donors.

Cross-talk Between NFkB and MAP Kinases

Biological stress, like oxidation, acutely activates two subcategories of MAPKs: ERK (extracellular signal-regulated kinase) and kinase p38. Stimulation of the surface receptors causes sequestration of small adaptive G-coupled proteins, which in turn induce a series of phosphorylations in order to activate MAPKs in the

cytoplasm. Many factors which can activate MAPKs can also activate NFkB and this means that there is cross-talk between the two pathways (Fig. 5.1). Activated MAPKs translocate to the nucleus where they phosphorylate specific substrates, many of which are transcription factor subunits. This cooperation between MAPK and NFkB in controlling gene expression also occurs in transcriptional activation. Interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS), E-selectin and interleukin-2 (IL-2) are all genes which are transcriptionally controlled by both MAPKs and NFkB [15–19].

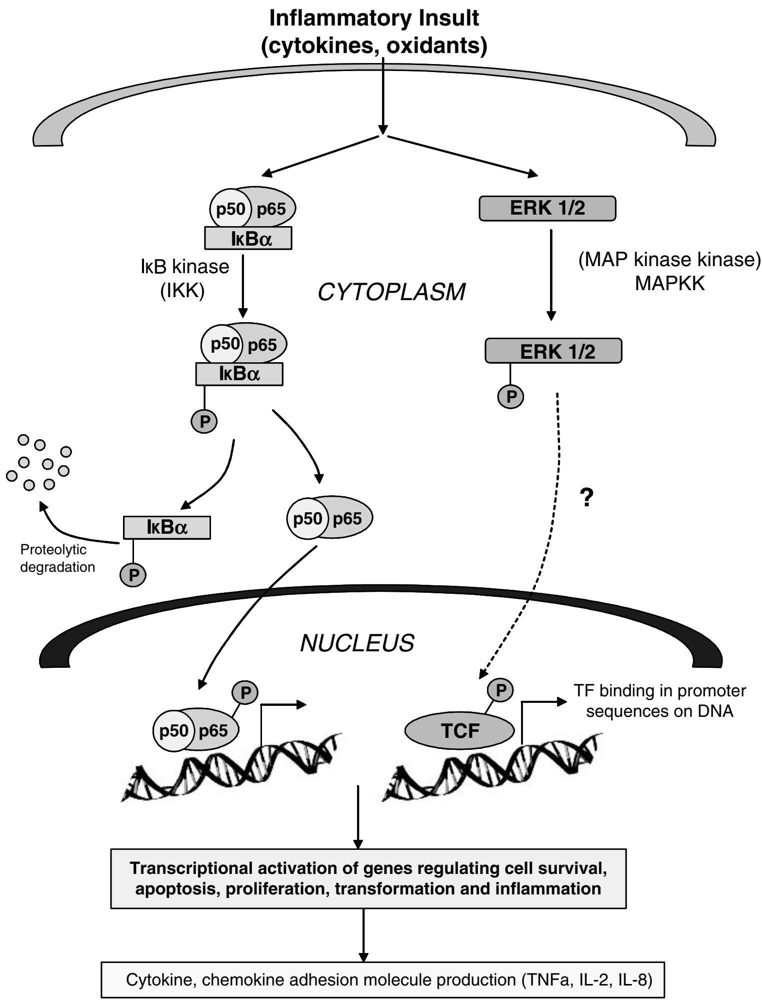

Fig. 5.1 Gene transcription phosphorylation cascade, important for cell survival, apoptosis, proliferation, transformation and inflammation

Activation of NFkB During Oxidative Stress: Role of the Antioxidative Inhibitors

The reactive oxygen species (ROS) act as second messengers leading to the activation of the transcription factor NFkB [9, 20, 21]. However, many scientists seem to disregard the importance of the role of the reduced condition of the intrinsic cell environment for activation of NFkB through oxidative pathways [22]. The most common antioxidative inhibitors in laboratories during cell investigation under conditions of oxidative stress against ROS are N-Acetyl Cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) [9, 20, 21]. However, it has been shown that NAC can inhibit the activation of NFkB when TNFα is used as the inducer, due to the fact that NAC can bind and block TNF receptors, having no effect on the activation of NFkB (for example through interleukin-1 (IL-1) or TPA). This shows that both NAC and PDTC may inhibit NFkB activation, regardless of their antioxidative action.

It is known that the oxidase complex Rac/NADPH is responsible for endogenous ROS production during NFkB activation [23, 24]. In the signal transduction pathways and hydrogen peroxide production by the NADPH-oxidase complex, the small protein Rac is absolutely necessary for guanosine triphosphate to be able to link with the process. The answer came from studies where the levels of endogenously produced ROS were investigated by blocking or activating the NADPH oxidase complex. The results suggested that endogenously-produced ROS does not lead to NFkB activation. This answers the question how these very effective but non-specific molecules such as ROS can determine the well-coordinated signaling of NFkB.

As mentioned, NAC can inhibit the NFkB signaling cascade induced by TNFα. This is carried out by the ability of NAC to cause structural changes to the TNF receptors, resulting in the modification of the disulfide bridges, by reducing the thiol groups within its molecule.

Effect of Cigarette Smoke Oxidants on NFkB: Activation of Intracellular Adhesion Molecule-1 (ICAM-1)

The intracellular adhesion of molecule-1 (ICAM-1) is an important mediator of inflammation and it is expressed in the broncho-epithelial cells [26, 27]. Several cytokines can influence ICAM-1 expression [28, 29]. TNFα can positively control the expression of the ICAM-1 gene in normal human differentiated broncho-epithelial cells through the mechanism which connects homodimeric (p65/p65) and heterodimeric (p65/c-Rel) complexes of the nuclear factor NFkB with the promoter of the ICAM-1 gene. Since the activity of transcription factor NFkB is controlled by oxidation reactions, (e.g. it can be activated by cigarette smoke), its activity can also be influenced by the intracellular redox state of the cell [30, 31]. Similarly, TNFα induction produces low levels of ROS within the cell [32, 33], which can act as intracellular messengers for NFkB activation, which in turn controls the increased

expression of ICAM-1. NFkB activation following TNFα induction occurs as a response to the oxidative substances produced within the cell (i.e. hydrogen peroxide (H_2O_2)) [34].

Conclusion: ICAM-1 expression through TNFα in the epithelial cells of the pulmonary airways is based on the activation of at least two transcription factors through the intracellular pathways.

Oxidative/Anti-oxidative Interventions in the Activation Mechanisms of NFkB and AP-1

Aerobic organisms can anticipate the side-effects due to the reactive oxygen species (ROS) by increasing the production of biochemical antioxidants such as glutathione, tocopherol, b-carotene, pyridine nucleotide [35–37], or by increasing the production of endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase, thioredoxin (TrX), glutathione peroxidase (GPX) and glutathione reductase (GR) [36,38–43]. The transcription factors NFkB and AP-1 are the main targets of oxidants [31,44–48]; NFkB can become activated by ROS, especially H_2O_2 in a variety of cell types [35,49–55] and this activation is stabilized by glutathione and its converting enzymes [49, 56, 57]. The reactive oxygen species can also activate AP-1 and it is thought that this activation is the result of activation of the MAPKs pathway as a response to oxidative stress [59]. The AP-1 binding with the nuclear DNA that follows its activation is promoted through a redox mechanism, i.e. Redox factor-1 (Ref-1) in cooperation with TrX [44, 60]. TrX increases the DNA binding activity of AP-1 and NFkB. TrX exerts its effect on the NFkB by reducing cysteines such as Cys-145: c-Fos, Cys-272: c-Jun, and Cys-62: p50. All cysteines are located in the DNA binding domains of transcription factors [61–64]. The factor Ref-1 regulates the redox state in the presence of TrX and thioredoxin reductase [65, 66]. Under oxidative stress, TrX translocates to the nucleus where it can cross-react with Ref-1. This is evidence that Ref-1 participates in the redox signaling cascade, from the cytoplasm to the nucleus, in order to activate specific transcription factors [67, 68].

NFkB Regulation by Nitric Oxide (NO)

There are several interesting interrelated activities between NO and NFkB and some evidence suggesting that two activities are coordinated. Factors that can block NFkB can also block the expression of the inducible nitric oxide synthase (iNOS) which is responsible for NO production [69]. Nitrogen oxide produced by iNOS acts as a negative feedback loop for NFkB, inhibiting its extensive activation and transcription of the NFkB- regulated genes [70–73]. Increased levels of NO have an inhibitory effect on NFkB due to the nitrosylation of cys-62 on the p50 protein that inhibits its DNA binding activity [74, 75]. The dependence of NFkB activation on

NO concentration has been extensively investigated. It has been shown that TNFα induction in exposures of low concentrations of NO promotes activation and increased DNA binding of NFkB, probably through the increased activation of Ikk-α [76]. It is not clear how NO mediates its positive effects. However, it has been observed that activation of Ras by S-nitrosylation is followed by the activation of NFkB [77, 78].

Mechanism of Oxidative Substance Action on NFkB Regulation

In order to evaluate the relationship between oxidative stress and NFkB, it is of critical importance to take into account the site where the oxidative substance is produced, the kind of substance (e.g. NO as opposed to $ONOO^-$) and the time-frame within which NFkB activation occurs. For example, oxidants acting on the cell membrane can activate cell membrane receptors or Ras in order to trigger the MAPK cascade, thus contributing to NFkB activation. On the other hand, oxidants produced by the mitochondria or easily moving through membranes by diffusion, do not attack proteins which can activate the cascade. According to Mossman [79], NFkB activation is much more complicated than its dissociation from its inhibitor IkB. Beyond degradation of IkB, phosphorylation of the NFkB subunits which leads to the transcriptional activation of genes regulated by NFkB, is also of great importance. NFkB subunit phosphorylation is independent of IkB degradation, thus offering an additional control point for NFkB regulation [80]. It is unclear whether the subunit activation occurs in the cytoplasm or the nucleus.

Oxidative Stress Through Receptor Molecules

Oxidative stress increases the activity of the signalling cascades controlling cell growth by stimulating the growth factor receptor phosphorylation [81, 82]. This is important since receptor activation seems to play a significant role in the cell response to oxidative stress [83, 84], as it increases the enzymic activity which is regulated by receptors (i.e. phospholipases A2, D and C) [85, 86]. Therefore, exposure to oxidants increases the activity of many protein kinases which play a role in mitogenesis, as is the case with PKC, many tyrosine kinases, MAP kinases and c-Jun N-terminal kinase [32,87–89]. From all the above it is clear that increased kinase activity due to oxidants, as well as the redox control within the cell, are important mechanisms. Through these mechanisms, alterations in gene expression may happen as an integrated response to redox imbalance. It seems as if oxidative stress reflects the true induction of the signal cascade by the tyrosine kinases and by the loss of the control by tyrosine phosphatases. These true balance influences are kinds of cell response towards functional changes such as cell death or cell differentiation. The scavenging of oxygen free radicals by antioxidants may inhibit the signalling cascade stimulated by the oxidants themselves [90–92].

NFkB and Immune Function

NFkB activation regulates a certain number of genes which are required for physiological T-cell response. These include IL-2, IL-6, IL-8 genes as well as a number of receptor genes (Fig. 5.1). There is evidence that the intracellular redox condition is a very important regulator of NFkB function. ROS have been denoted as universal second messengers of the T-cell signalling cascade, which can activate NFkB [46,93–95]. However, oxidation is unable to fully activate NFkB in T-cells without interference from other inducers. It seems as if oxidation is important for the phosphorylation and degradation of the IkB inhibitor while the reduced state is essential for NFkB DNA binding.

Reactive Oxygen Species (ROS) and Mechanisms of Immune Cell Activation

The primary events which take part in T-cell activation due to the intracellular signalling cascade are increased calcium influx, protein phosphorylation and mobilization of the cell membrane phospholipids. Intracellular ROS production (H_2O_2, $\cdot OH$ and possibly $O_2^{-\cdot}$), play a significant role in this mechanism [96–101]. Cell signalling via ROS specifically upregulates the nuclear complex of the transcription factor NFkB or that of the nucleus bound transcription factor AP-1, which is sensitive to redox substances [102]. T-cell activation is greatly influenced by the presence of "cooperative" cells especially monocytes and macrophages which offer various co-stimulating signals. ROS activity in T-cells is very sensitive to the "cooperative" cells. This suggests that in order for H_2O_2 to be produced by T-cells, a signal from another type of cell is required [103].

There is evidence suggesting that the presence of CD2 on monocytes can counteract the constant signal for ROS production by T-cells. The physiological ligand of monocyte CD2 is the ligand CD58 (LFA-3). The ligand CD58 activates CD2 through the co-stimulated pathway, and this is the required signal provided by the monocytes to the T-cells. In order to induce T-cell activation through receptors, it is important that two stimulants be used simultaneously. The use of one stimulant only inhibits T-cell activation through the receptor CD2 [104, 105].

Ras Gene Activation

There is evidence that ROS production is part of a mechanism which leads to Ras activation [106]. In T-cells, it appears as if there are at least two mechanisms for Ras regulation, one of which is through the protein kinase C (PKC) pathway. Phorbol myristate acetate (PMA) (one of PKC inducers) is an inducer of ROS production in these cells and therefore one of the suggested mechanisms for Ras regulation. Another mechanism for Ras regulation which is independent of PKC

activation is the stimulation of ROS production through the CD2 antigen/antibody complex [107].

Oxidative Signals and Redox Pathways in T-cells

T-cell lines express only the inducible form of NFkB [93, 108]. Several stimuli that can activate T-cells can at the same time activate NFkB. However, as previously noted, full T-cell activation requires at least two distinct signals, one of which is the T-cell receptor complex (TCR). Therefore, the most favourable activation of NFkB in T-cells is dependent on dual signalling cascades [109, 110]. Although exogenous ROS can actually activate NFkB in some cells, this is not the case in T-cells; NFkB activation occurs when its inhibitor IkB becomes phosphorylated and degraded, following its dissociation from the activated NFkB dimer complex [111–116].

Although *in vitro* studies have shown that lkBα is capable of binding to the p50 subunit of NFkB, masking its NLS [117], the binding of IkBα with p65 and c-Rel is believed to be the most important mechanism by which IkBα inhibits the activity of NFkB through nuclear translocation inhibition [117, 118]. The free IkBα molecule has a short life of 70 seconds to one hour and can be detected either in the cytoplasm or the nucleus [119].

Many factors such as phorbol esters [119], phytohemagglutinin (PHA) [120, 121], TNFα, lipopolysaccharides (LPS) [123, 124], calcium ionophores (ionomycin) [111, 125] and hydrogen peroxide (H_2O_2) [93] can induce NFkB activation in T-cells. However in most T-cells, the maximum NFkB activation has been observed in response to a combination of inducers, which in turn fulfil the prerequisites of dual stimulation [9, 109, 126, 127].

Although H_2O_2 and other ROS are considered as absolute second messengers which can associate the activities of the two inducers by acting synergistically or by activating both pathways in order to activate NFkB, we should not consider them as specific intracellular messengers in all kinds of T-cells. In fact, oxidative stress can control NFkB activation in T-cells by functioning as an auxiliary signal.

Endogenous ROS produced as a response to extracellular signals which react with the cell surface receptors, act as signalling molecules for NFkB activation [9, 128]. For example TNFα stimulation can produce ROS which can activate NFkB [46, 95] The antioxidant substance, NAC [129] can inhibit IkBa phosphorylation, leading to NFkB inhibition. Since NAC can offer precursor molecules for glutathione synthesis within the cell, along with the fact that NAC can modify NFkB activation in the cells, it was thought that this observation indicated the major role of the glutathione system in the activation of NFkB. It has already been noted that glutathione acts as the primary intracellular anti-oxidative system. In this system, glutathione cycles between its reduced (GSH) and oxidized (GSSG) form, playing a major role in the defense mechanism against oxidative stress [130]. Intracellular oxidative conditions are required for the phosphorylation of IkBα and its release from NFkB. On the other hand, a reducing environment is required in order for the NFkB dimer to bind to DNA. Several reducing substances, especially those attacking the

cysteine groups, can increase the binding ability of the transcription factor to the DNA. The modifications seen on cysteine 62 of the p50 subunit, the primary DNA binding subunit, suggest the requirement of a reducing environment [131–133]. The oxidative signal is able to cause an increase in the DNA binding ability of NFkB, as long as the cells sustain their ability to return to a reducing intracellular condition. The GSH system is thought to play a primary role in the preservation of a reducing intracellular environment. For this system, a model of dual function has been suggested in order to accomplish the double requirement, the intracellular oxidation and reduction, as well as the NFkB signaling. In this model, GSSG is considered to be indispensable to NFkB activation, while GSH, the indispensable requirement to NFkB binding to DNA [134]. The role of intracellular GSH in the activation and the DNA binding activity of NFkB have been further investigated using inhibitors of glutathione synthesis [135].

The conclusion from this study is that GSH seems to be a prerequisite for the regular binding and transcriptional activity of NFkB.

Oxidation of the Transcription Factor ERK

Apart from the transcription factor NFkB [46], oxidative substances can also cause phosphorylation of the extracellular regulated kinase (ERK) family, which is regulated by extracellular stimuli, as well as the mitogen activated protein kinase (MAPK) which is regulated by mitogenic stimulus. This phosphorylation cascade is the key element for gene transcription which is important for cell survival, apoptosis, proliferation, transformation and inflammation (Fig. 5.1).

The ERK family of kinases is typically activated through a series of phosphorylations which follows the cell surface receptor phosphorylation (e.g. the epidermal growth factor receptor [EGFR] phosphorylation). Phosphorylated members of the ERK family (ERK1, ERK2, ERK3, ERK4 and ERK5) have a transcriptional function in activating specific genes. For example the phosphorylated ERK2 translocates to the nucleus in order to phosphorylate the ternary complex factor (TCF). TCF binds to the c-Fos factor, which in turn causes transcription of proto-oncogenes such as the c-fos gene.

Phosphoinositide Pathway Activation

Role of Protein Kinase C (PKC)

Protein kinase C is a serine/threonine kinase and is a member of an 11-member kinase family whose activity depends on the presence of phospholipids. Each individual PKC is involved in specific cellular functions. PKC activation is considered to be an important regulator of cellular homeostasis due to its property to be activated by various biological inducers, such as growth factors, neurotransmitters, various hormones and phorbol esters, which relay important information to the cell. PKC is a cellular receptor of major importance for phorbol esters which promote tumour

growth. Therefore, there are serious indications that PKC plays a significant role in carcinogenesis [136–140]. Cells that overexpress PKC are apt to transformation due to the activation of the Ras oncogene which subsequently activates PKC [141]. Activation of PKC by Ras is a significant factor for the hydrolysis of phosphatidylcholine and the synthesis of diacylglycerol (DAG). PKC regulates various cellular functions such as mitogenesis, cellular adhesion, apoptosis, infiltration and metastasis [142–145].

Structure and Function of the PKC Molecule

Structure

PKC isoforms consist of two functional domains: a C-terminal catalytic subunit and an N-terminal regulatory subunit. The function of the catalytic subunit is inhibitory. Inhibition can be expressed either through proteolysis which releases the catalytic subunit, or by the addition of the appropriate cofactors such as calcium ions (Ca^{2+}), anionic phospholipids, phorbol esters or sn-1,2-diacylglycerol (DAG). PKC regulates one part of the signal (information) relay system of the cell which includes hydrolysis of phosphatidylinositol-4,5-diphosphate for the production of sn-1,2-diacylglycerol and inositol-1,4,5-triphosphate. Thus, the intermediate sn-1,2-diacylglycerol is produced; it can also be produced by hydrolysis of phosphatidylcholine [146].

Function

PKC participates as a significant factor in carcinogenesis, not only as a receptor of a specific category of tumour promoters, but as a factor that when stimulated, increases (directly or indirectly) the activity of the nuclear proto-oncogenes c-fos, c-myc and c-jun. At a biological level, it participates very actively in the control of cellular processes related to proliferation and differentiation. It is the key that regulates cellular homeostasis. This fact defines the role of PKC in carcinogenesis and the various mitogenic regulations. PKC activation takes place on the cellular surface [147]. There are serious indications that stimulation from an agonist that is derived by degradation of various membrane phospholipids is important for the relay of information from extracellular signals via the membrane. Many results show that PKC is a part of such a process. Ca^{2+} ion transport and PKC activation act synergistically in order to produce various cellular responses (Fig. 5.2). There is a close relationship between the mobilization of Ca^{2+} and phospholipid degradation, and occasionally there is a mutual complementarity. In the presence of a high concentration of Ca^{2+} ions, PKC activation requires less phospholipid degradation, while in the presence of high phospholipid degradation, less Ca^{2+} is required for the activation of the enzyme.

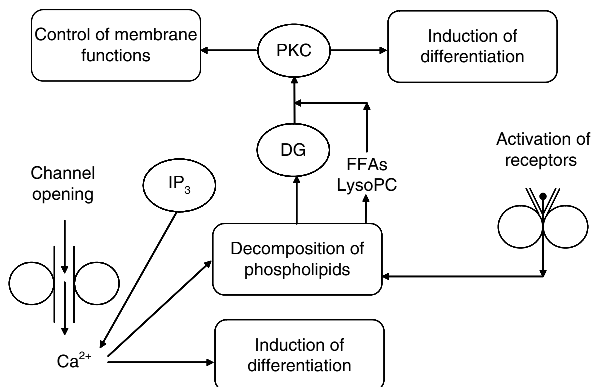

Fig. 5.2 PKC pathway functions to the various cellular control loops. IP3 = 1,4,5-Inositol-triphosphate, FFAs = Free cis unsaturated fatty acids, LysoPC = Lysophosphatidyl-choline

Intracellular Distribution of PKC

In steady state, a diffuse cell cytoplasmic stain for α-PKC can be observed. After stimulation by thyrotropin-releasing hormone (TRH) for 15 seconds, immunofluorescence accumulates at the cellular membrane [148]. Subsequently, it gradually returns to the cytoplasmic space. During this redistribution of the isoenzymes, a rise in the cytoplasmic calcium levels is observed. Therefore, the subcellular localization of PKC in vivo is regulated by calcium and diacylglycerol. There are serious indications that a part of cellular PKC is attached to the cytoskeleton. Many proteins of the cytoskeleton are thought to act as a substrate for PKC. Cytoskeletal PKC-α is located on the cytoplasmic side of the membrane together with the focal adhesion protein talin [149]. Talin together with another focal adhesion protein, vinculin, are known PKC substrates. PKC diffusion is a significant factor of signal relay and substrate recognition. It is obvious from the above that when a cell is stimulated, diacylglycerol is detected in various cellular compartments during cellular response.

Effect of Oxidation on the Structure of the PKC Molecule

The main targets of oxidative substances are the cysteine-rich zinc fingers. PKC is the first protein kinase which connects 4 zinc atoms, regulated by the thiol groups [150], making it a sensitive target for oxidation [151, 152]. The bond of zinc with the thiol groups is affected by the redox system [153, 154]. The catalytic domain of PKC is rich in cysteines, which are necessary for the functional activity of the kinase. In contrast with the zinc-thiol groups of the regulatory domain, the cysteines of the catalytic domain are unregulated and free to react with alkylating factors and antioxidating substances. Nitrogen monoxide inactivates PKC, reacting with cysteine sulfhydryls [155].

Effect of Oxidation on Membrane Bound PKC

The reactive oxygen species (ROS) are created on the membrane surface; therefore, the initial targets of the oxidative substrates are membrane-bound proteins. Protein modifications due to oxidation initiate the signaling processes before a depletion of glutathione occurs; a depletion which will occur later in the process of oxidative damage. Due to the central role of glutathione cellular homeostasis-redox, it is logical to define the way by which glutathione affects the modifications of PKC by oxidation in the cellular environment. The current belief is that PKC experiences the damaging effects of ROS at the scaffold of the cytoskeleton, reacting with membrane-bound proteins and the lipids of the cellular membrane [156–158]. Hence, PKC reacts with lipophilic factors and peroxides and not with the water soluble glutathione of the cytoplasm. Although the redox state of PKC does not seem to be regulated by glutathione, there are indications that glutathione suppresses PKC with a novel, non-redox mechanism [159]. This fact proves that glutathione impoverishment during oxidative stress removes, in an effective manner, the mechanism of negative regulation of PKC and therefore offers an acceptable environment for PKC activity and the promotion of carcinogenesis [160].

Effect of Oxidative Stress on PKC Function

During oxidative stress, the cysteines of different proteins are oxidised irreversibly and this is the first defense mechanism against oxidation. Reducing substances inactivate proteins of great importance by differentiating their regulatory thiols. Protein kinase C is the one and only protein that is activated by oxidation during endogenous oxidative stress. Protein kinase C constitutes a family of corresponding enzymic isoforms, which are related to cell proliferation, differentiation and apoptosis. The isoforms are divided into three classes, and this depends on the cofactors which participate in their catalytic activity:

(i) conventional PKCs which depend on calcium and are stimulated by DAG (diacylglycerol)
(ii) novel PKCs which are not dependant on calcium but are stimulated by DAG
(iii) atypical PKCs which require neither calcium nor DAG for their activation.

Today, there is evidence supporting the view that PKC is a cell-signaling molecule which is activated by oxidative substances (superoxide) and which reacts selectively with specific molecules in the regulating regions. Its action is inhibited by anti-oxidative substances when they react with the catalytic subunits of PKCs.

Phospholipase Activity

During agonist activity, diacylglycerol is initially produced as a result of inositol phospholipid hydrolysis, especially that of 4,5 phosphatidylinositol derived from the activity of phospholipase C [161, 162]. This production of diacylglycerol is naturally transient and momentarily leads to the formation of 1,4,5-triphosphate inositol (IP3). It is often accompanied by a phase of a steady increase of DAG levels [163–173]. This second phase of DAG production is attributed to the hydrolysis of phosphatidylcholine by the stimulated cells. The steady increase of DAG occurs as a response to mitogens and growth hormones [174, 175], phorbol esters [176, 177] and the oncogene Ras [178, 179]. All of these substances induce PKC activation.

Inositol phospholipid hydrolysis by phospholipase C commences with receptor stimulation or with the opening of the Ca^{2+} channels (Fig. 5.3). Initially it was thought that this was the sole mechanism for diacylglycerol production which connected the extracellular signals with the intracellular processes through protein kinase C. Hydrolysis, however, through agonists (signals) of other membrane phospholipids, particularly choline phospholipids, from phospholipase D and phospholipase A_2, seems to be involved in cell signaling. The products from the hydrolysis of these phospholipids promote and lengthen protein kinase C activation. Such a lengthening of protein kinase C activation is substantial for long-term responses, such as cellular proliferation and cellular modification (Fig. 5.3). Phospholipase A_2

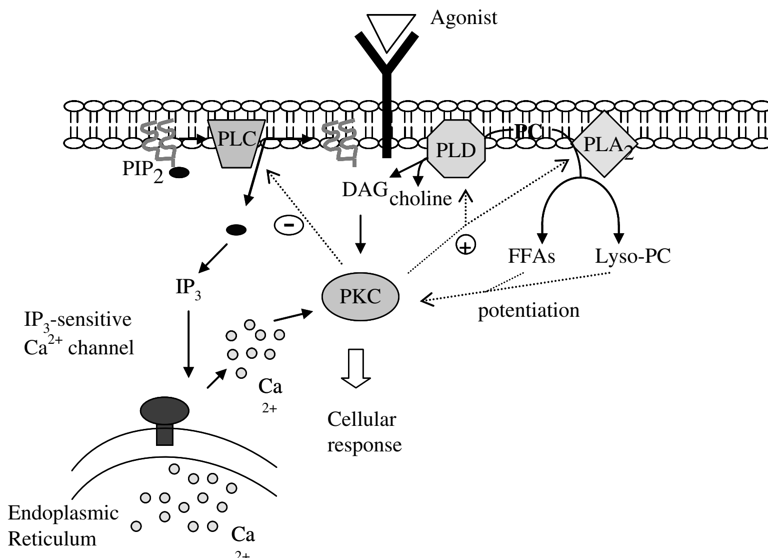

Fig. 5.3 Schematic representation of the degradation of the cell membrane phospholipids, caused by agonists for extended PKC activation. (IP_3 = 1, 4, 5 inositol triphosphate, PIP_2 = 4, 5 bisphosphate phosphatidyl inositol, PC = phosphatidyl choline, DAG = diacylglycerol, FFAs = free cis unsaturated fatty acids, Lyso-PC = lyso-phosphatidyl choline)

is activated by most agonists (hormones, neurotransmitters, growth factors) which induce inositol phospholipid hydrolysis [180–183]. Many *cis* unsaturated lipid acids and lysophosphatidylcholine, products of hydrolysis of phosphatidylcholine by the catalytic activity of phospholipase A_2, support PKC activation, thus contributing to signal relay through the PKC pathway [184–188].

Activation of the Phosphoinositide Pathway from Cigarette Smoke

Acetaldehyde

It has been proven that acetaldehyde, which is contained in significant concentrations in cigarette smoke, damages the mucociliary catharsis of the lungs [189, 190]. From one point of view, acetaldehyde directly obstructs the ciliary function of the bronchial epithelium, producing a slowdown of ciliary beating through the inhibition of dynein- ATPase activity, following linkage to ciliary proteins (considered to be of significant importance for their mobility). These proteins include dinein and tubulin. Reports suggest direct inhibition of ciliary ATPase and adduct formation with ciliary dinein and tubulin [189]. Other researchers suggest that acetaldehyde attains its bioactive result through the formation of covalent adducts with the nucleophilic amino groups of target-proteins [191–193]. This result significantly increases with cyanamide which eliminates acetaldehyde dehydrogenase [194].

Reactive Oxygen Species

The reactive oxygen species in cigarette smoke might also inhibit ciliary function. This is proved by the fact that the vapor phase of cigarette smoke becomes less toxic when introduced to cell cultures in the presence of peroxide dismutase and catalase [195].

Anaphylotoxin C5a

Complement-derived anaphylotoxin C5a is a glycopeptide which induces the secretion of cytokines from human bronchial epithelial cells which have been exposed to cigarette smoke [196]. This glycopeptide is capable of mobilizing cells which have C5a receptors (C5aRs) on their membrane and which induce cytokine synthesis. C5a receptors are constitutively expressed on the human bronchial epithelial cell surface. When these cells are exposed to cigarette smoke they become more active with regard to the secretion of cytokines through C5a, using a mechanism which is mostly unknown. Some researchers believe that there are intercellular signaling processes in relation to the C5a receptors, due to the fact that cigarette smoke increases the responsiveness of the C5a receptors to C5a. The element of intercellular

signaling related to the C5a receptors in lung cancer cells as well as in the epithelial cells of the airway tract is protein kinase C (PKC) [197–199]. As previously mentioned, acetaldehyde activates PKC [200]. Due to the fact that acetaldehyde is a known compound of the gas phase of cigarette smoke, the latter has been regarded as increasing IL-8 secretion in the bronchial epithelial cells through C5a by activation of PKC by acetaldehyde.

Malondealdehyde-acetaldehyde Combination

The low concentrations of acetaldehyde and malondealdehyde which coexist in cigarette smoke, act together and activate PKC in the bronchial epithelial cells [197]. The manner of action of the two aldehydes is through bonding with the amino groups of the nucleophilic proteins, thus forming malondealdehyde-acetaldehyde (MAA)-protein adducts (MAA-adducts) [201]. The MAA-protein adduct stimulates (phosphorylates) PKC and in a small span of 1–2 hours, IL-8 is secreted from the epithelial cells of the airway tract. According to Wyatt et al., free aldehydes are able to induce a severe intercellular signaling effect, whereas aldehyde adducts promote a chronic PKC activation extracellularly through the membrane surface receptors [202]. Protein adducts are linked with receptors and relay information intercellularly through scavenger proteins-receptors, such as protein P-37 (Fig. 5.4) [203–205].

Hydroxyl Radicals

A different interpretation of the DNA damage mechanism through in vitro PKC activation in rats has been proposed by Maehira, et al. [205]. These authors prove that in vitro activation of protein kinase C by reactive oxygen is avoided in the presence of $OH^{\cdot}$ radical scavengers, holding the $OH^{\cdot}$ radicals responsible for the activation of PKC. During the exposure of rats to cigarette smoke, significant changes at an extremely high level were observed in the PKC in cells of the

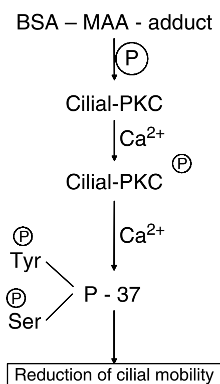

Fig. 5.4 Activation of serine and tyrosine in the P-37 polypeptide by phosphorylated protein kinase C (PKC)

pulmonary epithelium [205]. During chronic exposure of the animals to cigarette smoke, an accumulation of 8-hydroxy-diguanosine in the cells of their airway tract was observed. The authors believe that cigarette smoke causes DNA damage and contributes to the initial activation of PKC and its subsequent negative regulation as an inducer. These researchers also reported serious indications that during rat exposure to cigarette smoke there was 8-OHdG accumulation and activation of K-ras, (which are considered as DNA promoters in cells of the pulmonary epithelium), as well as a prolonged activation of PKC in the cellular cytoplasm.

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Chapter 6
Cell Protection Mechanisms from Oxidation

Definitions and Interpretations

Before analyzing the term oxidation, it is appropriate to define 'reductive and oxidative agents' as well as 'antioxidant and pro-oxidant agents'.

Reductive and Oxidative Agents: Definition and Interpretation

The addition of electrons to a chemical compound is defined as reduction while the loss of electrons from the compound is termed oxidation. The reducing compound renders electrons and thus causes the reduction of another compound. The oxidative substance accepts electrons and causes oxidation of a reducing substance. It is not possible to have oxidation in a system without simultaneously having a reduction. For example, when a Na atom transports an electron to a CI atom to form NaCl, Na is oxidized to Na^+ (Na-e$^-$ $\rightarrow$ Na^+ $=$ oxidation), while Cl is reduced to Cl^- ($\mathrm{Cl} + \mathrm{e}^- \rightarrow \mathrm{Cl}^-$ $=$ reduction). In this reaction Na is the reductant and Cl the oxidant. When reduction and oxidation characterize a chemical reaction, it is called a redox reaction. A redox reaction is the center of biological oxidation in the chain of chemical reactions, where the oxygen from the air is used to oxidize chemical substances from the breakdown of food substances and to supply energy for life.

Antioxidant and Pro-oxidant Substances: Definition and Interpretation

'Reductive' and 'oxidative' are chemical terms, while 'anti-oxidant' and 'pro-oxidant' are biological terms. An anti-oxidant is the substance which, when it exists in low concentration, (in comparison to the concentration of a prospective substrate for oxidation), either prohibits or delays a pro-oxidative initiation of the oxidation of the substrate.

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A pro-oxidative substance is toxic and it can cause oxidative damage to the lipids, proteins and nucleic acids, leading to pathological conditions. Pro-oxidants are synonymous to potent substances and therefore are chemically oxidative in a pathological sense. These compounds can drastically reduce another pro-oxidant substance, so that the final product has low toxicity.

Every anti-oxidative substance is reductive whereas the reductive substance is not necessarily anti-oxidative.

A pro-oxidative substance causes oxidative damage (to the substrate) and this damage can be inhibited by the presence of anti-oxidants. These inhibitions are measured and relate to the presence of an anti-oxidant. The measured anti-oxidative capacity can have physiological importance, because the pro-oxidative substance used in these systems is highly significant from a pathological point of view.

The existence of various damaging pro-oxidants in vivo, such as the ROS ($O_2^{-\cdot}$, H_2O_2, ROO^- and OH^+), makes anti-oxidants important for a healthy way of life. Therefore, the substances that are identified with inhibitive methods are antioxidative and they can reduce the pro-oxidants and protect the substrate from oxidation. These anti-oxidants are, of course, reductive agents.

Antioxidative Substances

Human cells use great number of antioxidants (Table 6.1) which react directly with oxidative agents and neutralize them.

These substances are called ‘scavengers’ and they are self-destructive. Vitamin C is the largest water-soluble antioxidant, while vitamin E is the major membrane-bound lipophilic antioxidant [1, 2].

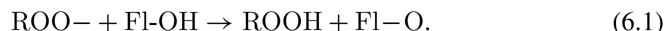
The ß-carotins and the ubiquinones are lipophilic antioxidants [3–6]. The hydrophilic agents are uric acid, glutathione and ceruloplasmin [7–10]. Uric acid is the final metabolic product of purines and it acts as a self-destructive antioxidant as well as a chelator of transition metals [7–9]. Uric acid binds to iron or copper and inhibits the oxidative reactions that are catalyzed by the metals without itself being oxidized [9]. The facile manner in which the transition metals act as oxidative reductive catalysts, makes them ideal components in many antioxidative enzymes where their position is functionally significant. Different members of the hyperoxide dismutase family use copper, zinc, or manganese as active position catalysts [11, 12]. Most glutathione peroxidases use selenium as an active position ‘catalyst’ [13].

Table 6.1 Antioxidative substances of the cell

Selenium	Ceruloplasmin
B-carotin	Vitamin C
Ferritin	Ubiquinone
Glutathione	Zinc
Uric acid	Copper, Iron

Antioxidative Substances Which the Cell Acquires from the Environment

The most common potent antioxidative substances which exist in nature are the flavonoids, which are active in both the hydrous and lipo systems [14]. The protective mechanism of flavonoids is of great interest. As multi-phenates, the flavonoids have the capacity to act as antioxidants, with free radical clearance mechanisms [15–17] to form less active flavo-phenoxyl radicals.



$$\mathrm{ROO-} + \mathrm{Fl\text{-}OH} \rightarrow \mathrm{ROOH} + \mathrm{Fl-O.} \quad (6.1)$$

$$\mathrm{HO-} + \mathrm{Fl\text{-}OH} \rightarrow \mathrm{H_2O} + \mathrm{Fl-O.} \quad (6.2)$$

On the other hand, these substances with the capability of binding transition metals, [18–21] inactivate iron ions thus inhibiting Fenton's reaction (Eqs. 6.3, 6.4) which is believed to be the most important path for the production of ROS.

$$\mathrm{O_2} + \mathrm{Fe(III)} \rightarrow \mathrm{O_2} + \mathrm{Fe(II)} \quad (6.3)$$

$$\mathrm{Fe(II)} + \mathrm{H_2O_2} \rightarrow \mathrm{Fe(III)} + \mathrm{HO^-} + \mathrm{HO^{\cdot}} \quad (6.4)$$

The antioxidant capacity of flavonoids depends mostly on their capability to provide hydrogen atoms and to bind the free radicals that are formed during lipid peroxidation.

The substitution of the ß-ring seems to be the most significant factor that contributes to the antioxidative effect of flavonoids. The hydroxyl groups enhance the antioxidative effect while the methoxy groups suppress it (Fig. 6.1). The presence of O-dihydroxy-substitution on the flavonoids promotes the antioxidative effect. A hyrdoxyl group in position C^3 enhances the capacity of the flavonoids to inhibit lipid peroxidation.

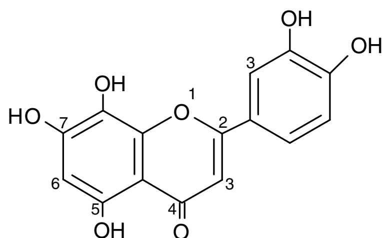


Fig. 6.1 The hydroxyl groups strengthen the antioxidant capacity of flavonoids

Antioxidative Effect and Toxicity of Flavonoids

Flavonoids are derivatives of diphenyl propane and include flavonols, flavonons, anthocyanidines and flavones. More than 4000 flavonoids are present in plants, fruits and vegetables. The vegetable flavonoid compounds play an active chemo-protective role against cancer. This beneficial result is due to their antioxidative action [21–23]. However some flavonoids are known to be mutagenic [24, 25] and co-carcinogenic [26, 27].

These detrimental mutagenic and co-carcinogenic actions are attributed mainly to the pro-oxidative action of flavonoids which, under some conditions create free radicals, (for example, in the presence of some metal ions, [28–32] such as those that exist in cigarette smoke).

Some phenols such as catechols, hydroquinones and catecholamines have been characterized as mitogens in *E. coli*, in the absence of the OxyR protein [33]. This protein is sensitive to reduction and regulates genes which encode antioxidative enzymes such as the catalase and the reductase of akyl-hydroperoxidase [34, 35]. Therefore, cells poor in the OxyR protein have decreased protection against oxidative stress. The oxidation of phenol substances and the conversion of quinones are catalyzed by nitrogen-active substances (NO, $ONOO^-$, NO_2). Substances poor in electrons, such as quinines, are capable of reacting with cellular nucleophilic substances (proteins, DNA) and they create toxicity via different pathways [36].

Flavonoids have an ortho-trihydroxyl group, (in the ß-ring) and they can cause breakages to single-strand DNA in the presence of NO. The pro-oxidative action of some flavonoids, in the presence of NO, is perhaps due to different active forms of nitrogen such as peroxynitrite, nitroxyl and NOx which are formed from the reaction of NO with the quinone/semiquinone/hydroquinone system.

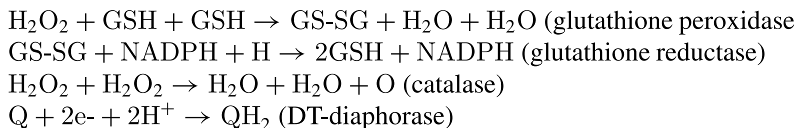
Antioxidative Enzymes and Proteins

The greatest discovery in the field of free radicals, is the family of enzymes called superoxide dismutases (SODs).

There are three kinds of superoxide dismutases in the human organism: SOD1 (known as cuzn-SOD), SOD2 (known as Mn-SOD) and SOD 3 (known as ec-SOD). SOD1 is an endoplasmic enzyme, SOD2 mitochondrial and SOD3 extracellular. A great number of other agents participate in the defence against ROS and in the consequences they cause in various cell functions. These agents are the antioxidative amino acids (e.g. arginine), the thiols (especially glutathione) the polyphenols of tea, the enzymes bound to metals (e.g. selenium and zinc) and the antioxidative enzymes (e.g. glutathione reductase and glutathione peroxidase).All of the above agents play an important role in the prevention and reconstitution of the damage to amino acids (proteins) and lipids [37].

Superoxide dismutase catalyses the reaction $O_2^{-\cdot} + O_2^{-\cdot} \rightarrow H_2O_2 + O_2$ and all of the members of this family use transition metals at their reactive site [12].

The genetic deletion of SOD is a fatal mutation in lower organisms, [12] a fact that strengthens the importance of this family of antioxidative enzymes. The final product of SOD is H_2O_2 which is toxic and must be removed quickly. In mammalian cells, this is accomplished by two families of enzymes: the glutathione-peroxidases and the catalases (Fig. 6.2).



$H_2O_2 + GSH + GSH \rightarrow GS\text{-}SG + H_2O + H_2O$ (glutathione peroxidase
$GS\text{-}SG + NADPH + H \rightarrow 2GSH + NADPH$ (glutathione reductase)
$H_2O_2 + H_2O_2 \rightarrow H_2O + H_2O + O$ (catalase)
$Q + 2e\text{-} + 2H^+ \rightarrow QH_2$ (DT-diaphorase)

Both, the glutathione peroxidases [13] and the catalases [38] render H_2O_2 non-toxic reducing it to H_2O and O_2 (Fig. 6.2). Glutathione peroxidases use the glutathione-reducing properties (GSH) in order to render H_2O_2 nontoxic [13]. The cysteine sulphydryls of GSH provide the reducing equivalents that are required for glutathione-peroxidase activity. The enzyme, glutathione-reductase, uses NADPH for reducing one GS-SG molecule to produce two GSH molecules, thus enabling the glutathione peroxidase reaction to continue (Fig. 6.2). In most mammals, catalase is found solely in peroxisomes bodies where their distinct function is to eliminate the H_2O_2 produced by the oxidation of long-chain fatty acids [39]. Since catalase is not present in the cytoplasm of mammalian cells and since the diffusion of H_2O_2 from the cytoplasm to the inner part of the peroxisomes is not possible, it is most likely that the glutathione-peroxidases will neutralize the cytoplasmic H_2O_2.

Another important antioxidative enzyme is DT-diaphorase which is called quinone reductase [40]. DT-diaphorase directly catalyses the bivalent reduction of many dihydroquinones to dihydroquinols: $Q + 2e^- + 2H \rightarrow QH_2$.

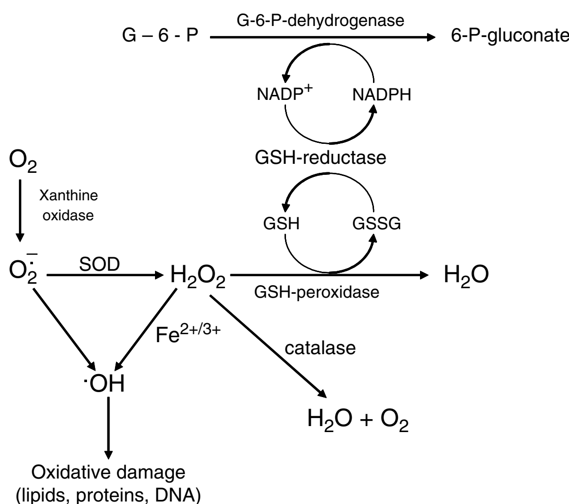


Fig. 6.2 Oxidative mechanism of the cell

The catalysis of a direct reduction of electrons of quinone substrate, the DT-diaphorase obviates the production of effective semiquinone radicals.

Reduced Glutathione in Broncho-epithelial Cells

Reduced glutathione (GSH) is a tripeptide with three amino acids (L-glutamine, cystein and glycine), and it contains sulphydryl groups. Its biosynthesis is shown in Fig. 6.3. It is an extremely protective antioxidant against oxidative and electrophilic compounds and it is the key-substance of the cell oxidoreduction mechanism. Its reducing property is of great significance in various biological functions such as: transcription activation of specific genes, signal transduction, cell proliferation, apoptosis and inflammation which are regulated by the reductive state of the cell [41–43]. The antioxidative effect of glutathione plays a very important role with regard to the antioxidative defence of the lung tissue, particularly in protecting the air-duct epithelium from oxidative damage by tobacco smoke components and from inflammation in general [44–46]. For example, glutathione is reduced in the mucous lining of the lung epithelium in smokers and in idiopathic pulmonary fibrosis [47–49].

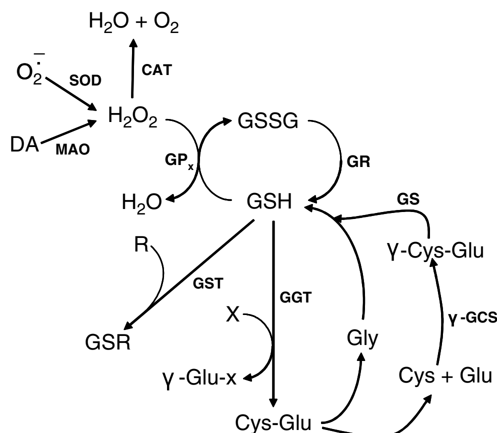


Fig. 6.3 Shows the GSH biosynthesis and its metabolic pathway. The γ-carboxy group of glutamic acid (Glu) is bound covalently with cysteines (Cys) by γ-glutamyl cysteine synthease (γ-GCS) and with glycine (Gly) by glutathione-synthease (GS) for the production of the reduced form of glutathione (GSH). GSH can also be used in the reaction with H_2O_2, in a non-enzymatic way, or in the presence of glutathione peroxidase (GPx). The oxidized GSH (GSSG) reproduces GSH with the aid of the glutathione reductase (GR). Glutathione-S-transferase (GST) catalyses the reaction of GSH with various substances. γ-glutamyl-transpeptidase (GGT) binds the glutamylic group to different substrates (X), including some peptides, amino acids, water and even GSH. Among other sources H_2O_2 can be produced intracellularly by the superoxide ion, through superoxide dismutase (SOD) or dopamine (DA) by mono-amino oxidase (MAO). H_2O_2 can also be detosified by the catalase (CAT)

The reactive oxygen species (ROS), (one of which is super-oxide anion [$O_2^{-\cdot}$], which is produced by phagocytes accumulated at the site of inflammation), are of major importance to the cell and tissue damage [50, 51]. Superoxide radicals are removed from the cell by superoxide-dismutase (SOD) which uses two superoxide radicals and two protons for the production of hydrogen peroxide, a less effective substance.

Hydrogen peroxide is then converted, by catalase, to water and oxygen. There is superoxide dismutase activity in the mitochondria, the cell nucleus and the extracellular matrix.

Glutathione-reducing homeostasis controls the level of ROS which is continuously created during cell metabolism. Lung cells are sensitive to the damaging effect of oxidants. They release cytokines such as TNF-α, IL-1 and IL-8 in response to oxidative stress, [52, 53] resulting in the development of serious chronic lung diseases.

Since they are sensitive to the reductive agents, the transcription factors NF-kB and reactive protein-1 (Ap-1) as well as c-Fos/c-Jun, play a key role in cytokine gene transcription and the transcription of other genes that regulate the protective action of antioxidants in a positive manner.

Smoking and the Protective Role of Reduced Glutathione

Smoking is associated with a high human health risk because it causes, among other effects, oxidative stress. Oxidative stress is associated with a reduction of antioxidants, protein alteration, DNA damage and down-regulation of the systems that depend on endogenous glutathione [54–58]. Glutathione is a major antioxidant which is used for the removal of peroxides and for maintaining the good condition of the antioxidants [59]. The ranges of the constant relation of GSH to its oxidized bisulfide form, are expressed as a Redox ratio of the GSH/GSSG pair. Cigarette smoke contains substances such as acrolein which react with glutathione [60], as well as the reactive oxygen species (ROS) which oxidize glutathione in GSSG.

The exposure of different cells to tobacco smoke causes a fast reduction of the intracellular concentration of GSH [51, 61], and this reduction is in accordance with the activation or the toxicity. Exposure to cigarette smoke increases the permeability of the epithelial and endothelial cells and this permeability is inhibited by GSH [62, 63]. Moreover, exposure to cigarette smoke inactivates many enzymes that contain thiols such as glyceraldehyde triphosphate dehydrogenase, creatine kinase, plasma paraoxanase and acyl-transferase of the lecithin cholesterol. In most cases GSH is capable of avoiding these inactivations [64–67]. Finally, exposure to cigarette smoke reduces the chemotaxis of the phagocytes and promotes the emission of pro-inflammatory cytokines. GSH is a major substrate of glutathione-S-transferase (GSTase) and it is depleted very quickly when GSTase eliminates the toxicity from the xenobiotic substances of cigarette smoke. The gas phase of tobacco

smoke contains a large number of drastic substances such as 10^{14}–10^{16} alkyl, alkoxy and peroxy-radicals per puff [68], up to 1000 ppm of NO [69], as well as high levels of drastic aldehydes, like acetaldehyde, formaldehyde, acrolein, crotonaldehyde and propanol [70], which are considered to be responsible for cell damage by cigarette smoke because they cause the depletion of thiols [71, 72].

Glutathione Transporting Pumps (GSSG) During Oxidative Stress

Some cells or tissues are able to release GSSG as a result of the hydroperoxide activity in the cytoplasm or during oxidative stress. This release shows that the path of GSH reductase is insufficient. Some substances are coupled with S-glutathione and are released into the extracellular environment. This emission depends on the presence of ATP and is promoted by some cell membrane glycoproteins that belong to the family of multi-drug resistance proteins (MRP) (Fig. 6.4). MRPs are transporting proteins that transport lipophilic components coupled with glutathione when oxidative mechanisms develop in the cell. These lipophilic components are substrates for MRP transporting pumps, when coupling promotes their transportation through the cell membranes. MRP transporting proteins play an important role in the control of the level of GSH while the transportation of GSSG to the extracellular environment serves as a compensatory mechanism of oxidative stress when GSH-reductase potential exceeds its limits. The coupling of lipophilic substances with glutathione and their transportation to the extracellular matrix, with the aforementioned pumps, is a process of major importance in reducing the implications by oxidative stress and cell homeostasis.

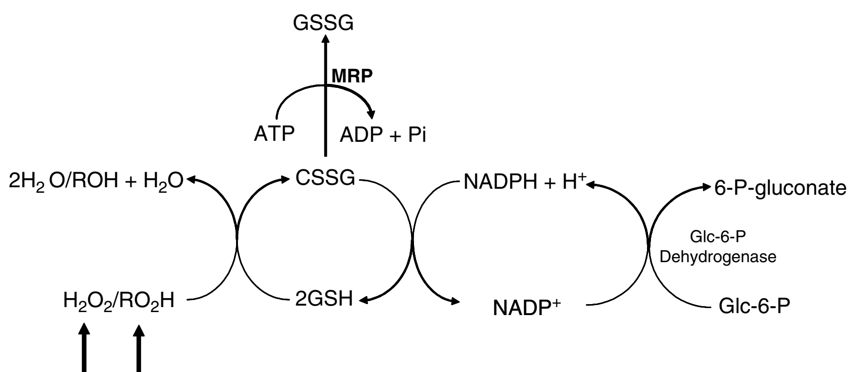


Fig. 6.4 Transportation of glutathione bisulfide to the extracellular matrix by MRP protein pumps. GSSG is the substrate of MRP proteins. Under conditions of oxidative stress the reduction of GSSG by glutathione reductase is problematic. This leads to the exodus of a great amount of GSSG from the cell

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Chapter 7
Role of DNA Adducts in Carcinogenesis

Central Role of DNA Adducts in the Mechanisms of Carcinogenesis

Most of the carcinogens in tobacco smoke products demand metabolic activation before reacting with DNA, although some, such as ethylen-oxide, formaldehyde and acetaldehyde, react directly with DNA [1]. The metabolic activation starts off from the endogenesis of cytochrome P450 which, in mammals, constitutes part of the normal system designed for responding to substances foreign to the human organism. Most of the metabolites which ensue from the reaction catalysed from P450 are excreted from the kidneys, and are devoid of toxicity; some metabolites are electrophiles which react with DNA-forming adducts [2–5]. The reconstitutive systems of the cell eliminate the adducts by restoring the DNA structure to its normal condition [6–9]. However, if the adducts are not affected and escape the restorative process, mutations may ensue. At present, there is conclusive evidence that DNA adducts due to cigarette smoke carcinogens can induce miscoding, and very often, the G-T and G-A mutations [10–12]. If these permanent mutations happen in regions of critical importance, like those of ras and myc, or in tumor suppressor genes, like TP53 and CDKN2A (which code P16) the result will be a loss of the normal control mechanisms of cell development, causing cancer [13]. A few cells which have experienced permanent damage are eliminated though apoptosis, a cell protection mechanism. Of course, the carcinogens contained in cigarette smoke, as well as their metabolically-activated forms, induce mutations by forming DNA adducts. These mutations are associated with a disarray of the checkpoints of the cell cycle, with chromosomal instability and other important damage [13]. The central role of DNA adducts in carcinogenesis has been recognised world-wide and has been established as a biological incident [14].

The metabolic formation, activation and elimination of the DNA adducts takes place continually, with the final result, the achievement of equilibrium/stability. Long-lasting DNA damage is compatible with multiple genetic alterations noticed in the lung tissue, while normal tissues proceed from hyperplasia to dysplasia, to cancer in situ and to infiltrative cancer [7, 15, 16].

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Endogenously-produced DNA Adducts

Further to exposure to the exogenous electrophile substances, the mammalian genome is also affected by substances which react endogenously with DNA. During normal cell function, very active electrophile substances which do not require metabolic activation, are produced. Endogenous damage of DNA is caused by DNA instability or by an error during replication and reconstitution of the base damage due to oxidation or to adducts originating from the reaction of bases with the aldehydic lipid peroxidation products. Lipid oxidation of the cell membrane leads to the production of a large number of aldehydes.

The three main classes of adducts derived from aldehydes are: the malondealdehyde adducts, the propano adducts from acrolein and crotonaldehyde and the ethino-adducts, from aldehyde epoxides [17, 18]. The master malondealdehyde adduct is pyrimidinopurine (MIG) which can be found in the human liver, white blood cells, pancreas and breast. The MIG levels are 1–120 adducts per 1080 nucleotides [17]. The acrolein and crotonaldehyde adducts (Acr-dG and Cro-dG, respectively) are detected in mice and humans. Exhaustion of endogenous antioxidative glutathione following lipid peroxidation in old age, lead to increased levels of acrolein and crotonaldehyde adducts in rat liver DNA [19, 20]. This shows that these adducts may originate endogenously. Since acrolein and crotonaldehyde are environmentally-polluting substances derived from cigarette smoke and car emission chemicals, we presume that Acr-dG and Cro-dG, detected in the human body are produced from both exogenous and endogenous sources. Aldehyde epoxides are sources of another class of adducts, the ethino-adducts, such as 1,N-ethino-deoxy-adenosine (ethino-dA) and 3,N4-ethino-deoxy-cytidine (ethino-dC).

DNA Adducts and Nitrosamines

Oncogenesis and Metabolism of 4-(methyl-nitrosamino) -1-(3-pyridil)-1-butanol (NNK) and its Metabolites

NNK is a strong carcinogenic substance, which is formed from nicotine during the tobacco leaf process [21, 22]. Smokers are exposed to large quantities of such substances which are contained in cigarette smoke, and which play an important role in the etiology of carcinogenesis associated with the use of tobacco products [21–30]. NNK is a strong lung carcinogen in mice [21–24]. During its metabolic process, it is immediately and very quickly converted to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in mice and in humans. NNAL is also a very strong carcinogen in mice and in rats. The metabolism of NNK and NNAL is summarised in Fig. 7.1.

NNAL is converted to its O-glycuronide [4-(methyl-nitrosaminol-1-(3-pyridyl) but-1-yl] β-O-D-glucosiduromic acid (NNAL-Gluc), which is detected together with NNAL in the urine of all those who are exposed to cigarette smoke [30–37]. The pyridine-N-oxidation of NNAL and NNK leads to NNAL-N-oxide and

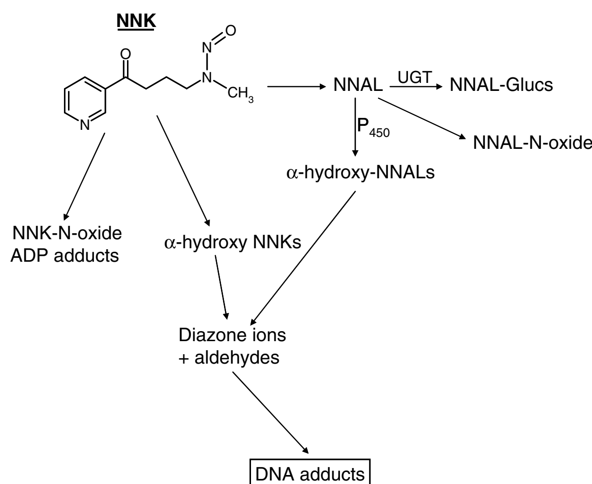

Fig. 7.1 Metabolic activation of NNAL and NNK

NNK-N-Oxide, respectively. Furthermore, NNAL and NNK can form adducts with ADP, in vitro [38, 39]. The metabolic activation of NNAL and NNK can take place during hydroxylation of the carbon atoms in the N-nitroso-groups producing intermediate compounds which are associated with DNA and proteins. a-hydroxylation of the NNK methyl-group produces 4(3-pyridyl)-4-oxobutano-1-diazohydroxide (like pyridyl-oxobutilate DNA). a-hydroxylation in the methylene group leads to methane-diazo-hydroxide which subsequently methylates DNA. In a similar manner, some intermediate substances (pyridyl-hydroxybutylate and methylate DNA) are produced, due to the a-hydroxylation of NNAL. In the DNA tissue analysis of rats in which NNAL was supplied, the existence of pyridyloxobutylated and methylated DNA has been observed, which indicates the formation of DNA adducts. Adducts which originate from NNK and from tobacco-specific nitrosamine NNAL are found in high levels, in the lung tissue of patients with lung cancer, as compared to controls [40]. The most frequent form of lung cancer in rodents exposed to cigarette smoke and/or nitrosamines, is adenocarcinoma versus exfoliative cancer [24, 41]. NNK and some other nitrosamines in cigarette smoke are very active carcinogenic substances such as furan in the liver tissues in rats [42, 43]. NNK and its major metabolite NNAL are the only cigarette smoke carcinogens related to the pancreas [44, 45].

Apart from the mechanisms of carcinogens due to DNA-adducts, there are some other mechanisms which act through the receptors on the cell surface. Nicotine, NNK and possibly NNAL are associated with nicotinic-cholinergic and with certain other receptors which activate kinase AKT (protein-kinase B) and protein-kinase A [46, 47]. This activation leads to apoptosis, increased production of arachidonic acid metabolites, increased cell proliferation and transformation. Gene deactivation through the promoter hypermethylation, is an important cigarette smoke

mechanism of carcinogenesis [48]. Recent studies have shown that the inactivation of the tumor suppressor gene CDKN2A and some DNA repairing genes, such as O^6-methyl-guanine-methyl-transferase are often detected in micro-cell-lung carcinomas [49].

DNA Adducts and Lipid Peroxidation

DNA adducts are also formed from the active derivatives of lipid peroxidation [50]. Hydroxy-alkenales such as trans-4-hydroxy-2-nonenale (HNE), produced during lipid peroxidation, react with the nucleic acids, forming DNA adducts by epoxidation: the 1,N^6-ethino-deoxy-adenosine (EdA), 3,N^4-ethino-deoxy-cytokine (EdC) and 1,N-ethino-deoxy-guanosine (edG) [51]. Ethino-adducts are formed during superoxidation of arachidonic acid and also, in the microsomal membranes with participation of iron ions (II), or coumenic hydroxy-peroxide [52]. The increased levels of ethino-adducts (e-adducts) are due to well known risk factors for cancer development, and there is a progressive increase of these adducts during pre-neoplasias, in patients predisposed to cancer, suggesting that these pro-mutagenic damages may lead cells to malignancy. The pro-mutagenic adducts are the ruling index of oxidative stress (lipid peroxidation) for DNA damage. Peroxynitrite plays an important role in DNA modifications, particularly in ethino-adduct production during lipid peroxidation. Long-standing oxidative stress induced either by exogenous factors such as smoking, or by superexpression of the “emergency” enzymes, is an important event in cancer development. Ethino-adducts are the key-index for the increasing load of DNA oxidative damage, which subsequently may increase the gene mutation rate, related to cancer and to gene-instability. Ethino-adducts constitute biomarkers which are very useful in determining the role of the oxidative stress and lipid peroxidation in human cancer.

Aromatic Amines and Amides

Aromatic amines and amides are well known environmental polluting compounds [53]. A large number of these compounds (about 12) are found in tobacco products and tobacco smoke [54]. Aromatic amines are carcinogenic substances in experimental animals and humans, are for the most part, metabolically transformed before reaching the DNA and forming adducts. DNA purines are the main targets of the aromatic amines. The reaction between purines and aromatic amines usually takes place at atom C^8 of Gua and rarely at the exo-cyclic aminofunctional positions (AF) [55]. Oligonucleotides transformed by aromatic amines are structured inside viruses or in the plasmid genome and used in studies of mutagenesis. In in vitro studies it has been shown that when oligonucleotides include C^8-Gua-AF, the DNA-polymerase can bypass the damage [56]. The incorporation of the oligomer creates a whole (100%) mutation provided both DNA double bands are being altered. The explanation for this phenomenon is that the natural immediacy of the already

mentioned oligonucleotides, bypasses the repairing excision. Mutations produced in the system C^8-Gua-AF are exclusively deletions (predominantly of the one base).

O^4-alkyl-thymines

The formation of O^4-alkyl-thymines, plays a role in mutagenesis from alkylating agents and probably carcinogenesis [57–59].

Cyclic Adducts of the Nucleic Acids

Certain chemical compounds are able to form covalent bonds in two atoms of the same base, and create cyclic adducts [60], such as certain bicarboxylate compounds, (i.e. glyoxal methylate and malondealdehyde) and also a,b,-unsaturated carbonyls (such as acrolein, crotonaldehyde, alogens and their metabolites). The carbamidic esters, are included in this group. A large number of these substances are contained in the gas phase of cigarette smoke and they are mutagenic and some are carcinogenic [60].

Significance of the Adduct Determination

DNA adducts are well known as active biological markers which express exposure to carcinogens and also susceptibility to cancer [61, 62]. In general, the detection of total adducts in DNA is considered as a strong marker of genotoxicity. Even if only certain of these adducts exhibit special interest in mutagenesis (i.e. O^6-adducts of guanine), their overall determination (calculation) can be correlated with the quantity of the most important adducts, which constitute reliable biomarkers of genotoxicity. When circulating lymphocytes are exposed to substances which enter the human body through inhalation, DNA adducts are created and these reflect the DNA adduct load of the whole body but not the DNA load of a particular organ [63]. In other words, the DNA load in lymphocytes is the same as that of the lung tissue [64]. Lymphocytes are the appropriate tissue for the study of DNA reconstitution capacity. Thus, the prospective of cancer development in smokers can be estimated. In experimental animals there is a strong correlation between different classes of carcinogens, such as nitrosamines, aromatic amines, heterocycle amines and polycycle aromatic hydrocarbons, and the levels of DNA adducts [65–67].

Cancer Biomarkers

Cancer biomarkers can assist in the prediction of cancer in a smoker. There are three categories of cancer markers: DNA adducts, protein adducts and metabolites excreted in the urine [68–73]. The levels of the biomarkers increase in those individuals who are exposed to tobacco products.

Among the cancer biomarkers, DNA adducts offer a prompt connection with cancer. Protein adducts are alternatively useful as compared to DNA adducts. Urine metabolites are probably the most practical biomarkers since they offer interesting data about the quantity and metabolism of the carcinogen.

Protein Adducts

When the measurement of hemoglobin-carcinogen adduct takes place instead of DNA-adduct, it determines the exposure levels to carcinogen substances, since most of metabolites that react with DNA, also react with proteins [74, 75]. The advantages of hemoglobin-adducts are the easy availability of large quantities of hemoglobin from the peripheral blood and the relatively long life (120 days) of human red blood cells which accommodate the aggregation of the adducts. Hemoglobin adducts with aromatic amines are a decisive form of carcinogic biomarkers [76]. Adducts which are formed with the amino-terminal valine of hemoglobin are disclosed in relation to the carcinogen dose taken by smokers [77, 78]. Examples of great importance constitute adducts due to ethylene oxide (Fig. 7.2), acrylonitrile and acrylamide [79, 80]. Ethylation of the hemoglobin amino-terminal valine is increased in smokers as compared to non-smokers [81]. The levels of ethyl-thymidine are higher in the smoker's lung DNA as compared to non smokers, as are the levels of ethyl-adenine in the urine.

These findings show that tobacco smoke contains some other ethylating agent, that has not yet been identified [82–84].

Excretion of Metabolites in Urine

Biomarkers of the urine offer information about the carcinogen dose and also the metabolic activation of the carcinogens. The t,t-myconic acid (benzene metabolite), 1-hydroxypyrene (marker of DAH intake), NNAL and its glucuronides

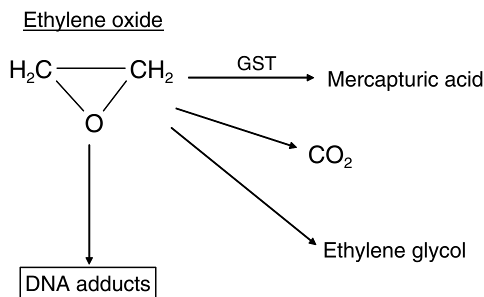

Fig. 7.2 Ethylene oxide is a nucleophilic carcinogenic substance which creates DNA adducts. When joined with GSH it is excreted in the urine as mercapturic acid or as further degradation products

(NNAL-Glucs) which are NNK metabolites, are extremely useful because of their presence exclusively in tobacco products (NNK is a specific tobacco carcinogen) and because they exhibit a high degree sensitivity. Another advantage is the relatively long life of the NNAL and NNAL-Glucs as compared to other metabolites.

Other Biomarkers

Different carcinogens or their metabolites, such as benzene, cadmium and NNAL, are increased in the smoker's blood. F2-isoprenes, which are biomarkers of oxidant damage are also increased in the smoker's blood [85]. Benzene and many other volatile organic compounds have been measured quantitatively in inhaled air [86].

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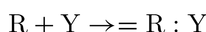
Chapter 8
Transmolecular Reactions of Genetic Toxicity: Alkylation and Methylation of DNA

Nucleophilia and Genotoxicity of Some Chemical Substances

Genotoxic activity of a chemical substance can be defined as the activity of this substance on a particular nucleophilic DNA group [1]. Nucleophilia is essential but not a prerequisite for a substance to be characterised as genotoxic or carcinogenic. Alkylation, for example, of the most DNA-nucleophilic center, guanine N-7, does not participate in mutagenesis or carcinogenesis, while alkylation of some less active groups such as guanine-O^6, can induce genetic alterations. Almost all well known genotoxic factors are nucleophilic or they are metabolically or chemically transformed in vivo to nucleophilic active substances. Most of these factors are nucleophilic alkylating agents, even if there are some other different groups of nucleophilic substances which are not mutagenic or carcinogenic. When a nucleophilic substance reacts with a nucleophilic atom, a new covalent bond is created by the uncoupled electrons of this nucleophilic substance:



$$R + Y \rightarrow = R : Y$$

The principle nucleophilic centers in biological macromolecules are thioles and thioethers, nitrogen atoms in the amino groups, rings and oxygen atoms. The formation of alkylating agents is related to genetic toxicity, based on the kinetics of alkylating and mutational results. Amino acid alkylation (i.e. histidine and cysteines) of the hemoglobin molecule can be used accurately as a control of exposure to the alkylating agents. Alkylation of the macromolecules is a useful final point of qualitative detection of genetic toxicity.

Alkylating Agents

Every alkylating agent is at once a mutagenic substance. In general, alkylating agents are mutagenic, carcinogenic or both, in a large number of pro- and eukaryotic organisms. Even if the existent molecular mechanisms in mutagenesis are not well known, there is strong evidence that mutations due to base substitution originate

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from the alkyl-DNA-adducts which misguide incorporation of the nucleotides during DNA replication [2]. O^4-methyl-deoxythymine (O^4-Medt) is an important intermediary substance of mutagenesis [3–7] and carcinogenesis [8, 9], produced by the nitroso-alkylating agents. From a theoretical point of view, alkylation at the position O_4 of thymine disrupts the base hydrogen bonds in favour of the formation of a couple of O^4-alkyl-T-G bases, leading to a mutational AT $\rightarrow$ GT transversion [10]. Furthermore, it has been shown that quite often DNA-polymerases incorporate dG against O^4-alkyl-dT adducts in synthetic polynucleotides in vitro. In Escherichia Coli and in the presence of nitro compounds [11] in the induction of AT $\rightarrow$ GC transversions, there is indirect evidence that O^4-alkyl adducts possibly play an important role in mutagenesis in vivo. Today it is well known that O^4-Medt adducts are mutagenic and play this role together with the alkylating agents and that O^4-Me nucleotides induce AT $\rightarrow$ GC transversions, while O^6-Me dG induce GC $\rightarrow$ AT transversions [12]. Thus, it has been shown that both adducts contribute to mutagenesis but O^6-Me-dG makes a major contribution [7, 11].

DNA Methylation

The role of DNA methylation in the promoter's regional area is important for normal cellular function and oncogenesis. For this reason, it is interesting to know the responsible mechanisms for alterations in the broncho-epithelial cells when they exist in a sensitive regional area of the genome, due to cigarette smoke.

The main targets of DNA hypermethylation are the non-methylated CpG islets which are located in the gene promoters. The dense methylation which exists on both sides of the CpG islets protects them from methylation. In neoplastic cells, this protection is lost because of long lasting exposure to the increased activity of DNA-methylation by DNA methyl-transferase (DNA-MTase) and also because of the mis-regulated regional protective mechanisms. Gene supermethylation may take place following the start of the neoplastic process. The dominating function of DNA methylation in mammals facilitates genome organisation to active and inactive regions for gene transcription. There are two forms of DNA methylation:

1) methylation de novo, which is established during embryogenesis
2) methylation which develops in some regions where DNA has been differentiated into adult cells [13–15].

In vertebrates, DNA-MTase catalyses de novo methylation and intervenes in maintaining the established model of DNA methylation. Studies in homozygous deletion of the DNA-MTase in mice during embryogenesis have shown that this enzyme with modified methylation is of vital importance for foetal development and survival [16]. DNA-MTase in vertebrates has an increased preference for semi-methylated DNA but not for non-methylated DNA [17]. This property of the DNA-MTase offers protection from the installation of irrelevant methylated regions

in DNA adult cells, particularly in the non-methylated CpG regions, and it may facilitate the maintenance of the normal regions of methylation. During DNA replication, DNA-MTase is able to recognise the normally methylated CpG regions and to catalyse the addition of one methyl group to cytosine in the corresponding CpG region.

Methylation in Eukaryotes

If the major role of DNA methylation in higher eukaryotes is to help chromatin organisation in the transcriptional active and inactive regions, then, the distribution of candidates for methylation CpG sites is as important as the role of DNA-MTase activity. During evolution of the DNA space, the CpG series is progressively limited in genomes so that in humans, as well as in the other eukaryotes, this binucleotide exists at a small ratio (5–10%) of its originally anticipated incidence [18, 19]. Most of the lost CpG regions represent a transversion by deamination of the methyl cytosine to thymines [18, 19]. In humans as well as in vertebrates with complex genomes, the remaining CpG regions contain methylated cytosines at a proportion of 70–80% [18, 19]. These methylated regions constitute the main part of chromatin which is a section of the non transcribing DNA and the main body of a large number of genes. The chromatin model, related to methylated DNA, represents a replicating DNA which is relatively inaccessible to the transcription factors [20]. Smaller DNA regions named CpG islets which appear every 100 Kb, include an expected number of non-methylated CpG at the expected frequency, associated with the 5′-adjusting gene regions. This means that the maintenance of the CpG frequency is essential for the gene transcription process. Research in the area of the specific mechanisms which protect the CpG islets from methylation has only just begun. It is extremely important that these DNA methylation changes in cancer become completely understood. Half of the genes in mice and humans (40,000 to 50,000 genes) carry 5′ CpG islets [18, 19] and these genes have a wide tissue pattern of gene expression. The methylated condition characterizes the CpG islets and is related to the majority of these genes. The absence of methylation in the CpG islets is essential for active gene transcription [21]. Every category of transformed cell loses its methylation in the normally methylated regions, as well as its increased DNA-MTase activity [22].

DNA Sub-methylation in Cancer

The most important modification of DNA methylation in neoplastic cells is the overall reduction of methylation [23]. This reduction of the number of methyl groups indicates the start of early tumor evolution, even before the real establishment of the tumor [24, 25].

In rodents, the exhaustion of the methyl group donors by restriction of S-adenosyl-methionine in the diet, leads to liver carcinogenesis and to DNA

hypomethylation [24, 25]. In hypomethylation in human tumors some specific genes, (such as the K-ras in lung cancer and cancer of the large intestine) get directly involved. The hypomethylation model concerns a certain number of oncogenes in humans [24]. The inter-relationship between the two prototypes of DNA methylation and gene expression includes the CpG regions located in the two promoters. In normal genes, the promoters include CpG islets; the CpG positions are not methylated and therefore are not targets of hypomethylation. DNA hypomethylation seems to deeply affect the structural integrity of chromosomes.

Local DNA Hypermethylation

Neoplasmic cells with gene hypomethylation very often include some confined hypermethylated areas. This change in neo-cells normally has non-methylated CpG islets at a proportion of 20%. The normally non-methylated CpG islets in the areas of genes constitute the primary target of a diffused hypermethylation in cancer cells. Such a hypermethylation is a landmark for the cancer cell and it is also the strongest relationship in the changes of DNA methylation and changes in gene expression in neoplasia. CpG islets, for example, in the calcitonin gene promoter's area in the 11P chromosome are non-methylated in normal tissues, while in human solid tumors, they are intensely methylated [26]. Many other areas rich in CpG regions in the same limb of the same chromosome, which accommodate a large number of suppressive genes [27], are in these conditions, hypermethylated simultaneously [28]. It seems possible that the 11P area behaves like a "hot spot" during methylation of the CpG islets in neoplasia. This change in DNA constitutes an important inactivation mechanism for tumor suppressor genes.

The Von Hipperl-Lindau gene in the 2P chromosome is the first suppressive oncogene which correlates transcription inactivation with the hypermethylation of CpG islets [29].

In mice and rats, exposure to different tobacco smoke carcinogens, leads to lung cancer, which is consistently the same as with human lung adenocarcinoma [30]. The hypermethylation rate of the P16 CpG islets (45%) and homozygotic gene deletion (40%) are very high in cultured lung cancer cells [31] unlike in solid tumors where the lost function of the p15 genes and hypermethylation in the CpG islets prevails in hemopoietic cell tumors. In adults and children, acute myelogenous leukemia cells (44% and 65%, respectively) include the p15 gene. In these cases, there is no simultaneous hypermethylation of the p16 gene [32]. The loss of p16 gene function is a prevailing picture in T-cell tumors. All of these tumor cells have inactivated their p15 or p16 genes, and more than 50% of these cells have both genes inactivated. In T-cell tumors, the p16 gene is always deleted, while the p15 gene is mostly hypermethylated. In high malignancy tumors, the p15 gene is rarely altered, while a proportion of more than 80% of these tumors exhibits hypermethylation of the p16 gene.

DNA Methylation Mechanisms in Neoplasmic Cells

The role of DNA methylation and in particular, methylation of the promoter's area, is of critical significance for oncogenesis. For this reason, it is important to understand the mechanisms responsible for the changes in neoplasmic cells.

Role of DNA-methyl-transferase (DNA-MTase) Activity Alterations

Changes in the DNA methylation rate in neoplasmic cells is related to changes of DNA-MTase activity. The activity of DNA-MTase is important for the DNA synthetic process and for the cell cycle. Some researchers believe that this relationship is a tool for inducing the alterations existing between normal and neoplasmic cells. It is very possible that the cell transition from the G_0 phase to the active cell cycle is fundamental in starting the activity of the DNA-MTase during neoplasmic evolution. However, in the transformed cells, there is increased activity of DNA-MTase which is accumulated in cells which are not required to enter the cell cycle in order to synthesise DNA through the S-phase. This fact probably intimates the increased expression of the DNA-MTase gene, during oncogenesis, and its fundamental role in the neoplasmic process.

Evidence that Increased DNA-MTase Activity Plays a Decisive Role in Tumor Evolution

Cell enzymic activity is very much increased during the gradual evolution of cancer, starting from cell hyperplasia, adenoma and carcinoma. This activity co-exists with the increased gene expression of DNA-MTase, the enzyme which catalyses DNA methylation at the CpG sites. The increased expression levels of the enzyme constitute a biomarker for the initial stages of the malignancy. Such genetic abnormalities exist along the respiratory pathways of smokers and these are due to a diffused long-lasting lung exposure to the carcinogens present in both the gas phase and tar of cigarette smoke. For example, it has been shown that mutations in the p53 gene and the loss of heterozygosity in the 3P chromosome in the bronchial epithelium cells with moderate or serious dysplasia, exist in cell areas near the primary cancerous mass [33]. Chromosomal abnormalities have been shown in the normal segment of the bronchial epithelium, and also dispersed precancerous cells in the whole area of the lung tissue [34].

A puzzle to be solved is this: Why does the genome of cancer cells simultaneously accommodate hypermethylation and increased activity of DNA-MTase? The first reason is that changes can take place in the chromatin which can reduce its capacity to maintain methylation. This limits the normal access of DNA-MTase to DNA. In this sense, DNA-MTase could have been stimulated earlier in oncogenesis and reconstituted the normal methylation areas as a result of decreased methylation

in the whole genome. In spite of this anomaly different studies have shown that the levels of increased DNA-MTase activity in the neo-cells are able to influence, to some extent, DNA methylation and contribute to regional hypermethylation. Other studies have shown that the acute, even average, degree of increase in DNA-MTase activity is able to increase DNA methylation as a whole. In rodents the increased DNA methylation is followed by a transient increase of DNA-MTase in type II lung epithelial cells, due to acute exposure to cigarette smoke [35]. Moreover, other important studies have shown that the super-expression of human constitutive DNA-MTase is not well tolerated by human cells. The increased activity of DNA-MTase contributes to hypermethylation of the CpG islets, and this is connected with deactivation of the transcription activity of the essential suppressor oncogenes.

Other Risk Factors Related to Tumor Evolution Which Play an Important Role in the Hyper-methylation of CpG Islets

Metals as carcinogens such as nickel, which is located in the cell nucleus, may create reversible processes, connected with changes in super-methylation which alters the DNA structure [36]; these authors raise the fact that the metals induce alterations in the chromatin structure, followed by methylation changes and that carcinogens lead to the inactivation of certain suppressor genes. According to this model, different carcinogens induce specific alterations in certain areas of the genome and they play an important role in the hypermethylation processes.

Mutational Changes in the Gene Promoter Regions Constitute the Hypermethylation Background of the CpG Islets

Methylation changes in the gene promoter regions in the CpG islets of the suppressor gene can take place in the absence of mutations in the coding region of these genes. Thus, the areas of CpG islets which accommodate methylation changes in the suppressor gene p16 do not include methylation mutations in cancer cells [29, 37].

In Normal Cells, Regional Factors are Associated with the Defence of CpG Islets Which are Altered in Neoplastic Cells

One serious question is this: how, in normal cells, are CpG islets protected from methylation? It has already been noted that cell exposure to increased DNA-MTase activity may cause diffused methylation in the CpG islets. Even though the DNA-MTase increase can be tolerated by the CpG islets, the regional factors which protect the islets from methylation can be neutralised in neoplasias. The CpG islets of human genes have anatomical grounds characterised by distinct limits at both 5′ and 3′ which separate the normal non-methylated series of islets from these heavily

methylated side regions which include repeated series of Alu. Furthermore, a large number of Sp-1 positions is located in both 5′ and 3′ sides, as a strategic position which protects the islets from methylation which originates from the side regions. During tumor evolution, a progressive increase in DNA-MTase is noted; this can exceed the normal protective mechanisms which have the status of being non methylated in these regions. This shows that methylation of the rich CpG regions takes place following the diffusion of the methylation process provided to the cell centers in multiple cell cycles [38]. It also shows the time extension of cell exposure in the increased activity of DNA-MTase during tumor evolution in order to exert constant pressure and to disrupt the protective boundaries of the CpG islets from methylation. Of course, there are some more anomalies, apart from DNA-MTase deregulation, which contribute to the hypermethylation of the CpG islets during neoplasias. In these anomalies, regional mechanisms which create protective boundaries for the CpG islets are induced. An attractive target is the loss of the joining proteins which react with their Sp-1 sites at the edges of each islet. If these proteins are insufficient or if their function is ineffective, the CpG islets are easily affected by methylation.

Some of these proteins are transcription factors such as Sp-1; they maintain their chromatin structure around the islet regions, so that the access to the DNA-MTase becomes impossible. Essential proteins for the chromatin structure such as histones, participate in the structural "homologies" of other proteins, so that, when they function simultaneously, they are able to regulate the chromatin structure in the gene's promoter [39, 40].

It has been shown that the histone H1e has a very strong binding affinity to DNA rich in CpG and thus binds, in vitro, DNA-MTase activity when the latter associates with the base series. It is important that in mutations, CpG methylation start very early. This condition, for human lung cancer is of great importance from the prognostic point of view. It has been shown that hypermethylated alleles p16 are detected at the stage of epithelial hyperplasia which precedes adenoma, followed by adenocarcinoma [41]. This early methylation proceeds during oncogenesis. Initially, there is an increase in the density of methylation inside the damaged gene. In the long term it is increased further during the stages of adenoma and adenocarcinoma [42].

The meaning of progressive hypermethylation of the CpG islets during tumor evolution resembles the classical gene mutation [43].

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Chapter 9
Toxicity and Carcinogenicity of Hydrocarbons

Isoprene

Properties of Isoprene Metabolism

Isoprene is an important diolephin, called either 2-methyl-1,3-butadiene, or 2-methyl-buta-1,3-diene ($CH_2{=}CmeCH{=}CH_2$). In nature, it is in liquid form, b.p. 35°, at 60°C; in the presence of Na^+ it is polymerised to a substance quite similar to natural rubber. Like butadiene, it is released into the environmental air from different sources and from humans.

Its biological production takes place by photosynthesis, with sun radiation as a source of energy [1]; it is a hydrocarbon released from certain deciduous forest trees [2]. It is also a product of burning tobacco leaves [3]. In the atmosphere it undergoes photochemical degradation, reacting with numerous mechanisms. The products of isoprene photochemical degradation include methacrolein, formaldehyde, 3-methyl-furan, acetaldehyde and ozone [4–8]. Isoprene is metabolised to its isomeric monoepoxides by mono-oxygenase which is activated by the cytochrome P450. Monoepoxides are further metabolized to the mutagenic di-epoxides of isoprene, the (2,2′)-2-methyl-deoxyrenes. Isoprene metabolism in animals and humans is quite similar to that of butadiene. Isoprene oxidation produces two monoepoxides: 1,2-epoxy-2-methyl-butene and 1,2-epoxy-3-methylobutane, which are further oxidized to isoprene diepoxides [9, 10].

Molecular Toxicological Behaviour of Isoprene

Toxicological studies in animals have shown that isoprene is a carcinogen in mice [11, 12]; there is evidence that isoprene is also a carcinogen in rats [13]. The International Agency for Research on Cancer (IARC) has classified isoprene as a possible carcinogen in humans [14, 15]. The carcinogenic potential of isoprene differs from that of butadiene and this is due to the potency of its two metabolites. The addition of the methyl groups to the isoprene epoxides substantially affects their activity and this can explain the very low carcinogenic potential of the isoprene

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epoxides, as compared to that of the butadiene epoxides. The addition of the methyl-groups to isoprene suppresses the effectiveness of the cross-linking.

Pharmacokinetics of Isoprene

The pharmacological prototype of isoprene serves as a tool for detecting the mono-epoxide concentrations in the blood, kidneys, liver and lung tissues when rats are exposed to isoprene inhalations for one week's time (5 days exposure and 2 days non-exposure). These concentrations are compared with those of similar rat exposure to isoprene for a two-year period of time. The estimated concentrations of the isoprene monoepoxides return to the primary control values during the two non-exposure days. The cumulative concentrations of the substance in tissues during a two-year exposure corresponds to the one-week exposure values [16].

Mutational Activity of Isoprene

There is supporting evidence that isoprene diepoxides are highly strong bacterial mutagens [17]. Isoprene metabolites are evaluated on the basis of human or animal exposure to these substances and the resulting hemoglobin adduct creation [18]. This evaluation is based on the formation of adducts between diepoxibutane and hemoglobin valine [19]. The methyl group in the isoprene molecule is of critical importance to biological activities. Studies on carcinogenesis related to isoprene and butadiene have shown mutations in the ras-gene and in the form of $A \rightarrow G$ transitions [20, 21].

The ascertainment of a large number of mutations in the ras-gene during exposure to isoprene indicates that isoprene metabolites create DNA adenine adducts.

The a-purinic sites and mis-replication at these sites indicate a further step towards carcinogenesis [22]. However, according to Begema, et al. [23] N7-Gua-adducts as isoprene DNA adducts can be considered as a choice biomarker.

Studies on Isoprene Carcinogenesis

B_6C_3Fi mice exposed to isoprene inhalations for two years may develop liver and lung cancer. The carcinogenic activity of isoprene is well documented in experimental animals [2]. According to the IARC (1994), breast tissue is a selected target for isoprene, which acts through its epoxides [2]. The testicles are also a frequent site of carcinogenesis for rats exposed to isoprene. It has been shown that isoprene is more effective than 1,3-butadiene when compared for testicular cancer induction.

Isoprene Toxicity in the Gaseous Phase of Cigarette Smoke

The mechanism by which free radicals are continually formed in cigarette smoke is due to isoprene which reacts with other cigarette-smoke constituents such as NO_2. At the start of cigarette burning, tobacco smoke contains a minimal quantity of NO_2,

while it contains large quantities of nitrogen monoxide (NO), which is considered as a weak free radical (400–1000 ppm/cigarette) [24]. This NO radical undergoes slow oxidation by environmental oxygen. From this oxidation, the extremely active nitrogen dioxide (NO_2) will ensue. Nitrogen dioxide reacts with the active dienes such as isoprene, in the gaseous phase of cigarette smoke and forms carbon-centered radicals ($R^{\cdot}$). These radicals undergo a scavenger effect via the oxygen in tobacco smoke, and from this reaction, alcoxy-radicals, ensue (page 25, Fig. 1.7). This model of the isoprene action in cigarette smoke is perhaps one of many other similar chemical reactions, taking into consideration that tobacco smoke is a complex mixture of toxic compounds [25].

Toxicity of Isoprene Photochemical Products

Isoprene and butadiene, from a chemical point of view, are identical. However, they are substantially different as far as toxicity is concerned. Isoprene, unlike butadiene, is not a carcinogen. However, when it is released into environmental air, it reacts with molecules which are produced by sunlight, and also with different mitogen oxides, and it is thus converted into photo-chemical reaction products [4–6, 26]. The photochemical products of this compound promote the cytotoxicity and gene expression of interleukin-8 (IL-8). The oncoming isoprene changes in the environmental air are due to the fact that this hydrocarbon undergoes a transformation that can induce serious damage to human health, as compared to that due to its mother compound. The aforementioned substances, produced by photochemical reactions, are all toxic, producing irritation of the upper respiratory tract and to all sensory organs. Among these substances, ozone induces inflammatory reactions of the sensory nerves, inactivation of enzymes which are excreted from the alveolar macrophages, cytotoxicity to the bronchial epithelium, changes in the respiration rate, alterations in epithelial cell membrane permeability, thus affecting so many other lung functions [27–29]. Ozone is a well known carcinogen in animals inducing damage in both rats and mice [30].

Butadiene

Butadiene is an important chemical compound, which is used for producing polymers and synthetic rubber. It is found in tobacco smoke and in car exhaust gases. The non-complete combustion of benzene in cars constitutes the major source of butadiene in the exhaust gases released into the environmental air. It is a strong carcinogen when introduced in mice [31–34] and a weak carcinogen in rats [35]. Mice are 100 times more susceptible to the carcinogenic process than rats. This extreme sensitivity of mice, (especially of B_6C_3Fi mice), seems to be due to endogenous retroviruses. NIH Swiss mice, which are free of such viruses, are 4 times less susceptible to lymphoma induction. This difference in sensitivity can be interpreted

by the existing metabolic differences in the two experimental animals. The chronic exposure of mice to butadiene inhalation creates lymphocytic lymphomas, hemangiosarcomas in the heart, alveolar/bronchial neoplasias, exfoliative neoplasms in the stomach, lobular-cell carcinomas in the breast, granular cell carcinomas and ovary and hepatocellular neoplasms. Lymphomas develop in the thymus of B_6C_3Fi mice which are exposed to 625 ppm of 1,3-butadiene for 23 weeks.

Stereochemistry of Butadiene

The stereochemistry of butadiene affects both the biotransformation of butadiene and its cytotoxicity in the hepatic microsomes of rats and mice [36]. Butadiene oxidation creates butadiene monoxide (BMO) and butadiene dioxide (BBO), two very active epoxide metabolites which have proven mitogenics [37–40]. A metabolic activation is necessary in order for the toxicity of butadiene to be evidenced. Butadiene metabolism has been studied in the microsomal systems of rats, mice and humans [41–44]. The isoenzyme of the cytochrome P450 is probably responsible for the butadiene oxidation of MBO. Initial butadiene oxidation from the cytochrome P450 creates two stereoisomers of BMO (R and S). The stereoisomers R and S (BMO) are metabolised in a different way and produce different toxicity in the liver cells. For this reason, the butadiene molecule structure must be taken seriously into consideration when its toxicity is tested.

Metabolism of Butadiene

Butadiene metabolism (Fig. 9.1) was first studied in the subcellular liver cell constituents of Wistar mice. Following oxidation, in presence of the cytochrome P450, butadiene is transversed to 1,2-epoxy-3-butane [45]. This monoepoxide is inactivated following association with glutathione and in the presence of glutathione-S-transferase. Epoxybutene is further oxidised to 1,2-dihydroxy-3-butene, which is

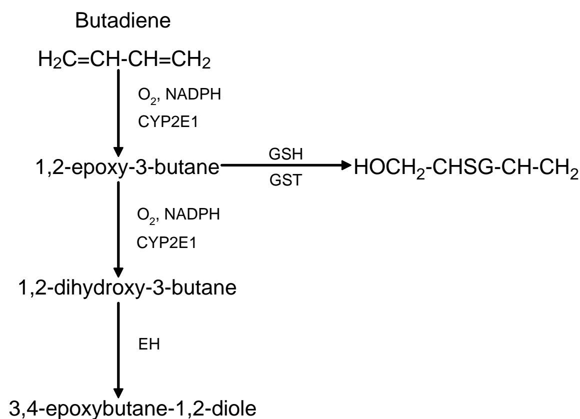

Fig. 9.1 Metabolism of 1,3-butadiene. GST = glutathione-S-transferase, CYP2E1 = cytochrome P450, EH = epoxide hydrolases

oxidised to 3,4-epoxybutane-1,2-diole [46]. Epoxybutene is inactivated by glutathione-S-transferase. The same processes that take place in the liver of mice may also induce the transformation of butadiene in the human liver and lung cell cytoplasm and microsomes.

During the inactivation of epoxybutene, metabolites which are detected in the urine of people exposed to butadiene, are created [47, 48], (as well as in the urine of mice, rats, hamsters and monkeys). Diepoxybutane metabolites are not found in urine tested as above. Diepoxybutane is more toxic and 12 times more mitogenic at the hprt sites of the splenic T-cells of mice as compared to epoxybutene. It is important to know that there is an equivalence between lymphoma induction in mice which have been exposed to butadiene and cancer induction in the hemopoietic system of humans. The epoxide metabolites of butadiene are responsible for the mitogenic and carcinogenic activities of butadiene [15, 16]. Epoxybutene and diepoxybutane, when introduced subcutaneously, induce neoplasias in mice and rats [49].

Mutagenic Activity of Butadiene

The epoxide metabolites of butadiene are alkylating agents. During the epoxybutene reaction with guanosine and double strand DNA deoxyguanosine, the formation of N^7-(2-hydroxy-3-butene-1-yl) guanine and its isomer N^7-(1-hydroxy-3-buten-2-yl)guanine, takes place [50]. These two alkylation products of N^7-guanine are found in the liver DNA of mice and rats [51]. The in vivo mutagenic activity of butadiene is related to K-ras gene activation by mutations in the codon 13 ($G \rightarrow C$ transversion) found in lung and liver neoplasias as well as in lymphocytic lymphomas. This butadiene activity is expressed by tumor suppressor gene inactivation. Genetic alterations in these genes constitute preliminary events during lung cancer development. In mice breast cancer, due to butadiene, there is allele loss in chromosome 11, at different sites of the neighbouring tumor suppressor p53 gene, and in chromosome 14 of the retinoblastoma tumor suppressor gene [52].

In lung cancers due to butadiene, a loss in heterozygosity (LOH) has been detected more often in the MTSI region of chromosome 4 in which an important gene that encodes for the inhibitor of the cycline D/CDK4 kinase complex, is located. Different kinds of allele loss express inactivation of the tumor suppressor genes in the carcinogenic process, and they are proportional to the genetic abnormalities found in human cancers. Loss of heterozygosity is a late event in multi-staged carcinogenesis and can take place at a later time of exposure to carcinogens.

Mutations of the DNA Bases

DNA has been considered as a suitable target for studying mutations in DNA bases because all changes in the base series are involved in the etiology of cancer [53]. This point of view has orientated research towards analysis of the series of DNA

bases at the hprt region of B_6C_3Fi mice splenic T-cells [54]. This type of research has evaluated the mutagenic potential of the in vivo exposure to chemical substances in order to determine various alterations in the DNA bases (mutational spectrum). By using butadiene and its metabolites, a +G mutation frameshift in a series of six guanine bases has been shown. The +G mutation frameshift is considered as a mutagenic "hot spot" for butadiene and its metabolites. Substitutions were observed at both the couple GC and AT during exposure to the epoxides EB and DEB. Ethylene oxide belongs to the alkylating agents. It reacts with DNA and produces two "adducts", 7-hydroxy-ethyl-guanine and N^3-hydroxy-ethyl-adenine. These adducts create a-purinic sites via automatic a-purination or via glycosylase action. The mutational spectrum for butadiene and the epoxides 1,2 epoxybutene and diepoxybutane is similar to that of ethylene oxide in B_6C_3Fi mice. Consequently, these substances act in experimental animals with a similar mechanism to that of ethylene oxide and induce the mutations observed in exon 3. In other words, 1,2-epoxybutene and diepoxybutane react with DNA deoxy-guanosine and produce N^7-(2-hydroxy-3-buten-1-yl) guanine and 7-(1-hydroxy-3-buten-2-yl) guanine [50, 55]. Thus, the epoxy-metabolites of butadiene are capable of producing a-purinic sites in DNA and possibly exercise a part or all of their mutagenic potential through these sites.

Loss of Heterozygosity in B_6C_3Fi Mice

The loss of heterozygosity in chromosomes 4 and 11 has been found in more than 70% of mice (exposed to butadiene) which developed breast and lung cancer. Alterations in chromosome 11 seem to reflect the inactivation of Trp53. The alteration prototype in chromosome 11 signifies the reduction of homozygosity at position Trp53 in almost all lung cancers. The human chromosome 17 is a position where allele loss is often observed in a large spectrum of cancers in humans [56]. According to the IARC 1,3-butadiene is carcinogenic in humans [49] while in mice it is a most "suitable" model for studying the carcinogenic risk of this substance.

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Chapter 10
Toxicity and Carcinogenicity of the Aromatic Hydrocarbons

Benzene

The general population is exposed to the toxic effect of benzene in areas in which atmospheric and indoor air exhibit high polluting values due to the benzene in regions with aggravated traffic, around the petrol stations, and in places where cigarettes are smoked [1–5]. Smoking has been incriminated as a unique and most important source of exposure to benzene for smokers [6, 7] and it is responsible for 50% of benzene exposure in the general population [6, 7]. Tobacco smoke in the gas phase, contains 30.3–50.89 μg of benzene per cigarette.

Metabolism and Distribution of Benzene in Tissues

When benzene is inhaled, it is distributed in four principal areas of the human organism: the liver, tissues with increased blood supply (brain, kidney, heart, spleen), tissues with poor blood supply (muscle, skin) and fat tissue [8]. Benzene is taken up through the lungs, the gastrointestinal system and the skin. Its metabolism takes place mainly in the liver and secondarily, in the bone marrow, where benzene exerts its toxicity. Its metabolism is dose-dependent and follows two pathways: the ring hydroxylation pathway and the open ring pathway [9–11]. Hydroxylated metabolites form glycuronides or sulfuric conjugations which are excreted in the urine as detoxification products. Sulfuric conjugations lead to the elimination of the toxic metabolites. The open ring pathway leads to the formation of toxic by-products, trans, trans-myconaldehyde and myconic acid which are also detoxified and excreted in the urine [12]. Benzene metabolism takes place quickly in the liver tissue where it is converted to benzene oxide, which reacts with glutathione to form promercapturic acid, or it is hydrated to form dihydrodiole and is oxidised to catechol (Fig. 10.1). Furthermore, there is a rearrangement of its molecule-forming phenol which forms hydroquinone.

Benzoquinone is also formed from its two dihydroxy-metabolites. Taking into consideration that benzene has no direct carcinogenic activity, and in relation to the fact that its metabolites are carcinogens, we can understand that benzene metabolic products are responsible for the real carcinogenic activity of benzene.

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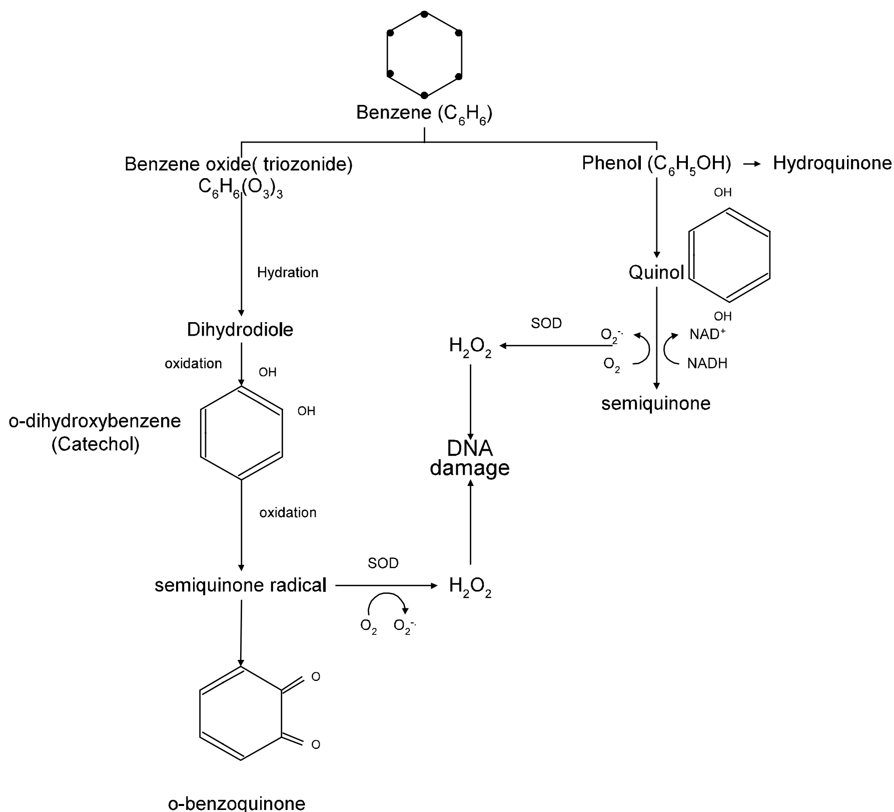

Fig. 10.1 The role of the benzene metabolites and H_2O_2 in DNA damage

In summary, the most important benzene metabolites are S-phenyl-mercapturic acid (S-PMA), trans-trans-myconic acid (t,t,-MA), hydroquinone (H_2Q), catechol (CAT) and phenol. The two acids (S-PAM and t,t-MA) are the most sensitive markers which demonstrate exposure to benzene [13]. Benzene metabolism is furthered through mono-oxygenase of the cytochrome P450, leading to the formation of benzene oxides, which are very active by-products of benzene metabolism. Also, malondealdehyde and hydroquinone are formed in the liver [12].

Toxicity Mechanisms

Benzene metabolites are transferred from the liver, where they are produced, to the bone marrow, where they perform their toxicity, associated with carriage molecules [14–16]. Hydroquinone and catechol are accumulated in the bone marrow where they become substrates for myeloperoxidase and form metabolites which are covalently connected with proteins [17–19]. Furthermore, hydroquinone and

o-benzoquinone are associated with the sulfhydryl groups of the tubulin, in the mitotic spindle, thus impeding the appropriate formation of microtubules [20]. If there is no physiological function of the mitotic spindle, the dissociation of chromosomes becomes unfeasible and, thus aneuploidy is created [21]. Benzene induces DNA degradation, chromosomal mutations, and aneuploidy in mammalian cells [22].

Benzene and its metabolites may block iron incorporation into precursors of bone marrow cells [23]. The decreased iron uptake is supported by the combination of hydroquinone and trans-trans-myconaldehyde [23]. Furthermore hydroquinone contributes to benzene toxicity by inhibiting cytokine production (ILα and ILß) from monocytes, which then negatively affect the regulation of hemopoiesis [24]. Also, hydroquinone decreases the cell content in calpain, the enzyme that is responsible for the conversion of the precursor forms of ILα and ILß [24]. In mice exposed to benzene, hydroquinone or benzoquinone, increases NO production by the animal's white blood cells in the bone marrow, as a response to inflammation messengers and to growth factors [25]. NO production represses cell proliferation, and forms intermediate by-products which react with primary and secondary amines and forms nitrosamines. Consequently, NO is important for the toxicity mechanism of benzene and its metabolites. Phenol stimulates myeloperoxidase enzymic activity, which uses phenol as an electron donor, and promotes phenoxy radical production. Phenoxy-radicals react further with hydroquinone to form 1,4-benzoquinone, a toxic intermediate, which inhibits significant cell processes [17]. Benzene metabolites are associated with DNA and RNA, thus inducing hyperphosphorylation of the genomic material, specifying the mechanism of the chromosomal damage and carcinogenesis [26]. Benzene metabolites are able to inhibit the function of enzymes that participate in DNA replication and reconstitution [27].

Clinical Significance of Benzene Toxicity

The exposure to a large quantity of benzene induces suppression of the bone marrow, leading to leukemia and aplastic anemia [11,28–31]. The cell- or chemically-mediated immune response during long-standing exposure to benzene is due to damage of the T- or B-lymphocyte "stem cell." Also, there is an increase in bone marrow cell abundance as well as an increase in thymus T-cells, as a compensatory response in those cells exposed to benzene activity. This compensatory increase in cell proliferation plays a role in the carcinogenic response of the experimental animal (C57BL mice). Moreover, benzene creates chromosomal abnormalities suggestive of genetic damage, in both the blood cells and other cell tissues [32–34]. Benzene is carcinogenic in animals and leukemogenic in humans [35–39]. It is able to decrease the number of stem cells and committed stem cells [40, 41].

Chronic exposure to benzene and its metabolites (hydroquinone, etc.) leads to a decrease in lymphocytes and to an increase in granulocytes in the bone marrow. Such exposure leads to leukemogenesis through activation of the protein kinase C, which participates in the proliferation of human myeloid cells. Furthermore,

activation of the protein kinase C by chronic exposure to benzene, causes hyperphosphorylation of the cell proteins, resulting in the activation and expression of certain proto-oncogenes.

It is interesting that a proportion 90% of the tumors resulting from exposure to benzene show a loss of the functional "locus" of the P53 allele. In $p53^{+/-}$ mice, benzene induces a high frequency loss of heterozygosity in chromosome 11. This is an important fact because the p53 gene encodes for the transcription factor, which supervises the gene transcriptions involved in different cell processes, such as cell-cycle regulation, reconstitution of DNA damage and apoptosis [42]. Loss of gene p53 function is associated with genome instability and unregulated development [43, 44]; it is the most common genetic alteration in human cancer [44–47]. Benzene inhalation produces a high incidence of thymus lymphomas in $p53^{+/-}$ mice [48]. PCR analysis has shown a loss of p53 allele function in most thymus lymphomas.

Biomarkers Which Detect the Exposure to Benzene

Phenol measurement in the urine of people exposed to benzene is the most common and most reliable method of detecting exposure to benzene. Moreover, this method determines the level of exposure to benzene [49–53]. Variations of the phenol content in the urine due to different agents unrelated to benzene exposure, may affect the phenol measurement if the levels of exposure to benzene are lower than 10 ppm. However, phenol measurement of the urine is useful in order to determine the benzene levels to which people have been exposed. In these cases, the biological markers of exposure are directly related to the marginal threshold value. This value has been defined as 50mg phenol/g creatinine in the urine [54].

Another marker of acute exposure to benzene is the presence of myconic acid in the urine [55–59]. The high sensitivity of myconic acid measurement is due to the fact that myconic acid levels in the general population are very low versus the high levels of phenol, catechol and hydroquinone. The measurement of myconic acid in the urine of female smokers and non smokers appears to be very interesting. The differences between the mean concentration values in the urine of the two groups are appreciable. Furthermore, smoking affects the levels of the t,t-MA in the smoker's urine (0.22–0.24mg/g creatinine in smokers versus 0.05–0.06mg/g creatinine in the non smoker's urine). Finally, the appearance of both DNA adducts and benzene metabolites in the urine is a useful biomarker of exposure to benzene. DNA adducts together with benzene metabolites have often been found in the urine of people exposed to benzene [60, 61].

Toluene

Toluene enters the lung tissue by inhalation and is excreted via the kidneys in the urine of humans, mice and rats [62–64]. It is also absorbed slowly by the skin [65]. Toluene absorption takes place through the double lipid layer of the cell

membranes [66]. When toluene enters the peripheral blood it is distributed among different tissues, such as the brain tissue, liver and lungs [67, 68]. The white matter of the brain, rich in lipids, selectively absorbs toluene while to the contrary, the gray matter does not absorb toluene [69]. Seventy-five to 80% of inhaled toluene is excreted in the urine, in the form of its metabolite, hippuric acid [64, 70] (Fig. 10.2). Another 7–20% of inhaled toluene is eliminated, unchanged by exhalation [64, 71]. Toluene metabolism takes place in the liver, catalysed by the isoenzyme CYP2E1. The same process occurs in the lung microsomes, but at a rate ten times lower than that of its metabolism in the liver [72].

Toxicity Mechanisms

Toluene toxicity is manifested in synaptosomes of the central nervous system and it is mainly due to the parent substance but not to its metabolites. Its disturbances are due to the reaction of toluene with the cell membranes (lipids and proteins) of the nervous system. The reversibility of the toluene action on the nervous system is presumed from its inducing transient anaesthesia.

Following the exposure of an experimental animal to toluene, some biochemical alterations can be found after about one hour. Toluene metabolites in the brain synaptosomes are able to reduce phosphatidyl-ethanolamine, alter the activity of the phospholipid methylation and increase the Na^{+}-K^{+}-ATPase activity [73, 74]. Furthermore, a reduced activity of the Mg^{++}-ATPase in the brain synaptosomes has been found [75] and an overall increase in concentration of some biogenic amines (dopamine, norepinephrine, 5-hydroxy-tryptamine) [76]. On a molecular basis, toluene enters the lipid bilayer of the cell membrane and reacts reversibly with the membrane proteins [77]. Chronic exposure to high concentrations of toluene, such as exposure to cigarette smoke (43–47 μg per cigarette) creates structural alterations in the lipid constitution of the brain. In the brain synaptosomes in mice, changes in the phospholipid constitution were found, during the acute exposure to

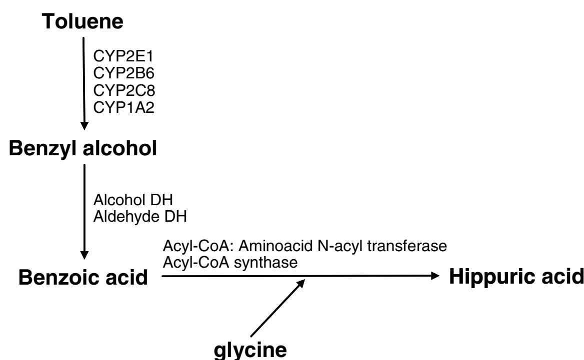

Fig. 10.2 Toluene metabolism to hippuric acid

toluene [73, 74]. In mice, a low concentration of brain phospholipids was found, following continuous exposure to toluene for 30 days [78]. Furthermore, a decrease in the number of the neurons at the hippocampus, after a six-month exposure to toluene at 1500 ppm, for 6 hours daily has been ascertained. It has not yet been clarified whether these changes are due to fragmentation or inhibition of the phospholipid synthesis. People exposed to long-lasting toluene action suffer from neurological disorders [79–81], hearing loss [82, 83], and color recognition anomalies [84–88]. Hearing loss, following the exposure of experimental animals to toluene inhalations, is due to the toluene itself but not to its metabolites. The molecular mechanism and pathogenesis of the color recognition impediment (dyschromatopsia) that is reported to those exposed to toluene are related to the dopaminergic mechanisms of the retina cells or to the toxic demyelination of the optic nerve fibers [84–88]. The repeated reaction of toluene with the proteins and phospholipids of the cell membranes, changes the activity of the enzymes which participate in neurotransmitter synthesis or degradation. The level of neurotransmitters in some special areas of the brain tissue is responsible for the production of sensitive neurological reactions.

Certain studies have demonstrated that there are increased concentrations of dopamine, nor-epinephrine and 5-hydroxytryptamine in mice that are exposed to 1000 ppm of toluene, for 8 hours [76] and reduced activity of the aromatic acid decarboxylase (enzyme which is involved in the neurotransmitter synthesis) in the rat's brain [89]. The point of view that repeated exposure to toluene may produce neurological reactions, by changing the joining capacity of the neurotransmitters with the cell membrane receptors, has been supported by the presence of constant alterations in the joining capacity of the D2 brain receptor and the increased level of prolactin in the blood serum. The increased level of prolactin in the blood serum may be related to the toluene reaction with the dopamine D2 receptors of the pituitary gland and the inhibition of the functional receptors. These receptors normally inhibit the release of prolactin in the blood serum. Additionally, the exposure of rats to 1000 ppm toluene, for 6 hours daily and for a few days only, produces a decrease in the glial fibrinoid acid protein (GFAP) of the brain [90]. A decrease in GFAP is observed in the thalamus and hippocampus – in other words, in the brain areas which control the levels of the serum glucocorticosteroids and which command high concentrations of the glucocorticosteroid receptors [90]. There is also evidence which maintains that a decrease of the brain GFAP is the result of the disorganization of the hypothalamus-pituitary-suprarenal axis and hormonal homeostasis.

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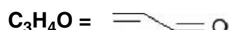
Chapter 11
Toxicity and Carcinogenicity of Aldehydes

During smoking, aldehydes originating from the burning of sugars added in tobacco, are detected in high concentrations in the lung tissues. Cancer may develop when formaldehyde contacts the broncho-epithelial cells. Formaldehyde is genotoxic and has been registered as an animal carcinogen. Acrolein decreases the respiration rate and inhibits the cilia movement of the respiratory epithelia. Propionaldehyde holds ciliotoxic and mucocoagulating properties. Rat exposure to the combined action of aldehydes leads to an important increase in lung tissue damage and to a decreased rate of respiratory movements. The gas phase of cigarette smoke contains high levels of active aldehydes, such as acetaldehyde, formaldehyde, acrolein, crotonaldehyde and propanol [1]. At present, it is well documented that the aldehydes in cigarette smoke together with GSH, form adducts. Furthermore, aldehydes inhibit the function of many enzymes, such as glutamyl-cysteine-synthase, GSH-reductase and GSH-peroxidase [2].

Acrolein



Acrolein is a volatile, inflammable liquid with a disagreeable odour. It is an $\alpha-\beta$-unsaturated carbonyl substance with carbon-carbon double bonding, carbonyl-group properties, and it also has properties due to the existence of a coupling system. A double carbon-carbon bond is able to undergo the electrophilic addition of acids and halogens, hydrogenation, hydroxylation and then rupture, while the carboxylic group undergoes nucleophilic substitution, typical of esters, or typical for acetone nucleophilic addition.

The above coupling system further increases acrolein activity [3]. Cigarette smoke contains 33–228μg of acrolein per cigarette and this content depends on tobacco quality and smoking habits [4]. Its ceiling value (based on a mean inhalation time of 30 minutes) is 25 $\mu g/m^3$, its permissible concentration value 0.5 $\mu g/m^3$, while a daily tolerable uptake is 0.5 μg/kg body weight [5]. During smoking, high local concentrations of acrolein are created in the respiratory pathway.

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Toxicity of Acrolein

Tobacco smoke is a source of different oxidants and very potent carbonyls [1, 6], among which the most potent is acrolein [1]. Acrolein is a potent water-soluble electrophile aldehyde and a ubiquitous environmental polluting substance. It is a highly dangerous substance to cells, inducing damage to lung tissues by inhibiting the defense mechanisms of the respiratory system [7]. This substance is formed in vivo, either following lipid peroxidation, together with some very active aldehydes, such as 4-hydroxy-2-nonenale, malondealdehyde and glyoxyale, or from the oxidative substances present in tobacco smoke, and from the release of oxidative substances during inflammation [8, 9]. These very active carbonyls may affect some amino acids (such as lysine, arginine and histidine), by inducing covalent modifications to some proteins which are considered as advanced end-products of glycolysis, or advanced end-products of lipoxidation [1, 6,10–12]. Protein modifications by acrolein constitute a marker for tissue damage due to oxidative stress, (such as arteriosclerosis, Alzheimer's disease) [13, 14]. Additionally, some end-products of glycolysis and advanced lipoxidation constitute agonists for the macrophage receptor. Macrophages are the predominant cell population responsible for emphysema development [15]. Cigarette smoke and its lipid peroxidation products such as different carbonyls (crotonaldehyde, acetaldehyde, acrolein, 4-hydroxy-nonenale, glycolaldehyde and malondealdehyde) are responsible for protein transformation. The transformed covalent proteins are bound to the scavenging macrophage receptor and subsequently adhere and accrue to the lung tissue. Acrolein is able to activate stress genes such as c-fos and heme-oxygenase [16], to intervene in the activation of transcription factors [17, 18] and to inhibit cell evolution [19, 20]. In low concentrations, acrolein inhibits cell proliferation by producing no cell death and it possibly induces apoptosis, while in high concentrations it may induce tumor development. It is possible that the reduction of cell proliferation due to acrolein is induced either by changes of certain stress-genes, or secondarily because of decreased glutathione (GSH), which is depleted following exposure to acrolein. The activation of the transcription factors NF-kB and AP-1 (activator protein-1) is inhibited by acrolein.

Acrolein is present in tobacco smoke, in exhaust-gases and in the vapour (fumes) of overheated cooking oil [21]. It is the most active among the $\alpha-\beta$-unsaturated aldehydes and it binds quickly with nucleophilic substances such as glutathione. This activity of acrolein constitutes its very toxic background [22]. A large gene number and transcription factors act on cell division and apoptosis. Acrolein affects some of these transcription factors, specifically the factors kB and AP-1 (Fig. 11.1). It also induces the expression of glutathione-S-transferase [14]. In vitro, 25–100μM concentrations of acrolein are lethal for the lung artery endothelial cells [23] and the broncho-epithelial cells [24]. The acrolein levels in the environmental air are 0.04–0.08ppm, whilst its tobacco smoke concentrations are very high at about 90ppm [25].

Variations in the thiol-redox are of critical importance for a large number of cell functions [26]. Consequently, alterations due to acrolein may affect transcription pathways like those induced by oxidative stress. As oxidative stress is determined,

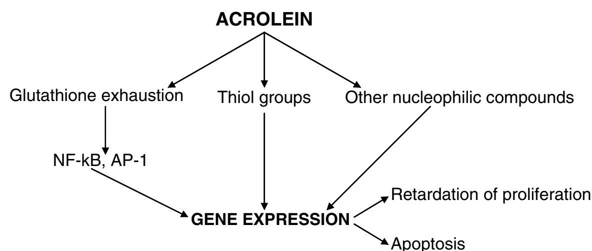


Fig. 11.1 Mechanisms by which acrolein affects the cells at the molecular level. Acrolein is a strong electrophile and reacts with the nucleophilic cellular compounds. This affects gene expression. Acrolein reacts directly with various genes and transcription factors. The net result of acrolein action is the retardation in cell proliferation rates and possibly apoptosis

disturbance of the pro-oxidative-antioxidative equilibrium in favor of pro-oxidation, results in remarkable damage [27]. The equilibrium of the cell-redox can be disturbed by glutathione loss due to acrolein and this can induce alterations in gene expression or the activation of transcription factors, which result in inducing damage in proliferation and in other physiological cell functions.

Effect of Acrolein on the Broncho-epithelial Cells

Acrolein induces reflecting activation of the broncho-epithelial cell receptors, resulting in an increased respiration rate, and it inhibits movements of the broncho-epithelial cell cilia, in vitro. Apoptosis of the broncho-epithelial cells is another mechanism of lung damage due to cigarette smoke. This cytotoxic activity is due to a reaction between the aldehydes and oxidative compounds in cigarette smoke [28]. The toxicity of aldehyde mixtures has revealed that histopathological alterations and cell proliferation of the olfactory epithelial cells due to mixtures of formaldehyde, acetaldehyde and acrolein, have been proven to be the most serious and most extensive for both the respiratory and olfactory epithelia, as compared to alterations due to single-aldehyde toxicity.

Acrolein is able to alkylate the free suflydrilic groups in the cytochrome P-450 and it inhibits the activity of enzymes which carry free sulfydrilic groups on their active site. It can also promote or induce complexes between DNA helixes and DNA proteins.

Effects of Acrolein on the Liver and Pulmonary Cells

When acrolein is inhaled for 4 hours and in concentrations of 14.7 mg/m^3, an important increase in alkaline phosphatase and tyrosine aminotransferase activities takes place in the liver of rats. These activities are significantly alleviated if preceded by adrenalectomy or hypophysectomy, or if the inhibitors of protein synthesis such as

actinomycin-D or puromycin are given to the animals earlier. This indicates that acrolein stimulates the pituitary-adrenal system which produces and releases glucocorticosteroids which may increase the synthesis of adaptive liver enzymes.

Acrolein may affect the inflammation mechanisms by aggregating neutrophiles and reducing their clearance by apoptosis in vitro [29]. Tobacco smoke acrolein contributes to causing pulmonary emphysema by inhibiting the contraction of the collagen jelly and by the production of fibronectin by the fibroblasts.

Acrolein, Glutathione and Apoptosis

Glutathione is a major target of acrolein when involved in the control of apoptosis and it is a factor that relates acrolein with death. A deficiency in GSH, due to diethylmaleic salt (DEM) (which alkylates GSH through glutathione S-transferase) increases susceptibility to apoptosis [30]. Even if GSH has been characterised as an important cellular molecule which plays a role in apoptosis, there is still the question of whether its variations are immediately effective or whether they act as a secondary influence.

Acrolein and Specific Genes Which are Related to Cell Proliferation and Apoptosis

The regulation of certain biological processes through redox-sensitive transcription factors has created substantial interest in the last few years. These proteins are connected with the promoter's region of the gene targets, thus promoting transcription. Regulations through redox-transcription can take place either in the cytoplasm, following activation of the transcription factors which are then transferred to the nucleus, or by affecting their joining with the promoter's region. This last process demands cysteine located in the DNA joining region. When acrolein is joined with these nucleophilic amino acids it is able to directly affect the transcriptional process, and cause disarray to the process by inducing glutathione exhaustion.

Acrolein and NF-kB

Acrolein has an immediate and prompt effect on the cell GSH, and based on the redox regulation of the NF-kB, it is expected to influence its activation directly or indirectly, through changes of its content in GSH, and more directly through its joining with the nucleophilic cysteine present in the subunit p50 and/or the subunit p65 [31, 32]. It is also possible that exposure to acrolein may lead to an increase in endocytic calcium, which is able to activate NF-kB, irrespective of the thiol redox status [33]. NF-kB activity following exposure to acrolein varies according to the extension and duration of GSH exhaustion, as well as the subcellular location of this exhaustion (the endonuclear exhaustion of GSH is of critical importance) [17].

Acrolein and AP-1, Fos and Jun

The activating protein-1 (AP-1) is synthesised by two gene products of two proto-oncogene families, Fos and Jun. These gene products form homodimer (Jun-Jun) and eterodimer (Fos-Jun) complexes, joint with DNA, and thus lend to AP-1, a role of great significance in cell proliferation control [34]. AP-1 constitutes part of an overall gene regulation mechanism following disturbance of the redox-equilibrium. However, pathways that control these regulation mechanisms have not yet been clarified. Antioxidative compounds activate AP-1 [35, 36], while the activation of AP-1 by growth factors seems to depend on the existence of ROS [37]. While AP-1 and NF-kB respond inversely to antioxidative compounds, the oxidants play a positive role in the activation process of both factors [38]. Irrespective of the particular role of the oxidants and anti-oxidants, the activation of AP-1 seems to be associated with the control of cell proliferation by NF-kB, Fos and Jun.

Acrolein and p53

DNA damage induced by chemical substances is associated with the expression of negative regulators of the cell cycle. The most interesting among these controls is the product of the p53 gene. Cell rest in phases G1 and G2 of the cell cycle induced by p53, offers the necessary time for the DNA to be repaired, or in case of inefficient repairing, allows the continuance of apoptosis. The joining of p53 with DNA and all transcription activities depends on the presence of cysteines in the joining with DNA sites [39]. Furthermore, increased reduction of the cell thioles by N-acetyl-cysteine increases the expression of the p53 by increasing the transcription rate of the p53 mRNA [40]. Thus, p53 activity is controlled at many levels by the cell thiol-redox. Acrolein alters this control by its binding to cysteines, thus preventing p53 from binding to the specific target molecules or by altering the total of reduced thioles.

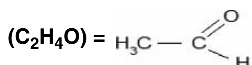
Acrolein and C-myc

An occasional increase in C-myc expression is an important message for cells entering the S-phase [41]. The function of C-myc in cell proliferation is complicated and there are many contradictions recorded in the literature. The C-myc gene plays a role in both the inhibition and the activation of transcription. The transcription factor NF-kB activates the promoter of the C-myc [42] and this creates a relationship between these two factors. There is also a role for NF-kB showing an up-regulation of the C-myc ascertained in apoptotic cells [43]. Two alternative transcriptional products, C-myc-1 (suppress up-regulation) and C-myc-2 (stimulates up-regulation) interpret this contradiction partially. However, the effect of acrolein on the C-myc gene is significantly necessary for proliferation and apoptosis.

Gene Expression Following Exposure to Acrolein

Acrolein is a very electrophilic substance and it is able to stimulate the gene family which is regulated by the electrophile response element (ERE) and which is referred to as ARE (antioxidant response element). ERE is a component of the DNA with cis-activity, which, for the first time, was identified in the gene promoter that encodes for the subunits of the glutathione-transferases of mice and rats [44]; the subunits consist of two AP-1-motives. Gene expression following acrolein activity has been studied with the cDNA micro array of Research Genetics (GF211) consisting of 4000 well known genes. The expression of 743 genes has been shown to be substantially up-regulated, while the expression of 282 genes was down-regulated more than three-fold. As was expected, ERE genes, including glutathione-S-transferase and γ-glutamyl-cysteine synthase, were gradually up-regulated. The reason that so many genes respond positively seems to be related to the overall activity of acrolein against nucleophilic molecules by infringing on a large number of pathways.

Acetaldehyde



Supporting evidence indicates that acetaldehyde is carcinogenic in experimental animals. It has been classified in the 2B group [45]. When inhaled, acetaldehyde creates malignant tumors of the olfactory tissue of rats and laryngeal malignant tumors in hamsters when exposed to toxic doses [40].

Direct exposure to acetaldehyde present in the gas phase of cigarette smoke can induce paralysis of the ciliary beat of the broncho-epithelial cells. Acetaldehyde is an effective substance and this is due to the polarity of the carbonyl groups. This fact may lend nucleophilic properties to it, when it is added to substances with amines, such as proteins and DNA, with which adducts are produced. In the different biological systems, acetaldehyde is reduced enzymatically to alcohol (ethanol) in the presence of the co-enzyme NADH. In the gas phase of cigarette smoke, acetaldehyde behaves as a base and this is due to its proton relationship. Cigarette smoke contains 879–1200 μg/cigarette of acetaldehyde, and this depends on the method used. At a temperature of 400°C acetaldehyde disintegrates forming methane and nitrogen oxide [46].

Acting Mechanism

Four hours of direct exposure to acetaldehyde which is included in the gas phase of cigarette smoke, is able to paralyze the ciliary beat of the bronchial epithelium [47]. This ciliary malfunction due to acetaldehyde is related to the direct inactivation of the cilia ATPase and also to adduct formation with the dienine and tubulin amino groups of the cilia [48–50].

This mechanism is very important from the point of view that when animals or humans are exposed to acetaldehyde or cigarette smoke [51] there is clearance inhibition of the airways [47, 51]. The result of this acetaldehyde action on the broncho-epithelial cell cilia is significantly increased by the action of cyanamide which is contained in cigarette smoke and which excludes the action of acetaldehyde-dehydrogenase and thus brings about some more intense cilia stasis [52]. In addition, the acetaldehyde of cigarette smoke can activate protein kinase-C resulting in IL-8 release under inflammatory conditions during which the activated component of the complement C5 (C5a) is increased in the air ducts, and which is responsible for the increased release of IL-8. Acetaldehyde increases collagen production in cultured asteroidal cells [53]. Furthermore, acetaldehyde in cigarette smoke inhibits the constriction of the collagen jelly of fibroblasts in vitro. This inhibition is due to a decreased production of fibronectin by fibroblasts, thus contributing to the inhibition of the damage repair, giving rise to emphysema [54, 55]. This aldehyde is able to inhibit DNA reconstitution and the strengthening of mutagenesis from nitroso-compounds in cultured human cells [56], and it also contributes to bronchogenic cancer development from cigarette smoke in humans [57].

Metabolism

The main metabolic pathway is the oxidation of its aldehyde component to acetic acid in the presence of aldehyde-dehydrogenase (ALDH), the action of which depends on NAD. Acetic acid enters the Krebs cycle for citric acid, in the form of acetyl-coenzyme-A, where it is metabolized to carbon dioxide and acetone bodies. In humans, it is metabolised mainly in the liver. Aldehyde-dehydrogenase activity is localised in the respiratory epithelium and the renal cortex of rats.

Mutagenesis

Acetaldehyde is genotoxic in vitro and it induces gene mutations in mammalian cells in the absence of exogenous metabolic activation. Indirect evidence from in vitro and in vivo studies indicates that acetaldehyde is able to induce cross-linking of protein with DNA (protein-DNA) or DNA-DNA.

The precise mechanism by which acetaldehyde can induce cancer is not well known.

Chemical and in vivo ***Reactions***

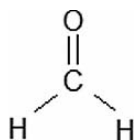
Acetaldehyde reacts with acid anhydrites, alcohols, ketones, phenols, NH_3, HCN, H_2S, alogens, P, isothiocyanate compounds, alkali and amines [58–60]. It is polymerised in the presence of trace metals or acids [59]. In vivo, acetaldehyde forms stable and/or unstable adducts with proteins. This prevents protein function

which is obvious from the enzymic action of the proteins and also prevents the joining of histones with DNA as well as inhibits the polymerisation of tubulin [61]. In the blood serum the protein adducts with acetaldehydes are increased in those persons who consume ethanol. These adducts are toxic for IL-2 excretion [62]. In the blood coagulation pathway, acetaldehyde forms adducts with the blood coagulation proteins, thus increasing or decreasing clotting time [63–66]. Acetaldehyde furthers the activity of the renin-angiotensin system (RAS) and creates adducts with proteins in the system [67]. It is not sure that acetaldehyde present in tobacco smoke is able to produce addiction. However, it is possible that some joining products of acetaldehyde with bio-amines contribute to addiction in smoking [68–75].

Conclusion

Exposure to acetaldehyde in cigarette smoke exceeds the "standards" of occupational exposure of 9 hours to this substance, by 10 to 100 times. In animals, chronic exposure to acetaldehyde inhalation creates tissue damage of the respiratory tract and bronchogenic cancer. Since acetaldehyde is metabolised in the respiratory epithelium, its systemic bioavailability is very low. Because of the direct damaging effect of acetaldehyde on the respiratory epithelium, a reduction of 10–100 times in cigarette smoke is advised.

Formaldehyde



Formaldehyde is a very effective and volatile substance, which is easily concentrated and is typically nucleophilic. It is included in cigarette smoke in a quantity of 45.2–73.1 μg/cigarette [76]. Concentrations of 60–130 mg/m^3 in inhaled smoke result in a daily uptake of 1 mg formaldehyde (daily smoking of 20 cigarettes). According to WHO (1989) there is evidence that formaldehyde does not have a high index of carcinogenesis. It can affect reconstitution of the damaged DNA even if it is not mutagenic [77].

Action Mechanism

Formaldehyde decreases the flow of mucous in two-thirds of the front part of the olfactory ciliated epithelium, while there is no activity in its posterior third part. This may indicate that formaldehyde is absorbed mainly in the front part of the

olfactory epithelium [78]. When rats are exposed to 18 mg of formaldehyde/m^3 (14.9 ppm), inhibition of the mucociliary function appears in designated sites of the nasal mucosa, and mucostasis is more prominent and extensive as compared to cilia stasis. Smoking 20 cigarettes a day corresponds to a daily uptake by inhalation of 2mg formaldehyde. Chronic exposure to formaldehyde by inhaling tobacco smoke creates tissue damage and cancer of the upper respiratory system.

Metabolism

Formaldehyde in both humans and animals is an important intermediate substance. It is produced endogenously from serine, glycine, methionine and choline and by the demethylation of N–, O– and S-methylate substances, in general. It is an essential intermediate product of the biosynthesis of purines, thymidine and certain amino acids [79]. A large number of enzymes catalyze the oxidation of the absorbed formaldehyde to formic acid. The most significant enzyme is formaldehyde-dehydrogenase; its activity depends on NAD, and as a co-factor it needs glutathione (GSH). There are at least seven enzymes which catalyze formaldehyde oxidation in animal tissues, like aldehyde oxidase and specific diphosphopyridine nucleotide (DPN)-dependent aldehyde-dehydrogenase [80]. Oxidation takes place in the liver and also in the red blood cells, brain, kidneys and muscles [81].

Carcinogenic Activity

There is evidence that the aetiological role of formaldehyde in cancer development has an effect only in rhinopharyngeal cancer.

Mutagenic Activity

Formaldehyde induces protein cross-linking with DNA (protein-DNA), DNA single strand breaks, chromosomal deletion and gene mutation in the human cells, in vitro [82]. In glutathione-deprived rats exposed to formaldehyde, increased cross-linking of DNA-proteins has been observed. This indicates that formaldehyde oxidation catalyzed from formaldehyde dehydrogenase is an important defensive mechanism against the covalent joining of formaldehyde with nucleic acids in the olfactory mucosa [81]. The concentrations of formaldehyde in inhaled cigarette smoke are higher than those used in animal studies.

Propionaldehyde

Propionaldehyde is a volatile liquid substance which consists of one carbonyl group and its characteristic functional group.

The main functional group is an aldehyde which classifies propionaldehyde in the carbonyls. The main trunk of this substance is a short aliphatic chain. The carbonyl group determines, to a large extent, its chemical properties and most importantly its nucleophilic property. Propionaldehyde is oxidized very easily. The gas phase of cigarette smoke includes 25–116 μg propionaldehyde per cigarette [82].Propionaldehyde is formed during smoking from precursor substances such as polysaccharides, pictines, proteins and possibly triglycerides. It forms, in vitro, cross-linking of DNA proteins, but it is less effective as compared to acrolein [83]. Propionaldehyde reacts with substances which have the amino group in their molecules, participates in nucleophilic reactions and reacts with oxidative substances. Acute toxicity following inhalation of propionaldehyde is mainly due to damage of the respiratory tract.

Combined Activities of Aldehydes

Toxicology studies of aldehyde mixtures have shown that histological alterations and cell proliferation of the olfactory epithelial cells caused by mixtures of formaldehyde-acetaldehyde and/or formaldehyde-acrolein, seem to be more serious and more extensive, in both the respiratory and olfactory epithelium, as compared to that observed during exposure to one aldehyde alone. The combined effect of the mixtures is the total action of the two parts, with however, a smaller final result. This is due to the competition of each of the two participants for the unique, common receptor.

Conclusion

Chronic inhalation of formaldehyde in tobacco smoke results in tissue damage and cancer of the upper respiratory tract (i.e. olfactory mucosa).

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Chapter 12
Toxicity and Carcinogenicity of the Nitriles

Acrylonitrile

Humans are exposed to acrylonitrile when it is used in the synthesis of different organic substances, such as acrylic fibers and plastics. Tobacco smoke includes acrylonitrile in such a quantity that it is able to damage human health. Chronic exposure of rats to acrylonitrile induces neoplasias in their brain and stomach [1]. People who work with acrylonitrile present a high incidence of lung cancer and malignancies of the hemopoietic and lymphatic tissues [2]. Recent studies have shown that acrylonitrile is a weak carcinogen [3, 4, 5] The International Agency for Research on Cancer (IARC) has classified acrylonitrile as a "possible carcinogen for humans" (group 2B) [6]. The mechanism of carcinogenesis for acrylonitrile in rats remains uncertain. Oxidative stress is a possible mechanism of tumor development due to acrylonitrile [7–9]. Cyanide induces lipid peroxidation in the rat's brain [10]. Oxygen radicals which are formed in these pathways may contribute to oxidative DNA damage and tumor development. Workers in the textile industry using acrylic fibers exhibit chromosomal abnormalities [11, 12]. Acrylonitrile is associated with hemoglobin both in vitro and in vivo [13, 14]. The acrylonitrile reaction with the N-terminal side of hemoglobin creates the CEVAL [N-(2-cynoethyl)-valine] adduct which is detected in the smoker's blood [15, 16]. Smoking contributes to acrylonitrile exposure [17]. The quantities of acrylonitrile in cigarette smoke vary from 10–20μg per cigarette [16].

Acrylonitrile is not directly carcinogenic/mutagenic, but rather it acts through its metabolites. Its toxicity depends on the kind of experimental animal, the tissue and its form of application. Consequently, it is important to understand its degradation pathways and the toxic, carcinogenic and mutagenic properties of the products of its metabolism.

Acrylonitrile metabolism is furthered via two different pathways: The first pathway leads to the direct joining with glutathione and epoxidation to form cyanoethylene oxide (CEO)—two reactions which are catalysed by the cytochrome P450 (Cyt P450). Furthermore, CEO is able to cause a variety of biological reactions (Fig. 12.1). N-acetyl-S-(2-cyanoethyl) cysteine is the main metabolite which is formed by the direct reaction of acrylonitrile with glutathione and it is excreted

J.C. Stavridis, *Oxidation: The Cornerstone of Carcinogenesis*, 175–179.

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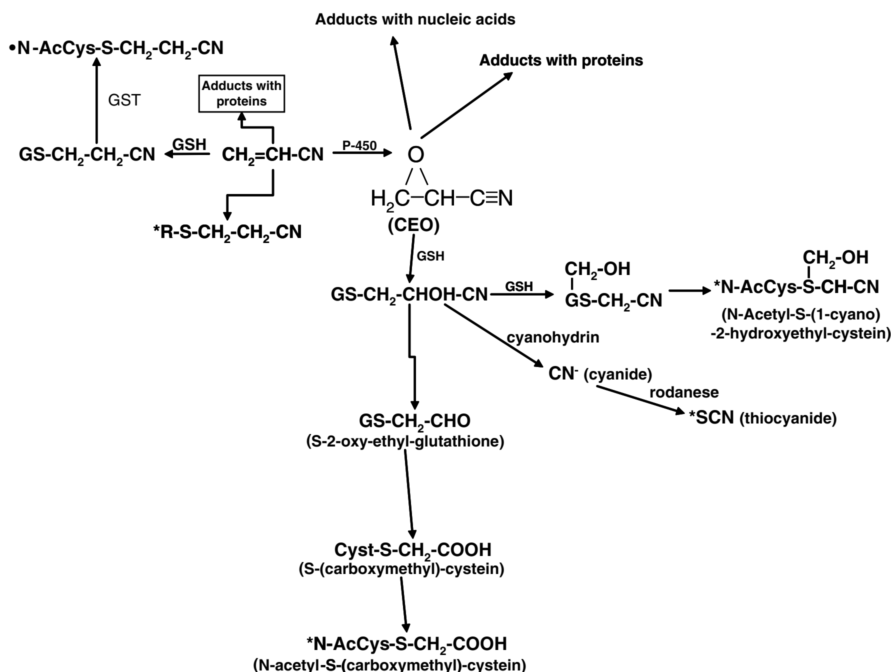

Fig. 12.1 The metabolic pathways of acrylonitrile. (CEO = Cyano ethylene oxide, GSH = Glutathione, GST = glutathione-S-transferase)

in the urine [18–23]. This reaction is enforced in the microsomes of the liver and it means that there is the participation of GSH-S-transferase [23].

The second important pathway of acrylonitrile metabolism that leads, via epoxidation, to 2-cyanoethylene oxide, is furthered by Cyt P450 [24]. In this case, cyanoethylene oxide, undergoing a series of reactions, results in the production of a large spectrum of metabolites excreted in the urine. For example, the nucleophilic joining of GSH to ethylene oxide creates a large number of metabolites. Cyanide is released from cyanohydrin and is converted into thiocyanide which is excreted in the urine [25, 26]. This release of cyanide lends toxicity to the acrylonitrile. The coupling product of both the acrylonitrile and the cyanoethylene oxide with GSH are further metabolised, in vivo, and excreted in the urine as mercapturic acid or as further degradation products (Fig. 12.1).

Such products of metabolism are both thioglycolic acid and the S-(2-carboxymethyl)cysteine in the urine of mice, while N-acetyl-S-(2-cynoethyl) cysteine is detected mainly in the urine of rats [27]. Acrylonitrile and ethylene oxide can be joined with proteins [28] and react with tissue thioles, resulting in a quick depletion of the induced glutathione reserves in different tissues.

Toxicity of Acrylonitrile in Humans

The toxicity of acrylonitrile is due to its two metabolic characteristics: cyanide formation and cell depletion of the glutathione reservoirs [29, 30]. Beyond the liver cells which are considered as major focuses of acrylonitrile metabolism, there are also the lung epithelial cells which are capable of metabolising acrylonitrile and ethylene oxide [31]. If inhalations of acrylonitrile are given to rats, glutathione depletion will ensue in their lung tissue cells [32, 33]. Lipid peroxidation has been observed in the red blood cells of humans who are exposed to acrylonitrile [34]. Acrylonitrile is able to create gene mutations, chromosomal deletions, non programmed DNA synthesis and cell transformation. All of these side effects demand previous metabolic acrylonitrile transversion. The acrylonitrile interchange of sister chromatids has been shown in cultured human lymphocytes [35, 36]. Recently, data in the literature based on studies in animals or humans, concerning the risk of cancer development after inhalations of acrylonitrile, have re-considered some older conclusions [37]. These data, as far as humans are concerned, indicate that there is no clear relationship between exposure to acrylonitrile and cancer, even if these studies were not able to positively exclude a certain small increase in the development rate of cancer [17]. In experimental animals, it has been clearly demonstrated that inhaled acrylonitrile is a carcinogen. The EU Commission comments on this subject as follows: The possibility that acrylonitrile is a lung or prostate carcinogen is not easy to exclude [38, 39].

Aliphatic Nitriles

Aliphatic nitriles, saturated or unsaturated, induce serious damage in fetuses during the gestation period. Saturated nitriles are acetonitrile, propionitrile and v-butylonitrile, while unsaturated nitriles are acrylonitrile, methacrylonitrile, cis-2-pentanonitrile and chloroacrylonitrile. Ten-days old rat fetuses have been cultured for 46 hours in rat serum, containing one of the above mentioned substances. All of these substances, according to their concentration, have caused limitations in fetal development and increased morphological abnormalities. There was no common damage pattern for all tested in vitro nitriles. All nitriles studied have induced the characteristic abnormalities shown in fetuses when they are exposed to sodium cyanate [40].

Methacrylonitrile

Methacrylonitrile is one of the major industrial aliphatic nitriles which are being used for the production of co-polymers, elastomers and plastics, as well as acrylic fibers. Both acetylonitrile and methacrylonitrile induce a similar increase in cell

proliferation, an increase in the apoptotic index of the exfoliative epithelia in rats exposed to methacrylonitrile (which induces stomach cell proliferation), and in parallel, an increase in apoptosis. At the experimental level, male rats are more sensitive than female to methacrylonitrile. Oral administration of the substance induces clinical symptoms such as spasms, ataxia and breathing abnormalities. It can also cause normocytic normochromic anaemia. In the peripheral blood a dose-dependent increase of thiocyanate and thyocyanide has been found. This is due to metabolism of the methacrylonitrile to cyanide. The liver is a target organ for methacrylonitrile toxicity. Another target of methacrylonitrile toxicity is the olfactory epithelium in which necrotic metaplasia has been detected.

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Chapter 13
Toxicity and Carcinogenicity of the Eterocyclic Organic Compounds

Furan: Toxicity and Carcinogenicity

Furan is a cyclic dien-ether in liquid form with an etheric odour; it is non soluble in water but soluble in alcohol, acetone, benzene and ether [1]. Furan enters the human organism through the respiratory pathway. It is present in tobacco smoke and exhaust gases and it is detected in the exhaled air of smokers and non-smokers. Smokers exhale 0.25–98 μg/h while non smokers exhale 0.33–28 μg/h.

Furan is considered to be a carcinogen in humans. Its carcinogenicity has been described in the literature [2, 3] which documents malignant tumor development in experimental mammals exposed to it. Two years of oral administration [3] of furan induces hepatic cholangio-sarcoma, hepatocellular adenoma and carcinoma [4, 5] as well as monocytic leukemia in rats F344N [6, 7]. It can also induce a dose-dependent hepatocellular adenoma and carcinoma in mice B6C3F1 [3]. There are not sufficient data to substantiate the carcinogenic activity of furan in humans. In bacteria, furan can induce gene mutations [8, 9]. In in vitro studies with mice lymphoma cells [10] furan induces gene mutations and in the ovary cells of Chinese hamsters, DNA damage [3]. In in vivo experiments in mammals, furan induces chromosomal deletion in the bone marrow cells of B6C3F1 mice [3]. Recent studies [11] have described its metabolic activation by the cytochrome P450 into a cytotoxic product which stimulates cell replication [12, 13]. The active metabolite of furan is cis-2-butadiene-1,4-diale [12]. Its metabolic activity may possibly clarify its joining capacity with proteins, *in vitro* and *in vivo* [14, 15].

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J.C. Stavridis, *Oxidation: The Cornerstone of Carcinogenesis*, 181–182.

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Chapter 14
Toxicity and Carcinogenicity of Metals

Certain metals which exist in cigarette smoke and which are inhaled in smoking cause serious diseases, including lung cancer [1–4]. Inhaled metals are not biodegradable and as a result they are deposited and remain for a long period in various areas of the pulmonary tissue. Some metals, including zinc, copper, iron, calcium and trivalent chromium actively participate in diverse important cellular activities, such as gene transcription control, neural conductivity, oxygen uptake from the lungs and transfer to peripheral tissues, various enzymic functions, cellular respiration, oxidation-reduction activities and control of cellular apoptosis. Some toxic metals are able to mimic the functions of "useful" bio-metals and, as a consequence, to substitute them into the various cellular processes, thus causing serious malfunctions to different vital cellular activities. In this way, toxic metals are able to activate and/or deactivate cellular functions controlled by other non-harmful metals which are useful to life. In some cases, biologically useful metals could, under certain conditions, become toxic such as chromium, the function of which is described in the following paragraphs. In addition, metals may cause cancer by disrupting genetic integrity, but their exact genotoxic mechanism is not yet known. Nevertheless, oxidative damage of genomic DNA has been extensively studied. The importance of the reactive oxygen species (ROS) in metal bio-toxicity results from the fact that they participate, in all cases, as cancer causing agents [5, 6] as well as in the multi-stage carcinogenic process that includes activation of carcinogenic compounds, oxidative DNA damage and tumor development [6].

Toxicity of Arsenic

Arsenic affects the normal operation of the cellular cycle: it splits the mitotic spindle in physiological human diploid fibroblasts [7, 8]. The rupture of the mitotic spindle causes delayed passage through the M-phase. In lymphocytes, mitotic rupture is accompanied by the termination of their proliferation. The examination of arsenic-treated cells under an ordinary microscope reveals an accumulation of mitotic cells and the exclusion of iodine propide. Their binding with Annexin-V implies the existence of a healthy membrane with the "flipping" of the membrane-bound phosphatidyl-serine (expected in the early stages of apoptotic cells). Apoptosis can

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also be confirmed by flow cytometry as well as with the TUNEL assay: positively stained cells with accumulations of DNA fragments (expected in the early stages of apoptotic cells). Arsenic disrupts mitosis and when the cells have a negative expression of the p53 phenotype, apoptosis is caused. Such cells are predisposed to the future growth of cancer. Indeed, chronic exposure to arsenic is related to lung, urinary, bladder and kidney cancer [9]. It has been shown that arsenic promotes the production of heme oxygenase—a fact which implies the presence of oxidative stress [10]. It also increases the level of intracellular superoxide [11, 12] and the production of NO which is suppressed by NO synthase inhibitors [13].

Mechanisms of Gene Damage

Arsenic causes complicated toxic cell damage [14] mainly due to oxidative stress, (chromosomal deviation and aneuploidy). These responses depend on changes in gene expression. In a study of 133 target-genes, 94 revealed a positive response to arsenic while 39 were suppressed [14]. These genes belong to the groups of signal transfer, cell cycle transcriptional control and stress response. Chronic expose to arsenic results in chromosomal damage of the peripheral lymphocytes [15–17]. There is a positive relationship between the chromosomal damage of the peripheral blood lymphocytes caused by arsenic and an increased risk of cancer [18]. Arsenic causes the termination of the cellular cycle [19], mitotic function disorders [19, 20], aneuploidy [19], cell skeleton rupture [21, 22] and apoptosis [12, 23]. There is also serious evidence that the reactive oxygen and nitrogen species are involved in arsenic-induced cell damage [11, 24].

Toxicity Mechanisms of Arsenic Compounds

Recent studies have shown that arsenic compounds induce the production of nitric oxide (NO), which causes DNA damage by activating poly-(ADP-ribose)-polymerase. Poly-ADP-ribosylation is a transcriptional modification of nuclear proteins catalyzed by poly-(ADP-ribose)-polymerase. Ribosylation is responsible for cell response to oxidation [25] that is responsible for arsenic toxicity [10, 12, 26] and the different cellular disorders caused in humans [27–30]. Polymerase activation leads to NAD consumption, which results in ATP depletion that finally leads to cell death [31, 32]. Ribosylation also plays an important role in DNA replication, gene expression [33] and carcinogenesis [27]. Arsenic acts as a promoter of cancer development [34]. The consequences of exposure to arsenic disappear when one is no longer exposed to it [35]. Elderly individuals are more susceptible to the carcinogenic action of arsenic than younger people [36]. Studies involving industrial workers have shown that there is a synergistic action between exposure to arsenic and smoking [37, 38]. At relatively high doses and short exposure times, arsenic stimulates JNK activity by inhibiting JNK phosphatase [39] or by stimulating mitogen-activated ERK kinase (MEKK) activity [40]. Arsenic activates the

mitotic extracellular signal-regulated kinase (ERK) cascade through a Ras- dependent pathway that possibly involves the participation of epiderma growth factor receptor (EGFR) [41]. It also activates the P38 cascade through oxidative stress, by a mechanism which is also Ras- dependent [42]. It has been recently reported that exposure to arsenic results in the expression of “stress” proteins through various signalling cascades [43, 44]. Many transcriptional control genes have shown increased activity—a fact that indicates that arsenic triggers the activation of signalling cascades causing increased levels of gene expression. High arsenic levels repress cellular response to stimulants such as inflammation cytokines [45] and growth factors [46, 47].

DNA Methylation by Arsenic

Inorganic arsenic is found in nature in two forms: the pentavalent (As^{5+}) and the trivalent (As^{3+}). The latter form is the most toxic. Both forms are subject to enzymic methylation. Methylation contributes to the control of specific genes, including certain tumor genes.

Since methylation of both DNA and arsenic share the same methyl donor, patterns of irregular methylation such as hyper or hypomethylation, have been detected in arsenic- treated cells [48–50]. These methylation patterns appear during DNA replication. A methylated double helix needs two successive replications in order to be completely de-methylated. Alterations caused by DNA methylation occur in the cells during their exposure to arsenic [48–50]. During hypermethylation in the promoter region of tumor suppressor genes, the suppressor function is lost because methylation of CpG islands (page 186) inhibits gene transcription [51–53]. The suppression of points which control tumor genes allows the accumulation of mutagenic material that is responsible for genotoxic activity which is usually related to exposure to arsenic.

According to Mass and Wang [48], since arsenic loses its toxicity when it is subject to methylation by methyl-transferase and S-adenosyl-methionine (SAM), (both are methyl group donors), it has been concluded that the mechanism of carcinogenesis by arsenic can be attributed to alterations in the methylation of suppressor genes. The binding of methyl groups by arsenic, does not allow DNA to maintain its methylated cytosines resulting in hypomethylation that leads to enhanced expression levels for certain genes, possibly tumor genes, and finally to cancer. DNA hypomethylation, is accompanied by an increase in chromosomal fragility which results in genetic instability, (which acts by itself as a stimulus that initiates or promotes carcinogenesis) [54]. Arsenic methylation catalyzed by MTase in the presence of adenosine-methionine, results in the production of reduced toxicity products such as methyl- and dimethyl-arsenic acid [55, 56]. Despite the fact that methylated arsenic is less toxic [57] there are no indications of low carcinogenicity for the methylated arsenic compounds. Arsenic-induced carcinogenesis includes alterations of DNA methylation patterns, probably in tumour suppressor genes, such as p53. Arsenic biotransformation also involves the SAM/MTase-cytosine pathway

through the transfer of methyl groups from SAM to cytosine and the production of 5-methyl-cytosine (5-Mec). The suppressor gene p53 that is also a target for arsenic, is as well involved in the aetiology of certain cancer types, such as lung cancer.

Arsenic also reacts with the sulfydryl groups; it easily binds with glutathione and other thioles and inactivates enzymes that possess active thiol sites [58]. The regulation expression of many genes is controlled by cytosine methylation levels in long cytosine rich DNA areas, (known as CpG islands), particularly in the promoter regions. Obviously, 5-methyl-cytosine alters the binding capacity of some transcription factors and the concomitant gene expression. Arsenic can cause DNA hypermethylation [48] and also hypomethylation [49]. Hypermethylation and hypomethylation co-exist in cells grown in the presence of sodium arsenate. There is an imbalanced situation which interrupts regular gene expression in cells exposed to arsenic.

Effect of Arsenic on the Cellular Cycle

It is well known that the cell cycle is regulated by kinase-engines and by regulating cyclines [59].

In order to avoid the reproduction of cells which suffer genetic damage, the cell cycle is controlled by a large number of control genes. These genes belong in categories such as signal transduction, transcription, cell cycle control, "stress response" and proteolytic enzymes [60].

Positive and negative cell cycle regulators are activated by arsenic—a fact which implies that cell exposure to arsenic results in deregulation of the cell cycle, which as a result, plays an important role in neoplasmic transformation.

Conclusions

The biotransformation of arsenic utilizes methyl groups from the cell. Chronic exposure to arsenic causes hypomethylation of DNA resulting in pathologic gene expression, which finally leads to cell transformation. This mechanism is the major cause of arsenic action.

Toxicity of Mercury

Mercury is an immunotoxic agent with a complex immunotoxicity which includes both immunosuppression and immunoactivation. Agents with genetic susceptibility related to the major histocompatibility complex (MHC) as well as genes not related to the MHC complex, contribute to the appearance of systematic autoimmune disease which is related to mercury. Mercury concentration, as well as its chemical nature, greatly affect its immunotoxicity. Both the cellular target (for example, B-cells are more susceptible than T-cells) as well as the activation state of the cells affect the immunotoxicity of a certain mercury concentration. The apoptotic necrosis pathway

CD95 is a major regulatory mechanism that controls the life span of activated lymphocytes. Malfunctions in this pathway are related to autoimmunity [61, 62]. Apoptosis occurs when the extent of damage overcomes the repairing capabilities of the cell. The suppression of apoptotic response facilitates the accumulation of cells that deviate from their physiological function and this fact is of extreme importance in the development of malignancy or autoimmunity.

Genetically susceptible rats exposed to inorganic mercury (Hg^{2+}) develop systematic autoimmune disease, which is characterized by pathologic lymphocyte proliferation and the accumulation of autoreacting lymphocytes [63, 64]. The inhibition of apoptosis by mercury (Hg^{2+}) via the CD95 pathway has been confirmed using criteria such as DNA fragmentation, nucleus condensation, phosphatidyl-serine externalisation and PARP degradation. Mercury either directly or indirectly is related to apoptosis which is caused via the CD95 pathway by down-regulating the element that signals the formation of the necrotic signaling complex near the membrane (DISC) and by upregulating via the activation of caspase-3.

Toxicity of Cadmium

Tobacco plants absorb large quantities of cadmium (Cd) from the soil. Tobacco leaves contain approximately 0.7μg/g Cd. Processed tobacco leaves contain 0.45–3.1μg of Cd per cigarette [65]. During burning and mainstream smoke inhalation, the smoker is exposed to significant quantities of the metal. Cadmium is among the most toxic compounds in cigarette smoke. The daily Cd intake by the smoker is dose-dependent on the number of cigarettes smoked [66]. The concentration of cadmium in the urine of smokers is almost the same as that of non-smokers [67]. Cadmium levels in the lipoid tissue of smokers is four times higher than that of non-smokers (10ng/g) [68]. Higher cadmium concentrations in smokers have also been found in the renal cortex and the prostate [69]. Cadmium levels of 3.0μg/g dry tissue have been found in the pulmonary tissue of smokers compared to 1.1μg/g in non-smokers. This finding can be used as an marker of pulmonary damage [70], since the life span of Cd in human lungs is calculated as equal to 9.4 years. It is well known that cadmium chloride is related to human lung and prostate cancer [71] as well as to damage in the kidneys, liver and testicles [72]. Catalase, D-mannitol (scavenger of OH radicals) and butylated hydroxytoluene (an antioxidant agent) reduce the chromosomal anomalies caused by cadmium [73]. Superoxide dismutase also reduces the single strand DNA breakage that is also of cadmium exposure aetiology [74].

Cadmium Genotoxicity

The basic mechanisms involved in carcinogenesis due to exposure to cadmium are gene deregulation, oxidative stress, caderin disruption and the inhibition of DNA repair and its contribution to apoptosis.

Gene Deregulation and Information Transfer

The deregulation of gene expression is considered as the most important factor in a multi-stage model of chemical carcinogenesis. In particular, the induction of cellular proto-oncogenes [75] and the stimulated proliferation of the cells involved [76], play a very important role in the proliferation process stage, after an initial mutagenic incident.

The early and immediate response genes (IERGs) are primary-oncogenes which undergo early transcriptional activation when resting cells are exposed to mitogenic compounds, such as cadmium. They code for transcription factors and play an important role in chemical carcinogenesis. Genes related to cadmium toxicity and carcinogenicity are the c-fos, c-jun, and c-myc. Cadmium causes hyperexpression of these genes [77, 78]. The products of IERG genes constitute mitogenic growth signals that stimulate the proliferation of cells and they are considered to be important factors in a multi-stage carcinogenic model [75].

Cell exposure to cadmium induces the expression of many "stress" genes, such as the genes that code for metallothionein, genes that code for heat-stress proteins (HSPs), those that participate in oxidative stress response or those that participate in glutathione synthesis (GSH). "Stress" genes are induced only at high cadmium concentrations.

Genes that code for metallothionein (a low mw protein comprised of 30% cystein) are induced by hypotoxic cadmium concentrations. Metallothionein abundance protects rats against cadmium-induced lung carcinogenesis, while the corresponding transcriptional loss is responsible for the susceptibility to lung cancer due to cadmium [79].

Heat shock proteins are cellular chaperones induced by hyperthermia and other environmental stress, such as exposure to toxic chemical compounds. The induction of these proteins is considered as an adoptive response which leads cells to perform only elementary survival functions under stressful conditions in which cadmium exposure is included. Inside the cell cadmium induces the production of denatured or pathologic proteins by reacting with adjacent thiolic groups or by substituting zinc (Zn) in protein molecules that contain zinc (HSP induction signal) [80]. Cell exposure to cadmium results in significant induction of genes HSP_{10}, HSP_{32}, HSP_{40}, HSP_{60}, HSP_{70}, HSP_{89}, HSP_{90} and HSP_{110}.

Genes that Control Glutathione and Thiol Proteins

Glutathione and other proteins that contain the thiol groups are key players in cellular defense against cadmium toxicity and carcinogenicity. The ionic cadmium [Cd^{2+}] which is considered responsible for its toxicity and carcinogenicity is eliminated by glutathione and thus its reaction with important cellular targets is prevented. The GSH reducing cycle which includes glutathione peroxidase and glutathione reductase (Fig. 6.2 page 121) renders cadmium-induced ROS atoxic,

protecting the cells from toxicity and carcinogenicity. Cell exposure to cadmium induces the genes for γ-glutamino-cystein synthase (γ-GCS), glutathione-S-transferases (GST-α and GST-π) as well as increased glutathione production which quickly and effectively eliminate Cd^{2+} toxicity [81, 82]. Frequent exposure to cadmium, however, may overcome the beneficial effects of glutathione and related defense mechanisms and cause toxicity and carcinogenicity [83]. In general, the expression of anti-oxidant genes such as those that code for superoxide dismutase and catalase, is suppressed by cadmium [84–86], and this fact can be considered as the main cause for cadmium-induced lipid peroxidation, oxidative stress and concomitant toxicity.

K-ras and P53 Genes

The K-ras and p53 genes are among the most frequently mutated genes in human cancers, especially lung cancer. Recent studies have shown that following exposure to cadmium, there were no mutations in the codons for K-ras and p53 genes, indicating that cadmium is a weak mutagenic agent [87–90]. It inhibits DNA repair and as a result induces genotoxicity [91, 92] without itself being genotoxic [93].

Transcription Factors

The proto-oncogenes c-Fos and c-Jun code for proteins which are members of the AP-1 element that functions as a transcription factor regulating the expression of a large number of genes which control cell development and division [94]. Cadmium is a strong inducer for the c-Fos and c-Jun genes and constitutes a major mechanism for cell metamorphosis and oncogenesis. Of course, other transcription factors are also induced by cadmium, such as NF-kB and the metal transcription factor (MTF1) [95, 96]. Cadmium limits the binding ability of transcription factors with DNA [97–99].

Translation Factors

The complete process of protein synthesis or translation (initiation, elongation and completion of amino acid chain synthesis) is regulated by the expression of a number of genes which are collectively called translational factors. Two of these genes, the translation initiation factor (TIF3) and translation elongation factor 1-δ are overexpressed in the BALB/c-3T3 cells which are transformed when exposed to $CdCl_2$ [100, 101].

Suggested Genotoxicity Mechanism

Cadmium activates the expression of many proto-oncogenes, including c-fos, c-jun and c-myc as well as the tumor suppressor gene p53 [102–108]. Today, it is believed that the mechanism of cadmium action is indirect, possibly acting through

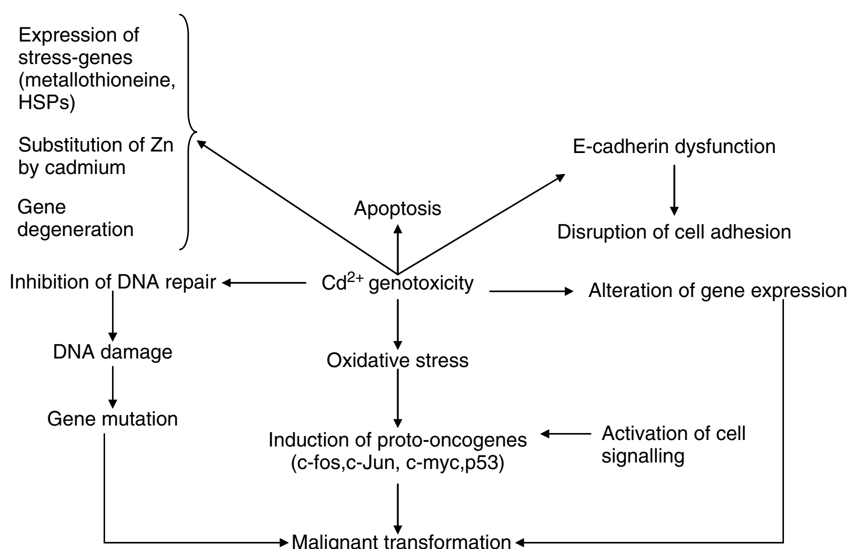


Fig. 14.1 Proposed genotoxicity mechanism of cadmium. (HSPs = Heat-shock proteins, ERGs = Immediate response genes)

metalloprotein [109], or through the substitution of Zn^{2+} by cadmium in transcription factors proteins. Another suggested mechanism is the induction of proto-oncogenes through the mobilization of intracellular calcium [110, 111]. In human cancer cells, gene shifting is observed. The shifting mechanism remains unknown.

It is supposed that the shifting creates a transformed phenotype through the expression of the corresponding gene. Both c-myc and c-jun RNA are cell nucleus transcription factors necessary for cell transition from the rest stage (G_0) to the proliferation stage (G_1), and consequently, their overexpression alters the cell cycle. The frequency and overexpression of c-myc and c-jun RNA levels are the same as the frequency and levels of the observed shift. While cadmium does not seem to exert transforming action through the mutation of H-ras and p53, cadmium-induced cell transformation can be attributed to gene shifting and to c-myc and c-jun overexpression (Fig. 14.1).

Mechanisms Which Alter Gene Expression in the Presence of Cadmium

Many mechanisms that involve secondary messengers such as the ROS, intracellular Ca^{2+}, transcription factors and cellular information cascades such as kinases and DNA cytosine methylation, are considered to be responsible for cadmium-induced deregulation of gene expression. Cadmium exposure results in increased intracellular Ca^{2+} levels [111, 112]. Ca^{2+} directly deregulates gene expression by reacting with specific response elements such as the CREB factor (c-AMP-response element binding protein) found in the promoter region of these genes [113]. Alternatively,

cadmium effects can indirectly be realized through the activation of protein kinases which cause overexpression by phosphorylation of the various transcription factors [114]. In addition, cadmium activates calmodulin-dependent target genes by its ability to mimic calcium ions [115]. Cadmium, although not a Fenton-metal, creates the ROS which can affect gene expression. For example, the AP-1 element which is comprised of proteins coded by c-Fos and c-Jun, is a transcription factor susceptible to the redox system of the cell, whereas the participation of the ROS in the transcriptional activation of AP-1 by cadmium is well known [111]. The activation of the protein kinases in the cell, which leads to the previously mentioned transcription factor phosphorylation, and the transcriptional activation of target-gene expression, are considered to be the main gene expression regulation mechanisms by cadmium. The kinases that are activated during the cell's exposure to cadmium include protein kinase C [99, 116, 117], stress-activated protein kinase [118], tyrosine kinase, casein kinase-II [119] and three more kinase types that are activated by the mitogens of the MAPK family, (such as the protein kinase that is activated by extracellular ERK signals, the JNK and the P38 MAP kinase) [120–122].

Disruption of E-cadherin-mediated Cell Adhesion

According to Pearson and Prozialesk [123], cadmium carcinogenesis involves E-cadherin, a molecule that participates in tumor penetration (Fig. 14.1). E-cadherin is a Ca^{2+}-associated transmembranic glycoprotein which participates in calcium-dependent cell-to-cell adhesion. The intracellular part of the E-cadherin molecule is bound to the actin of the cell skeleton through molecules called catenins. The extracellular part of E-cadherin has Ca^{2+} binding sites as well as an area through which the molecule adheres to the adjacent cell. Ca^{2+} adhesion to E-cadherin reduces the flexibility of the protein molecule and limits the space available for uniform cell-to-cell adhesion [124]. E-cadherin plays the role of signaling molecule which regulates cellular permeability and polarity [125]. The dissociation of cell adhesion through E-cadherin signals the activation of genes through ÿ-catenin, which functions at the early stages of tumor initiation. ÿ-catenin modifies the expression of many genes, including c-myc and c-jun.

Cadmium is associated with the polypeptide that corresponds to one of the extracellular regions of E-cadherin which bind to Ca^{2+} thus altering its 3D-structure [126]. In addition, it destroys the E-cadherin-dependent junctions between cells in epithelial cell cultures [127].

Cadmium and Apoptosis

Cadmium causes apoptosis through a mitochondrial- dependent pathway, a fact verified by the activation of caspase-9 in HL60 cells in the presence of cadmium [128] (Fig. 14.1). In contrast, it has been proven that cadmium induces apoptosis in normal human lung cells through a mechanism which is used in the mitochondria, but

independent of caspase activity [129]. The hypothesis that cadmium causes apoptosis through the mitochondria is verified by the fact that the presence of the anti-apoptotic protein Bcl-2 protects the cells from cadmium-induced apoptosis [130]. In addition, the pre-apoptotic protein Bax is induced by cadmium in primary lung epithelial cells [131].

Cadmium-induced apoptosis is not necessarily the only protection against malignant transformation. Only a fraction of the cells become apoptotic, while 25% show increased metallothionein and remain alive.

Suppression of DNA Repair by Cadmium

The inhibition of DNA repair has been suggested as a mechanism that contributes to cadmium genotoxicity [132]. There are 4 mechanisms of DNA repair: repair by base excision, repair by nucleotide excision, recombination repair and repair in erroneous base pairing.

Low cadmium concentrations repress base excision DNA repair [133]. As far as nucleotide excision DNA repair is concerned, cadmium suppresses the removal through the excision of thymine dimers. Low concentrations of cadmium suppress the special binding of the repair proteins with the DNA, while this malfunction is reconstituted with Zn. Cadmium reduces the cell's ability to repair the mis-pairing dGTP oxidation yields of 8-oxo-dGTP which are mistakenly incorporated into the DNA and cause AT $\rightarrow$ CG transitions. The cells are protected from 8-oxo-dGTP by 8-oxo-dGTPases. The 8-oxo-dGTPases are inhibited by cadmium and this fact constitutes an additional mechanism which contributes to the mutagenic and carcinogenic potential of this metal [134].

Reactive Oxygen Species (ROS) and Cellular Antioxidant System

The carcinogenic action of cadmium is related to the production of the ROS. Cadmium produces hydroxyl radicals [135], superoxide radicals, nitric oxide and hydrogen peroxide [136, 137]. It increases the levels of lipid peroxidation in the liver in humans [138] and in the mitochondria of the rat liver [84]. It reduces intracellular glutathione and significantly inhibits the activities of superoxide dismutase, glutathione peroxidase and catalase [139].

The cadmium-induced reduction of intracellular antioxidant levels, in combination with the production of free radicals during normal cell metabolism, can explain the increase of lipid peroxidation and DNA damage. Cadmium activates the expression of many genes, including genes which code for antioxidant proteins.

Cadmium and Emphysema in Smokers

Cadmium is an important polluting compound in cigarette smoke and it is believed that it plays a significant role in the pathology of emphysema in smokers [140]. It

seems that the mechanism of cadmium- induced emphysema involves the suppression of the production of proteoglycan and procollagen by the lung fibroblasts [141]. It is a well known fact that the proteins of the lung connective tissue are not inert proteins, but they form dynamic structures which are continuously composed and decomposed during a person's lifetime [141]. The imbalance between composition and decomposition results either in the deposition or the loss of connective tissue and these alterations are considered important in the pathology of lung fibrosis and emphysema, respectively [142]. Thus, a part of cadmium action in the lung is exerted on the above mentioned fibroblasts—the only connective tissue producing cells in the lungs. Cadmium suppresses fibroblast proliferation and procollagen synthesis [143]. This procedure is especially active in smokers who constitute the largest population group susceptible to emphysema. Smoking comprises the greatest cadmium source to which the smoker is continuously exposed. The mean life span of cadmium in a biological system is approximately ten years [144]. There are remarkable similarities between the damage observed in laboratory animals that are chronically exposed to cadmium and that in the centrilobular area of the lung tissue in smokers [145].

Cadmium Synergy with Other Metals

Some metals (cobalt, arsenic, nickel and cadmium) possess a weak mutagenic activity. Despite this, they have the ability to induce diverse damage in living cells, attributed to DNA oxidative damage [146, 147] or to gene silencing due to alterations in DNA methylation patterns [48, 148]. Metals suppress DNA repair processes, and this is caused by the co-mutagenic actions of arsenic (III), cobalt (II), nickel (II) and cadmium (II) in mammalian cells [149].

Nickel Toxicity

The amount of nickel that the tobacco plant absorbs from the ground is quite significant. As a result, tobacco leaves contain large amounts of nickel [150], with 0.64 and 1.15μg/g per dry leaf. During smoking, tobacco smoke contains approximately 75ng per cigarette [150]. Other studies have shown higher Ni content in inhaled smoke, varying between 2.78 and 4.84μg per cigarette. Epidemiological studies as well as experiments in rats have shown that the exposure of humans and laboratory animals to an environment with nickel-containing particles results in lung damage [151–153]. Following the inhalation of these particles, the accumulation of neutrophilic granulocytes, increased protein concentration [154] as well as prostaglandin and cytosine production are observed in the lungs. The response to these processes is the development of oxidative stress [155, 156]. Nickel (as well as other metals), catalyzes the formation of the reactive oxygen species (ROS) that cause lipid peroxidation. Carbonyl compounds, one of the lipid peroxidation byproducts, are produced from the lipids [157] and proteins [158] of the cell

membrane. Some carbonyl compounds such as acetaldehyde, participate as mediators to biological response, (which is the prostaglandin synthesis from the airway epithelial cells) [159].

Chromium Toxicity

Chromium salts, among which is chromium dichromate ($K_2Cr_2O_7$), comprise an example of mutagenicity, genotoxicity and carcinogenicity [160, 161]. Cr (VI) as chromate salt ($Cr^{vi}O_4^{2-}$), easily penetrates the cell membrane, probably through the ion channels [162]. Intracellular chromium reduction is considered as a necessary step for the carcinogenic action of the ion metal, while the intracellular reduction product of Cr (VI) is Cr (III) [163]. The intermediate products of the biological reduction of Cr (VI) include Cr (V), Cr (IV), sulfur radicals (RS·), carbon centered radicals (R·) and the ROS (·OH, $O_2^{-\cdot}$, H_2O_2) [164, 165]. Cr (VI) is reduced in the liver through the univalent reduction pathway [166] (Fig. 14.2). Low molecular weight cellular constituents reduce Cr (VI) in vitro at normal pH. These compounds are glutathione (GSH) [167, 168], cysteine [169], lipoic acid [170] and molecules that contain diols, NAD(P)H, ribose, fructose and arabinose [171, 172]. Only those components which react with Cr (VI) at a relatively high rate are capable of substantially contributing to Cr (VI) reduction in the various cellular systems. Among these compounds, ascorbate and GSH are the best non-enzymic candidate compounds. The reduction of Cr (VI) by GSH creates glutathionyl radicals (GS·) [167, 168], Cr (V) and Cr (IV) complexes [173]. These two stable compounds (Cr V) and Cr (IV) are used as models for the study of the role of intracellular Cr (IV) and Cr (V) in the mechanisms of carcinogenicity by Cr (VI). Another important reducing compound for Cr (VI) is ascorbate [174]. The reduction of Cr (VI) by ascorbate has many advantages over GSH [175]. The ascorbate reaction products, Cr (V) and Cr (IV) react with H_2O_2 and produce hydroxyl radicals (OH) which cause DNA fragmentation [174] (Fig. 14.2).

Reduction of Cr (VI) by Reducing Enzymes, Organelles and Intact Cells

A variety of enzymic and non-enzymic factors can function as Cr (IV) reducing agents. These factors include microsomes, the mitochondria [176] and many flavoenzymes such as glutathione reductase (GSSG-R), lipoyl-dehydrogenase and $NADP^+$ ferroredoxin oxidoreductase [177–179]. In the presence of NAD(P)H, glutathione reductase reduces Cr (VI) to Cr (V) which is identified as the Cr (V)-NADPH complex. During the overall reduction process, molecular oxygen is reduced to $O_2^{-\cdot}$, which is dismutated to H_2O_2.

The most direct indication of Cr (VI) reduction has been established by the production of Cr (V) in laboratory animals that were administered Cr (VI) [180]. Cr (V)

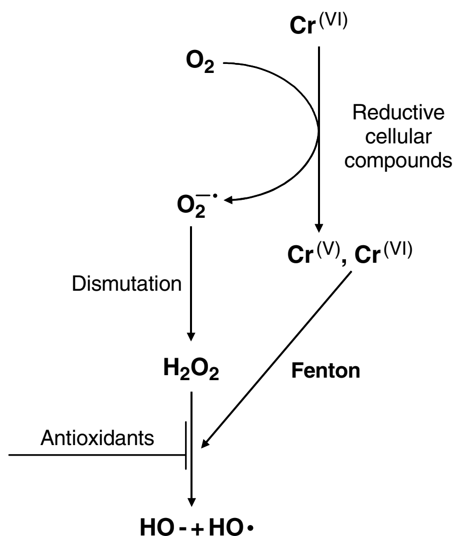


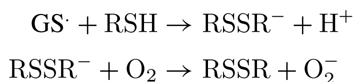
Fig. 14.2 Cr (VI) reduction in the liver through the univalent reduction pathway

was mainly detected in the liver and a small quantity in the blood. These chromium amounts were found in the form of Cr (V)-NADPH complexes with a hydrogen bond at Cr (V) [181].

Production of Free Radicals

Production of Thiol Radical (GS·)

With the use of EPR (spin trapping), the formation of Cr (V) and GS· radicals from glutathione and secondly from cysteine, has been verified. The GS· root causes direct cellular damage, by reacting with thiol molecules and producing O_2^-.



$$GS^{\cdot} + RSH \rightarrow RSSR^- + H^+$$
$$RSSR^- + O_2 \rightarrow RSSR + O_2^-$$

The formation of $O_2^{-\cdot}$ leads to the formation of H_2O_2. Superoxide radicals may further increase by reducing Cr (VI) to Cr (V) and subsequently reacting with H_2O_2.

Production of Hydroxyl Radicals by Cr (V) Reactions

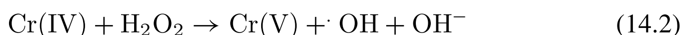
The production of hydroxyl radicals (·OH) is believed to be the result of the reaction between Cr (V) and hydrogen peroxide through a Fenton-type reaction.

$$Cr(V) + H_2O_2 \rightarrow Cr(VI) + {}^{\cdot}OH + OH^- \qquad (14.1)$$

The Cr (V)-NADPH complex which results from the reaction between Cr (VI) and NADPH is used to verify the above reaction [184]. The activity of Cr (V) depends on its structure (186). For example, tetra-peroxy chromate { $[Cr(O_2^{2-})_4]^{3-}$} has a tetrahedral structure with all covalent bonds totally occupied by half of its O_2^-. The complex $[Cr(O_2^{2-})_4]^{3-}/H_2O_2$ does not easily split H_2O_2 to produce $^{\cdot}OH$. On the other hand, a Cr (V) complex such as Cr (V)-NADPH is octahedral, with one free binding site. H_2O_2 binds to this resonance site thus forming a long-living complex that produces OH radicals.

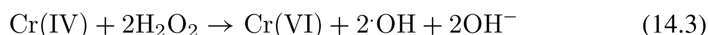
Production of Hydroxyl Radicals by Cr (IV) Reactions

Cr (IV) is another potent intermediate which is produced by the reduction of Cr (VI) by reducing compounds (such as ascorbate and GSH) in the cell. Cr (IV) can produce $^{\cdot}OH$ radicals from H_2O_2 through a Fenton-type reaction [174]:



$$\mathrm{Cr(IV)} + \mathrm{H_2O_2} \rightarrow \mathrm{Cr(V)} + {}^{\cdot}\mathrm{OH} + \mathrm{OH^-} \tag{14.2}$$

As long as Cr (V) is produced, it reacts with H_2O_2 and forms more OH radicals. By adding reactions (14.1) and (14.2), the final reaction is:



$$\mathrm{Cr(IV)} + 2\mathrm{H_2O_2} \rightarrow \mathrm{Cr(VI)} + 2{}^{\cdot}\mathrm{OH} + 2\mathrm{OH^-} \tag{14.3}$$

Production of Hydroxyl Radicals by the Reaction of Cr (III)

The lack of toxicity and carcinogenicity of Cr (III) in intact cells can be explained by the low uptake rate of this cation into the cell envelope. However, the existing Cr (IV) inside the cells is reduced to Cr (III). Cr (III) inside the cell can produce hydroxyl radicals from H_2O_2 in a pH-dependent process.

ROS Production in Intact Cells

When intact cells (Jurkat) are exposed to Cr (VI), hydroxyl radicals are produced [186]. $^{\cdot}OH$ as well as Cr (V) result from this reaction system. The Cr (V) produced is identified as the Cr(V)-NADPH complex. The addition of NADPH or glutathione reductase induces the production of Cr (V); this means that NADPH-dependent flavoenzymes participate in the formation of the Cr (V)-NADPH complex. The addition of hydrogen peroxide induces $^{\cdot}OH$ radicals. This fact verifies that Cr (IV) contributes to the production of $^{\cdot}OH$ radicals in the cellular system as well.

The Role of Free Radicals in Cr (VI)-induced Carcinogenesis – DNA Damage

When DNA is incubated with Cr (VI) and ascorbate, a significant number of DNA strand breaks is produced. The addition of hydrogen peroxide effectively promotes DNA damage. The number of DNA fragments is directly related to the number of free radicals produced. OH ions react with guanine at various positions producing a series of reaction products. Among them, the most well studied is 8-hydroxy-deoxyguanosine (8-OHdG) [187]. The formation of this adduct is considered as an marker of ROS implication in the toxic and carcinogenic mechanism of many compounds. It has been proven that the OH radicals created by Cr (V) and Cr (IV) cause the hydroxylation of 2-deoxyguanosine (dG) and the formation of 8-OHdG [188, 189].

NF-kB Activation

NF-kB is considered as a transcription factor found primarily in oxidative stress; it functions by promoting the transcription of many genes [190–192]. Cr (VI) can cause NF-kB activation in Jurkat cells [193]. ·OH radicals produced during the Fenton reaction from Cr (V) and Cr (IV) play an important role in the activation mechanism of NF-kB by Cr (VI). It should also be noted that the NF-kB binding sites are used as promoting elements for the c-myc oncogene, which is related to Burkitt's lymphoma [194]. Cr (VI) may also cause the expression of the c-myc oncogene through NF-kB activation.

AP-1 Activation

The AP-1 transcription factor is a dimer whose amino acid chain in its DNA binding site is comprised of Jun and Fos products. AP-1 activation leads to c-Jun and other oncogenes overexpression. A certain number of mitogen activated kinases (such as MAP kinase), participate in AP-1 activation through the regulation of kinase cascades. The amino-terminal end of c-Junk (JNK) and p38 kinases are activated by a special MAPK kinase (MAPKK) through the phosphorylation of threonine and tyrosine. MAPKK kinase is activated by another special MAPKK kinase (MAPKKK) through the phosphorylation of the amino acid threonine or serine. These results show that the ROS are used as an activation signal which initiates the activation of AP-1 and NF-kB in response to the Cr (VI) stimulus, while p38 and JNK act as executing kinases for the activation of AP-1 and NF-kB, respectively.

p53 Activation

The tumor-suppressor protein p53, plays an important role in protecting cells from oncogenic damage. In the literature it has been reported that 50% of all human

cancers involve a mutated p53 gene. This transcription factor is considered to be a response to oxidative stress and it is activated by various stimuli such as UV and γ-radiation or by the depletion of nucleotide reservoirs. Cr (VI) may activate p53 in human epithelial cells [165] (Fig. 14.4). Superoxide dismutase which produces H_2O_2 from superoxide radicals, increases p53 activity. Catalase which consumes H_2O_2 limits the production of ·OH radicals and represses p53 activation. NADPH which accelerates the one-electron reduction during transition from Cr (VI) to Cr (V) and increases the production of ·OH radicals, induces p53 activation. Consequently, the ·OH radicals produced during the reduction of Cr (VI) are responsible for P53 activation.

Tyrosine Phosphorylation

Tyrosine phosphorylation is a very important step in the regulation of many key cellular functions. It participates in the control of cell proliferation, differentiation, regulation of the cell cycle, signal transduction, metabolism, transcription, morphology, adhesion, ion channel regulation and cancer development. Cr (VI) increases phosphorylation levels in human epithelial cells in a time-dependent manner [195].

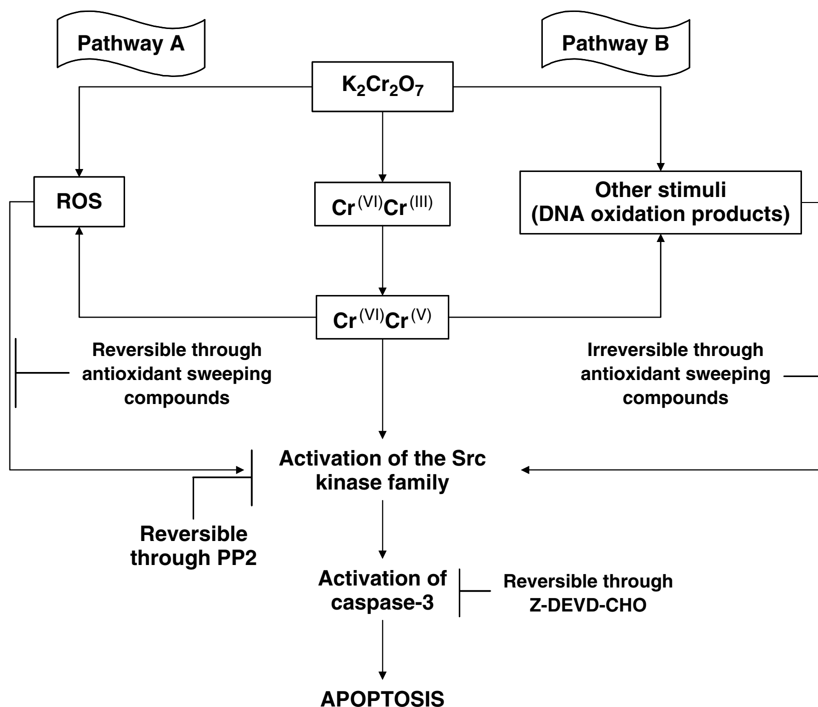


Fig. 14.3 Probable signaling pathway for the $K_2Cr_2O_7$ induced apoptosis. Pathway B is considered on the basis of antioxidants inability to block or inhibit the Cr (VI/V) induced apoptosis

N-acetyl-cysteine (NAC), a general antioxidant, inhibits Cr (VI)-induced phosphorylation. Catalase (H_2O_2 scavenger) and aspirin ($^{\cdot}OH$ radical scavenger) also inhibit Cr (VI)-induced tyrosine phosphorylation. There are strong indications for the increased activation of certain tyrosine kinases and caspase-3, as important steps towards apoptosis caused by $K_2Cr_2O_7$ (Fig. 14.3). The ROS do not seem to be the only stimulant in this process. Perhaps there are other stimulants, apart from ROS, that are involved in the activation of Src family tyrosine kinases and caspase-3 in Cr (VI/V)-induced apoptosis.

Apoptosis

Apoptosis is the process during which cellular death begins and ends in a predefined way through the activation or synthesis of gene products necessary for cell destruction. It is the response to physiological and pathologic stress which disturbs the balance between the rates of cell division and death, thus affecting the accumulation of neoplasmic cells. The two main mediators of apoptosis are p53 and ROS (Fig. 14.4).

It has been proven that Cr (VI) can cause apoptosis [186]. Chromium-induced apoptosis depends on the p53 state, since mutations in the p53 gene facilitate the development of resistance to apoptosis and increased survival of the damaged cells. The ROS produced from the reduction of Cr (VI) also activate p53 and, play an important role in the signalling of the apoptotic pathway (Fig. 14.5). The ROS produced from Cr (VI) initiate apoptosis before p53 activation. Although p53 is not necessary for the initiation of Cr (V)-induced apoptosis, it may induce apoptosis by activating the transcription of another redox gene. The ROS produced by Cr (VI) have a dual role in the mechanism of carcinogenesis: they cause genetic damage and apoptosis. Cr (VI)-induced carcinogenesis depends on the balance between these two processes. Apart from Cr (VI), other carcinogenic metals can cause apoptotic cell death [196, 197]. Although the apoptotic mechanism caused by the Cr (VI/V) complex has been extensively studied [198], an understanding of the chromium effect on cell signalling pathways is still incomplete.

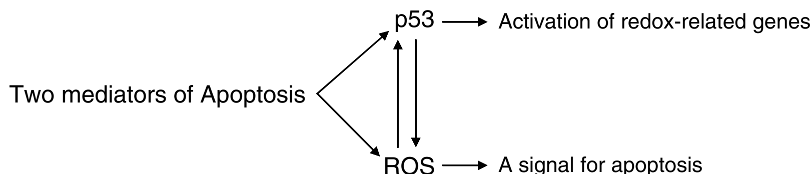


Fig. 14.4 The two most significant mediators of apoptosis, the ROS and p53

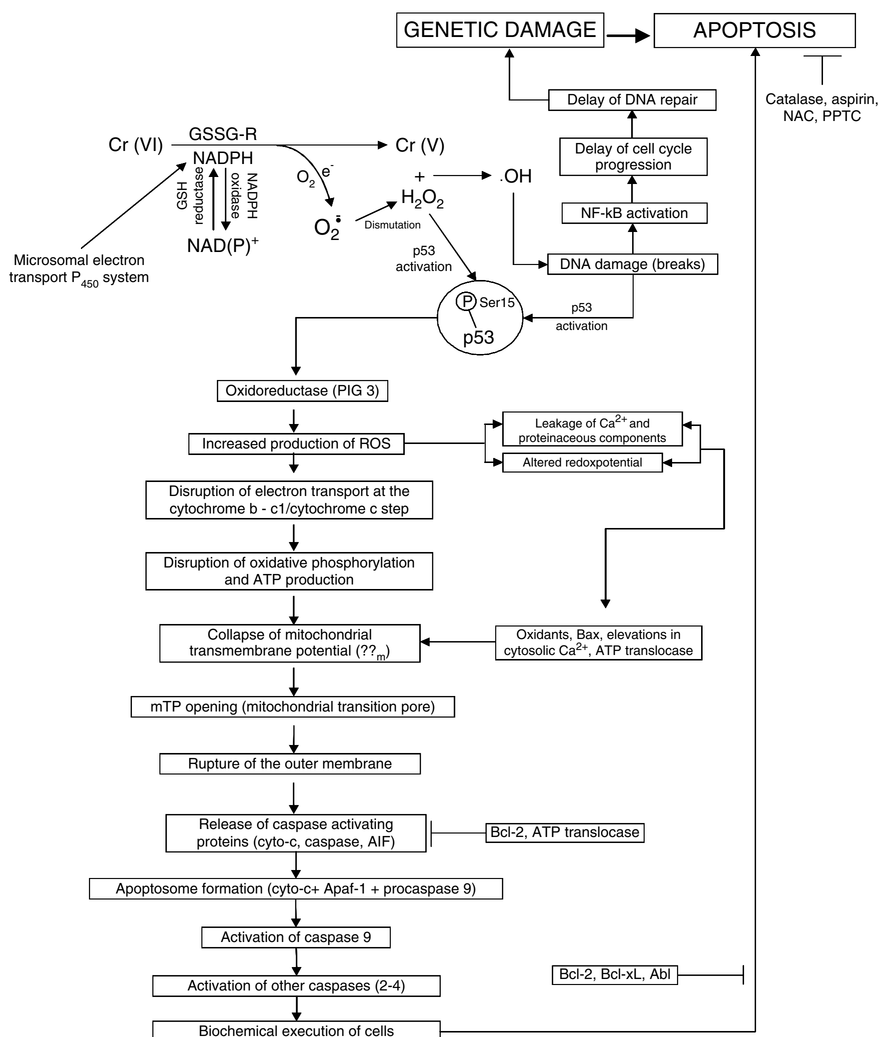


Fig. 14.5 The events in Cr (VI)-p53 activation leading to apoptosis and downstream gene regulation. The intermediate products of the biological reduction of Cr (VI) are Cr (V) and Cr (IV) and ROS ($^{\cdot}OH$, $O2^{\cdot -}$, H_2O_2). Hydroxyl radicals by ROS react with guanine of nuclear DNA and form 8-hydroxy-deoxyguanosine (8-OHdG). This DNA damage is recognized by a "sensor" molecule which identifies the lesion by the p53 protein. The sensor phosphorylates p53 and regulates a large number of downstream genes. Another route to apoptosis is through the collapse of the mitochondrial potential

Termination of the Cell Cycle

Under normal conditions, the cell cycle advances without interruptions. When there is damage, especially at the DNA level, most normal cells have the ability to stop their proliferation at the phases G_1/S or G_2/M and to resume when the damage becomes fixed. The cell cycle controls the initiation of DNA division and mitosis, in order to assure that the genome remains intact. Lack of fidelity during DNA division which may arise from a mutation, can lead to cell death or cancer. It has been verified that:

1) Cr (VI) can cause the end of the cell cycle at the phase G_2/M in human lung epithelial cells A549 [199].
2) Cr (VI) at relatively low concentrations causes the end of the cell cycle, while at high concentrations it causes apoptosis.
3) the ROS produced after cell exposure to Cr (IV) are involved in the ending of the cell cycle.

Hydrogen peroxide is a key molecule in this process.

The Portrait of Gene Expression

Many oxidative stress-related genes are activated in the presence of Cr (VI). These genes are Cu/ZnSOD, glutathione peroxidase and metallothionein IIA. Many calcium-related genes are induced by Cr (VI). Since calcium signalling goes along with the production of the ROS, the induction of genes related to calcium is an indication that the Cr (VI) causes oxidative stress to the cells. In addition, Cr (VI) induces many important genes in the intracellular kinase cascades. The proteins that correspond to these genes include the G-protein, Src and MAP kinases. The G-protein and Src kinase are involved in the regulation of cell proliferation and differentiation. Cr (VI) induces various oncogenes (such as Jun-B and raf) as well as genes involved in cell cycle regulation (such as cycline-dependent kinase genes).

Conclusion

Cr (VI) is reduced by various reducing agents inside the cell envelope towards lower oxidation states, such as Cr (V) and Cr (IV). During the reduction process molecular oxygen is also reduced to the superoxide radical ($O_2^{-\cdot}$) which dismutates to H_2O_2. Hydrogen peroxide reacts with either Cr(V) or Cr (IV) and produces $^{\cdot}OH$ radicals. Thus, Cr (VI) can produce a whole spectrum of the ROS. Biological systems are protected from the oxidative damage caused by free radicals by enzymic or non-enzymic antioxidants. When the balance between pre-oxidants and anti-oxidants yields towards the side of pre-oxidants, chromium-induced oxidative damage occurs. Through reactions induced by the ROS, Cr (VI) can cause DNA damage,

activate the nuclear transcription factors NF-kB, AP-1 and p53, induce apoptosis, cause overexpression of oncogenes, positively regulate antioxidant agents, activate certain enzymes which participate in MAPK signaling pathways, stimulate enzymes involved in cell cycle control and control mechanisms, and activate the enzymes responsible for the reduction of Cr (VI).

Lead Toxicity

Cigarette smoke contains lead at a level of approximately 13 ± 4ng per cigarette. Lead distorts cellular function and causes apoptosis. The exact molecular mechanism of this apoptotic process still remains unknown. Two probable triggering mechanisms have been proposed: an increase in Ca^{2+} concentration and production of the ROS. The mitochondria and caspases have a central role in the active and functional apoptotic phase [200–201]. During the active phase, the mitochondrial pores of permeability transition open by various apoptotic inducers, such as the increased calcium pre-oxidants of the mitochondrial matrix and the thiol reacting factors [202, 203]. The opening of the pores leads to depolarization and cytochrome c release from the mitochondria to the cytoplasm [200, 204]. Following the formation of the apoptosome, caspases which decompose the dead substrates and activate endonucleases which disrupt genomic DNA into high molecular weight fragments, that lead to the apoptotic nuclear morphology, are activated. The anti-apoptotic members of the Bcl-2 family suppress the opening of the mitochondrial apoptotic permeability pores and consequently the whole apoptotic process [200].

Metals as Signals for the Transcription of Information into Nuclear DNA

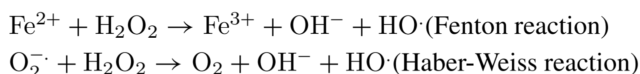
In vitro studies have shown that there is increased IL-6, IL-8 and TNF-α synthesis in human air track epithelial cells when exposed to metal mixtures [155, 205]. Further studies have shown that the exposure of broncho-epithelial cells to metals results in a constant accumulation of tyrosine-phosphorylated proteins [156]. This indicates the activation of signal transduction pathways which cause increased gene expression for these proteins. Acute exposure to arsenic [39] and chromium [206] at concentrations between 300 and 500 micromolar, activates phosphorylation-dependent components of the protein MAP kinase signalling cascade. The three MAPK pathways transfer signals which lead to various cellular responses, such as cellular development, differentiation, proliferation and apoptosis [207–213]. Each one of these major pathways is comprised of a three-stage cascade that involves a MAPK Ser/Thr, which is phosphorylated by a double specificity kinase (Thr/Tyr MAPK), which in turn is phosphorylated by another MAPK kinase. The pathway of the extracellular receptor kinase (ERK) transfers typical growth factor signals that lead to differentiation or proliferation [209], while the cytosine and “stress” (heat, inhibitor

synthesis) signals activate the C-Jun NH1-terminal and p38 kinase pathways which lead to a "stress" response – the end of growth- or to apoptosis [212–216]. Signalling through the MAPK pathways culminates with phosphorylation and the expression of cytokine genes [217, 218].

Iron Toxicity

Biological Oxidation and the Role of Iron in Oxidative Stress

Iron is an important component in the oxidative processes which cause free radicals. The accepted mechanism for its participation in oxidation is the Fenton reaction, as well as reactions catalyzed by the Haber-Weiss reaction:



$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\cdot} \text{(Fenton reaction)}$$
$$O_2^{-\cdot} + H_2O_2 \rightarrow O_2 + OH^- + HO^{\cdot} \text{(Haber-Weiss reaction)}$$

Oxidation by free radicals such as lipid peroxidation do not necessarily initiate the $HO^{\cdot}$ production of the Fenton and Haber-Weiss reactions, but a complex form of iron "Fe-O" [219].

The combination of iron susceptibility and the increased production of intermediate products of oxygen reduction such as hydrogen peroxide and the superoxide radicals create a pre-oxidative condition within the cell. As already mentioned, iron participates in the free radical production reactions and in the concomitant consequences at the various levels of cellular function. Iron (Fe^{3+}) is transported into the cell nucleus by a special ATPase. This transport is dependent on the presence of magnesium, a characteristic of the P-ATPase transport pump [220]. The various types of transformations that DNA elements undergo are caused by the reactive oxygen species (ROS). Among these transformations, base transitions are the most notable, since many of them are mutagenic. 8-oxo-guanine for example, creates horizontal modifications, such as G $\rightarrow$ T transversion [221, 222]. To cope with this damage, biological systems have developed specific repairing enzymes [223]. For example, 8-oxo-guanine-DNA-glycosilase is responsible for the repair of the above damage [224, 225]. This enzyme has two different activities: a glycosylating one that removes the modified base creating an abasic site and an abasic endonucleating one which cleaves the phosphodiesteric bond near the abasic site where the damage occurs. Other serious damage caused by the ROS originates from the presence of iron; this causes the splitting of DNA helices [226]. Hydroxyl radicals attack the carbon at position 4 in the middle of the sugar moiety [227]. By removing the hydrogen from this site, a carbon-centered free radical is created. The addition of an oxygen molecule creates a peroxy-radical. In this way, repeated rearrangements lead to the disruption of the sugar ring resulting in ÿ-emission. A propenal base is removed and the remaining bonds have a c-phosphoglyceric and a 5-phosphate end, respectively. The 3-phosphoglyceric end is a marker for the DNA break caused after

the attachment of the hydroxyl radicals (Fig. 2.2, page 69). When the H_2O_2 resulting from the iron in cigarette smoke enters the nucleus of the cell, it reacts with the chromatin-bound iron and creates hydroxyl radicals in situ (Fig. 14.6). These hydroxyl radicals will attack the adjacent DNA bases and create modifications [228]. Many of these modifications result from the action of the ROS and have been proven to be mutagenic [222]. Since mutagenicity is considered as an initial step towards cancer, the relation between the ROS, iron and cancer has been widely accepted.

Iron as a Possible Cause for Intracellular 8-oxoGua Accumulation

Iron has the ability to easily accept and donate electrons through transitions between the ferric (Fe^{2+}) and the ferrous (Fe^{3+}) form. For this reason, iron is a very useful component of the cytochromes and other oxygen-binding biomolecules such as hemoglobin and myoglobin. Despite this, cellular iron also exists in the form of non-protein-bound "free iron." The latter iron form is in dynamic equilibrium with the other iron forms inside the cell, bound to low molecular weight cytoplasmic ligands which have not yet been identified. This iron form is also catalytically active and participates in the ROS-producing reaction (Fenton reaction) [229]. Proteins isolate iron in order to reduce the risk of ROS production. Iron ions, for example, circulate in the peripheral blood, bound to plasma transferrin while ferritin helps in the iron accumulation in the cell. There are strong indications of a direct relationship between the iron overload of an organism and carcinogenesis. Iron overload maintains increased levels of the harmful free iron, through which the ROS catalyze the formation of the potential carcinogenic compound 8-oxodG in the cellular DNA [230]. Antioxidants reduce the amount of 8-oxodG in cellular DNA.

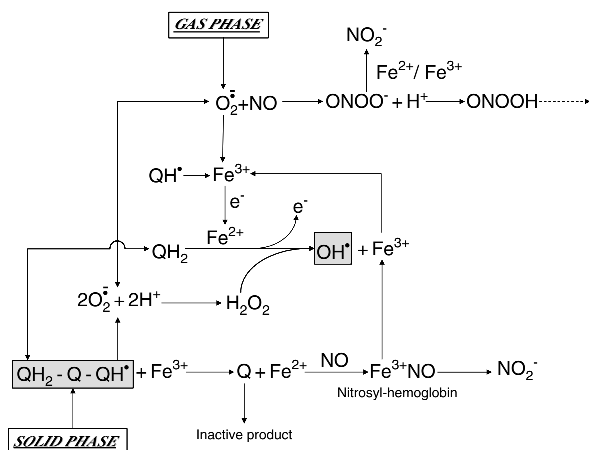


Fig. 14.6 The H_2O_2 resulting from the iron in cigarette smoke enters the nucleus of the cell, reacts with the chromatin-bound iron and creates hydroxyl radicals in situ

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Chapter 15
Oxidative Profile of Cigarette Smoke and Lung Cancer

While smoking constitutes a major cause of carcinogenesis, our knowledge concerning the acquired genetic changes caused by smoking which lead to lung cancer, still remain both elementary and unclear. The creation of a malignant phenotype needs, as a prerequisite, the creation of a large number of mutations in oncogenes. In lung cancer cells as well as in the nearby normal cells, an overexpression of growth factors and of a large number of regulatory peptides along with their receptors, is observed [1]. It is believed today that lung cancer is caused by a series of pathological alterations (preneoplastic) in the pulmonary epithelium; they are extensive and multi-focal, covering the whole pulmonary tissue and they are related to both lungs (cancerization field) [2]. During a prolonged exposure to cigarette smoke, oxidation product aggregates of nuclear DNA are formed on the cells of the bronchial epithelium (aggregates such as 8-hyrdroxy-diguanosine (8-OHdG)), the aggregation importance of which has been proven by the mutational transcription of the 8-OHdG bases of DNA as a frame of $G \rightarrow T$ transition and the incorrect integration of 8-oxo-GTP as a substrate for $A \rightarrow G$ transition [3]. Cigarette smoke contains large quantities of the reactive oxygen species (ROS) as well as other substances which create the ROS [4], some of which are capable of entering the cell and subsequently the cell nucleus, thus causing the aforementioned oxidative damage to nuclear DNA [5]. Auto-oxidation of the polyphenolic compounds that exist in cigarette smoke produce the oxidation substances $O_2^{-\cdot}$, H_2O_2 and $\cdot OH$. The gas phase of cigarette smoke contains large quantities of oxygen free radicals such as $10^{14-}10^{16}$ alkyl-alkoxy-peroxy-radicals per puff [4], as well as 1000ppm of nitrogen monoxide radicals (NO), which, alongside the volatile organic compounds, give smoke a large part of its toxicity and its carcinogenic activity [6] (Tables 15.1, 15.2, 15.3).

Hydroxyl radicals have to be created very close to DNA (2–3Å) in order to damage it, due to their short life span (10^{-6} sec). H_2O_2 is less potent in comparison to the OH radicals, but it has a much longer life span. It is easily diffused to the interior of the cell nucleus where it finds the chromatin attached Fe^{3+} with which it reacts. This reaction produces $\cdot OH$ radicals (Fenton reaction), which now act in situ, since the required minimum time for $\cdot OH$ to cover the distance of the few Å has now been achieved.

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Table 15.1 Toxic molecules in the gas phase of cigarette smoke

Hydroxyl radicals ($^{\cdot}OH$)
Superoxide radicals ($O_2^{-\cdot}$)
Hydrogen peroxide (H_2O_2)
Nitrogen monoxide ($NO^{\cdot}$)
Peroxynitrite ($ONOO^-$)
Alcoxy-radicals ($RO^{\cdot}$)
Volatile organic compounds

Table 15.2 Peroxynitrite toxicity ($ONOO^-$)

1. Strongly oxidative for proteins, lipids and DNA
2. Capable of dissociating into toxic molecules such as NO_2, NO_2^+, OH
3. Oxidises protein sulfhydrylic groups (−SH), including hemoglobin (Hb)
4. Changes the amino acid tyrosine to an oxidised nitric product
5. Inactivates human antitrypsin (a1-P1) via oxidation and nitration of methionine and tyrosine
6. Nitrates the amino acid tyrosine on the surfactant - A, whose ability to act synergistically with proteins of the surfactants – B and C is reduced, thus lowering the surface tension of the lipids of the surface-active factor.
7. Oxidises methionine in various proteins, thus reducing their biological functions
8. Reacts with the transcription factors of nuclear DNA

The solid phase of cigarette smoke (tar) contains stable radicals such as the quinone-hydroquinone-semiquinone complex, chatechol, polycyclic aromatic hydrocarbons (PAHs), alkenes, amines and nitrosamines [4, 7, 8] and mainly metals [9–12]. All of the aforementioned substances are known sources of the reactive oxygen species (ROS).

Tar particles contain large quantities of metals such as Fe^{3+}, As, Cd, Cr, Ni, (for metal toxicity see pages 183–204). When inhaled oxygen comes in contact with a metal particle (i.e. Fe^{3+}) which is embedded in the intercellular or lipoid tissue of

Table 15.3 Major groups of toxic and carcinogenic chemical substances in the gas phase of cigarette smoke

1. Hydrocarbons
Methane, ethane, propane, methyl-propane, methyl-butane, hexane, ethylene, acetylene, propylene, butadiene, isoprene, pentadienes, methyl-pentadienes, ethyl-pentadienes
2. Aldehydes and ketones
Formaldehyde, acetaldehyde, acrolein, crotonaldehyde, methacrolein, propionaldehyde, isobutyraldehyde, acetone, propanone, butanone
3. Nitriles and amines
Methyl-nitrile, ethyl-nitrile, acetonitrile, acrylonitrile, benzonitrile, methacryl-nitrile, ethylamine, benzylamine, phenylamine
4. Aromatic hydrocarbons
Benzene, toluene, ethyl-benzene, xylene, styrene
5. Oxygen and nitrogen heterocyclic compounds
Furan, methyl-furan, dimethyl-furan
Other volatile, non-organic compounds
Hydrosulphide, methyl-mercaptan, ethyl-mercaptan, sulphur dioxide, hydrocyanic acid, ammonia, nitrogen dioxide, nitrogen monoxide, carbon dioxide, carbon monoxide

the lung, it is reduced by the quinone complex (Q, QH_2, $\cdot QH$) to superoxide ($O^{2-\cdot}$), which is subsequently dismutated to H_2O_2. The latter reacts with Fe^{2+} and produces hydroxyl radicals ($\cdot OH$) (Fig. 14.6, page 204). The quinone complex is contained by the tar mould of the particles [13]. Tar particles contain another important tumour inducer, chatechol, which creates H_2O_2 and $O_2^{-\cdot}$ radicals [4, 14]. Each cigarette, when burnt, emits 20–35mg of tar which contains approximately 100μg of H_2Q and 300μg of chatechol. When the two substances act synergistically, they obtain a multiple biological result or a result at very low concentrations. Many of the smoke constituents and especially the ROS, induce serious damage to the structure and the function of biomolecules [15–23]. Greater emphasis has been given to oxidative stress (pages 41–57) which is related to cigarette smoke exposure and to the fact that its side effects can be prevented from certain antioxidants such as glutathione (GSH) [24, 17]. Indeed, exposure of the epithelial cells to cigarette smoke induces a rapid depletion of their reducing potential, especially that of intercellular glutathione [25, 26], resulting in inactivation of many enzymes which contain thioles in their molecules, (such as phosphoric glyceraldehyde dehydrogenase, creatine kinase and plasma paraoxanase). This inactivation can be prevented by glutathione [15–19].

ROS and Gene Toxicity

The ROS react with cellular DNA and induce oxidative damage in the form of single strand breaks [27]. This form of DNA damage is considered to be of great importance in the development of cancer. From this ROS reaction, more than 100 oxidative DNA-adducts have been identified [28–30]. The ROS cause, apart from single strand breaks, double strand breaks and purine, pyrimidine and deoxyribose changes as well as DNA-DNA coupling. The ROS cause damage to nuclear DNA via lipid peroxidation, during which potent electrophilic substances, such as epoxides and aldehydes, are created [31, 32]. For instance, malondealdehyde (MDA) is an extremely electrophilic and nucleophilic substance. These properties facilitate MDA in reacting with cellular nucleophilic substances as well as in forming dimers such as MDA-MDA, which are mutagenic [33, 34].

Oxidation Affects Various Cellular Functions

Low concentrations of the ROS induce cell proliferation [35]. The ROS and other free radicals, in large concentrations, affect the expression of certain genes and the signaling pathways. They also cause calcium release from intercellular storage, resulting in kinase activation, such as protein kinase C [36]. They also affect receptors which regulate proliferation and activate transcription factor pathways, such as MAPK, AP-1 and NF-kB [37]. From the above, it is obvious that DNA damage such

as mutations and altered gene expression, take part in the carcinogenic processes, with cellular oxidation as a common denominator.

Mechanisms

During long-standing exposure to cigarette smoke, an accumulation of 8-OHdG is observed in the lung epithelial cells and the cell anti-oxidative mechanisms do not function efficiently in order to offer protection against the existing oxidative load. Furthermore, the increase in 8-OHdG concentration suggests malfunction of the DNA repair systems which do not consider the increase in DNA damage from the endogenous side products of oxygen, due to metabolism. Innumerable tar particles which contain metals and some stable quinone complex radicals, are deposited on the pulmonary tissue (lipoid, lung interstitium) where they exist as pre-carcinogenic, harmful substances, which unremittingly give mutagenic stimuli (ROS) to the pulmonary cells. Therefore, mutations which are constantly repaired by the antioxidation and DNA repair mechanisms, are continuously produced. This shows that an unstable balance exists between oxidation and antioxidation mechanisms as well as between cellular repair mechanisms. Bronchial epithelial cells definitely have a limited life span and are renewable. The new cell generation will also be subject to the mutagenic action of the metals/radicals which are embedded in various parts of the pulmonary tissue. Thus, at every moment the presence of pre-neoplastic damage is confirmed throughout the whole extent of the pulmonary tissue (cancerization field) [38]. This fact is important and seems to be related to the cancer development mechanisms via oxidation in smokers. The deposition of metals in the microenvironment of the pulmonary tissue requires immediate, continuous and prolonged corrective responses from the bronchial epithelial cells which recruit all their defense mechanisms. Genes that encode toxicity removal enzymes (superoxide-dismutases [CuZnSOD, MnSOD], glutathione-peroxides, glutathione reductases, catalase, DT-diaphorase) play an important role in these responses [39, 40]. Glutathione-S-transferase (GSTM1) is also an acceptable defense system against oxidative stress [41–44]. The intercellular redox state regulates the antioxidants of the cell and defines the quantity of the oxidised and reduced form in which every redox pair exists. The intercellular redox state is transferred from the oxidation state to the reduction state. The ROS produced by metals negatively influence the redox state and via this mechanism, affect signal relay [45, 46].

Mitochondrial DNA Damage by Oxidation

Mitochondrial protein complexes that take part in the important mitochondrial processes, such as the opening of the mitochondrial permeability transition pores and the Ca^{2+} release pathway which depends on pyridine-nucleotides, are proteins with their thiols of the inner mitochondrial membrane (are readily oxidised from

peroxynitrite and are of great importance) [47, 48]. The protein adenine nucleotide translocase (ANT) (page 87) is also subject to oxidative substances [49]. These adenine nucleotides regulate the opening of the mitochondrial membrane pores during varying levels of oxidative stress [50, 51]. The mitochondria are constantly under the weight of an oxidative load which is related to mutations. Mutations in mitochondrial DNA [52, 53] and the changes in mitochondrial gene function, seem to be related to the development of neoplasia. Altered expression and mutations in mitochondrial DNA genes which encode complexes I, III, IV and V, and in the overchanged regions of mitochondrial DNA, have been found in human tumours. Mitochondrial DNA is more prone to oxidative damage of its bases than nuclear DNA. Mutations are twice as frequent as those of nuclear DNA. Mutations in genes that encode for oxidative phosphorylation damage ATP production. Reduced ATP production damages the cell cycle, thus prohibiting development via the cell cycle [54]. Fragments of mitochondrial DNA have been found amongst nuclear DNA. This fact has been considered as a mechanism of oncogene activation [55]. Hydrogen peroxide and other reactive oxygen species are involved in the activation of the cell nuclear genes which are implicated in mitochondrial biogenesis, transcription and replication of the mitochondrial genome. Low levels of hydrogen peroxide promote mutagenesis [56]. Mitochondrial biogenesis might be the cellular answer in order to balance out the malfunction of oxidative phosphorylation which is associated with the mutated mitochondrial DNA. Oxidative damage and the induction of mutations to mitochondrial DNA contribute to the multilevel process of carcinogenesis [57].

Role of the P450 Enzymes in the Toxicity/Carcinogenicity of Certain Cigarette Smoke Chemicals

The toxic and carcinogenic chemicals in cigarette smoke (such as isoprene, butadiene, PAHs) do not further their biological effect by themselves but are bioactivated to electrophilic forms (Fig. 15.1) in order to produce molecules capable of reacting irreversibly with tissue nucleophiles and form adducts with proteins and nucleic acids. Many of these reactions are catalysed by enzymes involved in the biotransformation of xenobiotic chemicals, such as epoxide hydrolase, glutathion-S-transferase, cysteine conjugate β-lyase, γ-glutamyltranspeptidase and UDP glucuronosyltransferase.

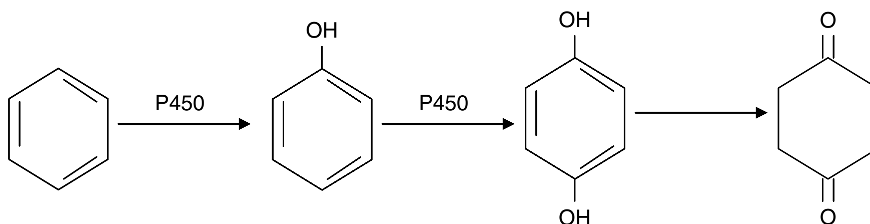

Fig. 15.1 A model of activation of chemicals to electrophiles catalysed by P450

The bioactivation reactions involve oxidations catalysed by the monoamine oxidase, monooxygenase and peroxidases (myeloperoxidase). Yet the majority of oxidative bioactivation are caused by the P450 enzymes [58].

A large number of human P450 enzymes are present in the liver and many of these are found in the endoplasmic reticulum, clustered as microsomes. The dominant mechanism by which P450 enzymes catalyse oxidations is the one-electron oxidation. P450 are capable of both one-electron transfer and hydrogen atom transfer when they oxidize substrates; the mechanism is probably dependent upon the use of FeO^{3+} instead of peroxy forms (Fig. 15.2). The substrates which evidence for one-electron oxidation include amines, sulfides, alkanes and polycyclic hydrocarbons. Chemicals, such as tobacco smoke PAHs are considered as substrates for one-electron oxidation. The mechanism for activation of PAHs is the formation of diol-epoxides.

Conclusion

The assault on DNA and RNA as well as other cellular components by the ROS, which are included or produced by cigarette smoke, is the base for cancer development from oxidation with metals playing the primary role. Free radicals (ROS) are implicated in chemical carcinogenesis via various metabolic pathways [59, 60]. However, no known immediate connection between mutations of oxidised DNA and cancer exists. This weakness is the consequence of the limited knowledge we posses in the understanding of the mechanisms of carcinogenesis and the probability that there might be a simple mechanism which explains the whole process [61]. Despite the fact that free radicals are the main cause of certain cancers, it is highly unlikely that they are necessary in all cases, since alternative initial pathways exist, as well as mechanisms for the promotion of carcinogenesis [62]. There are, however, serious indications with regard to the role of free radicals in the promotion of the carcinogenic processes [63, 64].

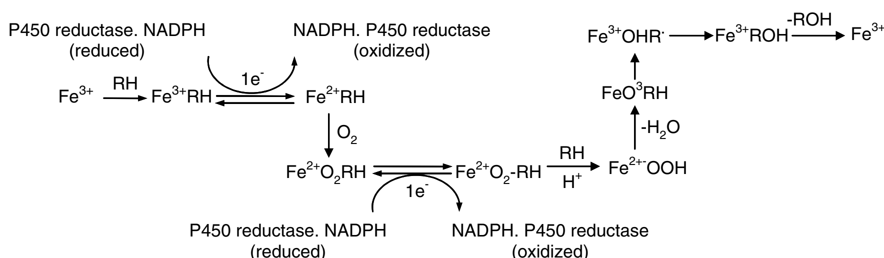

Fig. 15.2 P450 catalytic events. The events start with P450 iron in the ferric state. The substrate (RH) binds to the enzyme. The electrons derive from NADPH-P450 reductase. The FeO^{3+} complex rationalize the P450 reactions

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Chapter 16
Smoking and Lung Cancer

Pre-neoplastic Alterations in the Smoker's Lung

Tobacco smoke has been classified by the Environmental Protection Agency (EPA) as being among the carcinogens which damage human health. Different substances present in both the gas- and solid-phase of tobacco smoke may cause apoptosis to the broncho-epithelial cells and certain forms of cancer (Table 16.1). Special emphasis is being given to the fact that tobacco smoke is a rich source of the reactive oxygen species (ROS), as well as of ROS products. The ROS synthesis differs in the two tobacco smoke phases (gas- and solid-phase or tar). The solid phase contains large quantities of stable radicals, such as the quinone complex (quinone, semi- quinone, hydroquinone) and catechols, two compounds that can damage nuclear DNA [1, 2]. On the contrary, the gas phase components contain very active molecules which have a short half life; these enter the lung by inhalation and are quickly distributed among the major organs. Their toxic activity on the cells begins immediately, with the first puff, simply following the laws of gases.

Pre-neoplastic Alterations

As happens with other epithelial cell malignancies, the different forms of lung cancer are created following progressive histopathological alterations (pre-neoplastic changes) in the bronchial epithelium. Squamous cell carcinoma changes include hyperplasia, metaplasia, dysplasia, cancer in situ and invasive metastatic cancer [3–5]. However, such a successive evolution which precedes squamous cell carcinoma and adenocarcinoma is not known as yet. Pre-neoplastic alterations are often extensive and multifocal and are widespread throughout the bronchial epithelium of heavy smokers. This phenomenon is extremely important because it seems to be related to mechanisms of lung cancer development in smokers and is referred to as field cancerization [6]. Loss of heterozygosity (LOH) is ascertained in the short legs of chromosomes 3, 9 and 17 (3P, 9P and 17P, respectively) and is caused early in the multistep development of invasive lung carcinoma [7–11].

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Table 16.1 Consistency of whole cigarette smoke in various carcinogenic substances

Chemical substance Category	Number of substances per category	Representative carcinogens in inhaled smoke	Substance quantity in μg/cigarette
PAH	14	B(a)P	0.009
		Dibenzo(a, h) anthracenes	0.004
Nitrosamines	8	NNK	0.123
		NNN	0.179
Aromatic amines	12	4-aminodiphenyl	0.014
		2-naphthylamine	0.010
Aldehydes	2	Formaldehyde	16.0
		Acetaldehyde	819.0
Phenols	2	Chatechol	68.0
Volatile hydrocarbons	3	Benzene	59.0
		1,3 butadiene	52.0
Nitro-compounds	3	Nitromethane	0.5
Other organic compounds	8	Ethylene oxide	7.0
		Acrylonitrile	10.0
Inorganic compounds	9	Cadmium	0.132

There are some changes in the microsatellites (which signify changes in magnitude in the one or both alleles) which are established in many human cancers. In the lung cancer, these changes are found in all regions of the lung tissue. This can be used as a clone index for the lung tissue and for the early diagnosis of cancer [12, 13]. With regard to the loss of alleles, the same allele is deleted in biopsy specimens taken from the bronchial epithelium and this loss of allele is a phenomenon characterized "loss of special alleles" [7], with intent to show the dispersion of cellular clones, altered at a molecular level and dispersed broadly in the lung tissue [14]. Previous studies describe a unique kind of mutation with a similar constitution in many regions of the lung tissue in which the bronchial epithelium exhibits serious histological abnormalities in both lungs in smokers who do not have lung cancer. This fact shows that these particular clones are able to disperse progenic cells in large areas of the lung epithelium. By using the technique of fluorescent in situ hybridism, the existence of a large number of mono- and tri-somal clonal hypoclonal and small sized spots in the bronchial epithelium (which is damaged by smoking), has been ascertained. A small number of advanced clones acquire the capability to be widely dispersed. These clones are the immediate precursor of invasive tumors. A problem in explaining the above mentioned studies is possibly our limited potential to relate the damage to smoking. However, there is supporting evidence [15, 16] that the level of the PAH-DNA adducts in smokers is higher compared to non smokers. The DNA-adduct levels found in neoplasmatic tissue as well as in the macrophages, are higher in smokers [17, 18]. BPDE-DNA adducts exclude the transcription of an important gene [19], since they are not reconstituted sufficiently by the NER pathway (page 54).

DNA Adducts Due to Active Substances in Tobacco Smoke

DNA adducts are natural complexes which are formed by active chemical compounds, associated covalently with certain DNA sites. The formation of covalent adducts with DNA and with the active form of chemical carcinogens constitute an important step in the processes of chemical carcinogenesis. The carcinogenic effect of many chemical substances contained in cigarette smoke depends on their capacity to act as electrophilic substances [20–24]. Certain carcinogens are congenitally electrophiles, while some others are hydrolysed instantaneously to active molecules and many others are activated by the cell enzymes. The resulting active substances transverse DNA components, RNA and proteins, thus forming covalent adducts in which carcinogenic substances are associated with the nucleophilic atoms of nucleotides or amino acids. Adducts are important because they promote DNA replication and they become chemical progenitors of inherited genetic conversions. Mis-replication or mis-repairing of this damage may induce mutations which start with the malignant transformation of the somatic cells [25, 26]. Genotoxic factors create activated molecules [27]. DNA, being the target of these molecules, consists of different nucleophilic atoms. According to Loveless [28], the reaction of the O^6 of guanine (Gua) with alkyl, consolidates the enolic base tautomer and accommodates coupling with thymine (Fig. 16.1). This G $\rightarrow$ A transition is the result of the mutagenic potential of the O^6-methylguanine (O^6 MeGua). Genetic experiments have shown that alkylating agents prefer the GC $\rightarrow$ AT transitions and exceptionally the TA $\rightarrow$ CG transitions [29–31]. The role of O^6 MeGua in carcinogenesis became understandable by experiments which showed that methylation due to N-methyl-N-nitrosurea may induce a mutation to the Ras-gene and cause breast cancer, similar to a mutation due to methylation by the O^6 MeGua. The result is that O^6 MeGua represents the pattern of adduct mutagenesis [32–34]. Mutagenesis in cells and carcinogens in animals induced by alkylating agents, are related to the methyl-guanine in the DNA molecule [28, 35–39]. O^6 MeGua is able to form in vivo, a model of mutagenesis similar to the mutagenesis induced by alkylating agents [40]. The transition of G to A is the first mutation in the coupling of bases when alkylating agents or O^6 MeGua are used [41]. Alkylating agents, which contribute to lowering the O^6 Methyl-Guanine-Methyl Transferase (MeGuaMeTase) level, increase the levels of both O^6 MeGua and mutations [42]. Thus, it is shown that O^6 MeGua is responsible for the fact that the mechanisms related to mutagenesis concern

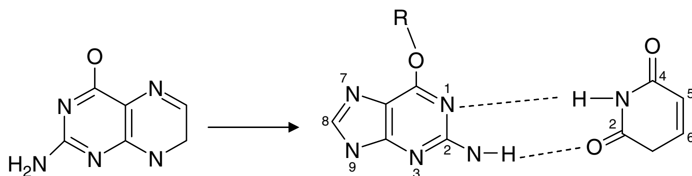

Fig. 16.1 Reaction of the O6 of guanine with alkyl, accomodates coupling with thymine

the base substitution and formation of alkyl-DNA adducts which guide the misincorporation of nucleotides, during DNA replication. Mutations start off when the DNA-polymerase encounters such a carcinogenic DNA adduct during DNA replication.

DNA Adducts and Carcinogenesis in Smokers

Clinical and epidemiological studies state that there is a relationship between an increased DNA adduct level and cancer. The relationship between the genomic polymorphism that can modify the degree of the cancer risk due to cigarette smoking, and the levels of DNA-adducts, is complicated. This complexity is related to carcinogen-metabolism and DNA damage reconstitution. Molecular studies of tumors have shown that there is a relationship between the p53 mutation and DNA adducts, as there is a correlation between the DNA adduct levels and the important alterations of 3P21LOH, which may take place in the very early stages of carcinogenesis due to cigarette smoke. It appears that the different response of individuals to tobacco carcinogens is by nature, multifactorial. Recent studies have shown that there is a linear relationship between the adduct level and the daily or life cigarette use [43–45]. An interesting fact is that smokers with higher-level adducts suffer from cancer because of cigarette smoking in a shorter time interval when compared to smokers with a lower adduct level. One year after giving up smoking the adduct level acquires an average value. An estimation of the half life of adducts in the lung tissue is about 1–2 years. This length of time is longer than that which is required for the biochemical processes of DNA repairing as well as that of the substitution rate of the dead cells with new ones. This can be due to the slow clearance of the cigarette smoke carcinogens (tar particles, metals) which are deposited on the lung tissue. Moreover, adduct formation due to smoking is allocated to many other organs of the human body, such as the cardiovascular system, peripheral blood, bladder, neck of the uterus, large bowel, pancreas, kidney, liver and stomach. Recent studies of five parameters have made known what plays a causative role in carcinogenesis as far as the DNA adducts are concerned:

- Circumstantial evidence that the genetic polymorphism existing in cancers and related to tobacco smoke is justified from the different concentrations of DNA adducts in human tissues.
- Evidence that DNA adducts are risk factors since they produce cancer development, as related with tobacco smoke.
- Correlation of the DNA adducts with molecular alterations in the oncogenes and tumor suppressor genes, in human tumors.
- Possible importance of the endogenous and chemically unstable DNA adducts in carcinogenesis due to tobacco smoke.
- Methodology concerns which affect research related to the role of DNA adducts in carcinogenesis from tobacco smoke.

Genes Frequently Implicated in the Carcinogenic Mechanisms in Smokers

K-Ras Genes and Smoking

Tobacco smoke includes a large number of carcinogens, co-carcinogens and tumor promoters. All of these compounds are able to contribute to the multi-stage process of neoplasia in the lung bronchi [46]. The term "field cancerization" indicates that tobacco smoke carcinogens are created over the whole surface of the lung airways, thus giving rise to an appropriate environment for cancer development. Proto-oncogenes and tumor suppressor genes K-Ras [47], L-myc [48] and p53 [49] are involved in this process in humans. The proto-oncogene K-Ras is involved in the pathogenesis of lung cancer [50]. The Ras family consists of three high homology genes, K-, H- and N-Ras. These genes encode for membrane-associated proteins which participate in the signal transduction pathways and control cell proliferation. The mutagenic activation of the K-Ras genes, near codon 12 has been associated with a rate of 30% of adenocarcinomas [51, 52].

In epithelial cell cancers such as lung cancer, the mutation rate is more than 80% in K-Ras genes which take place in codon 12 [53]. The first incidence of mutation in codon 12 of the Ras gene can be found in malignancies related or not to smoking. According to Feng, et al. there is a choice of DNA damage for codon 12 and non sufficient damage reconstitution, and this needs further consideration [54].

At present, it is well known that mutations of the K-Ras gene are detected in the normal part of the lung tissue in patients with lung cancer [55, 56]. Mutations of the K-Ras gene in lung cancer are referred to as the lateral $G \rightarrow T$ transversion, thus proving the significance and the consequences of BPDE-guanine adducts and some other DNA adducts present in the lung tissue of smokers [57].

Some studies have shown that there are two categories of adenocarcinomas which are related to smoking: those with a high level of chromosomal anomalies and those with a low level of such anomalies. Mutations of the K-Ras gene are more frequent in tumors with a low level of anomalies [58].

K-ras Genes and O^6-methyl-guanine-DNA-methyltransferase (MGMT) in Lung Cancer

In human lung adenocarcinoma, an alteration of two genes plays an important role in the pathogenesis of cancer: K-ras and MGMT genes. The mutations of K-ras take place predominantly in codon 12 and can be found at a rate 30–40% of lung adenocarcinomas in smokers [59–62]. The MGMT gene is inactivated in small-cell lung carcinomas [63]. MGMT is a reconstitution enzyme of the DNA enzymes which protect cells from the carcinogenic activity of alkylating agents, by eliminating adducts from the O^6 position of guanine.

In case of reconstitution failure, the adduct leads to mutational transversions in the K-Ras gene in particular. The fundamental inactivation mechanism of the

MGMT gene of the intermittent methylation of the CpG islets in its promoter region, that leads to transcription silence [63, 64]. This kind of cancer with silence in the MGMT promoter gene very often includes mutations in the K-ras gene [63] and this reveals that MGMT gene deletion is able to induce a subsequent K-ras gene mutation. Hypermethylation of the MGMT gene promoter takes place in the early stages in the evolution of lung adenocarcinomas [65]. K-ras mutations in codon 12 have been established in lung cancer (70%) in long-term smokers, evidenced by horizontal G $\rightarrow$ T transversions and uncommonly by G $\rightarrow$ A transitions (4 horizontal G $\rightarrow$ T transversions and 4 G $\rightarrow$ A transitions) [66].

K-ras Gene Mutation and the Role of Glutathione-s-transferse M1 (GSTM1) in the Incidence of Lung Cancer

The carcinogens which are related to tobacco smoke are oxidised by the enzymes of phase 1 that subsequently become atoxic by the phase II enzymes, such as glutathione-S-transferase. K-ras mutations have been tested in relation to the gene polymorphism of the cytochrome P450A1 (CYPA1) and the GSTM1 in smokers with lung adenocarcinoma. K-ras mutations are at a high frequency in patients with genotype GSTM1(−) in relation to patients with genotype GSTM1(+) [67]. In this study, mutations in smokers with lung adenocarcinoma were due partially to the accumulation of carcinogens which preserved their toxicity because of the GSTM1(−) genotype. It is well known that glutathione-S-transferases (GSTs), render the active metabolites of B[a]P, the diol-epoxides, atoxics. Numerous studies indicate that insufficient activity of GSTM contributes to genetic susceptibility to lung cancer from cigarette smoke [68–72].

Mutational Prototype of the Exons 1 and 2 of K-ras Gene

Mutations to genes of great importance, like K-ras and p53, are implicated in cancer development related to exposure to tobacco smoke [73]. The most frequently found genetic alterations in lung cancer are in the K-ras proto-oncogene and the tumor suppressor gene p53 [74]. The important role of these alterations in carcinogenesis has been shown in cell cultures (rat fibroblasts) which mutate p53 and activate the K-ras proto-oncogene, both of which have been exposed to the carcinogenic substances of cigarette smoke, resulting in complete cell transformation in vitro [75–77]. The chemical carcinogens selectively induce specific conversions to the base couples, such as those recognised in the codons 12,13 and 61 of the K-ras gene and in codons 249 and 273 of the p53 gene [78–80]. Mutations of the K-ras gene concern G to T transversions in codon 12, in a proportion 30–60% of adenocarcinomas, in smokers [81–83].

Role of DNA-methyl-transferase (DNA MTase) in Cancer Development

The role of the DNA-MTase gene superexpression and its increased catalytic activity in DNA methylation in the CpG region plays an important role in cancer development [84]. Hypermethylation of the CpG islets, (that normally are not methylated, especially when they are located in promoter areas), is associated with the inactivation of certain tumor suppressor oncogenes such as the p53 gene [85–90]. Methylated cytosines constitute hot spots for mutation [91] while DNA-MTase activity leads to mutagenic C-T transition [92]. If the relationship between the increased DNA-MTase activity and tumor development indicates that this enzyme plays a functional role in the processes, then the target cells, (for example those cells which have been damaged by the carcinogens of tobacco smoke) must show a significant increase in the enzymic activity of this enzyme. There is supporting evidence that this increase can take place in these cells, and an early increase of the DNA-MTase activity is directly associated with neoplasia development and constitutes a key-step in carcinogenesis [93]. The increase of DNA-MTase activity can also be associated with signal transduction through the Ras pathway. Mutation or superexpression of the K-ras gene stimulates the myc-gene and the complex Jun/fos, and this results in an increase in the AP-1 transcription factors [94] which regulate DNA-MTase gene transcription [95]. Increased DNA-MTase activity may affect tumor evolution via different mechanisms. Increased methylation in these DNA areas which normally are non-methylated, but rich in cytosines, is associated with alterations of the chromatin structure and this may cause DNA instability in the form of allele loss [96, 97] leading to deactivation of a large number of tumor suppressor genes [85, 86]. DNA methylation also leads to changes in a series of bases, because methylated cytosine is extremely sensitive to the gene mutations of eucaryotics [92]. Alternatively, the increased expression of the DNA-MTase gene may induce serious mutagenic transversions in the CpG regions, due to direct enzymic cytosine deamination [92].

These anomalies exist throughout the smokers' bronchial tree and start from chronic and diffused lung exposure to the carcinogenic substances in cigarette smoke. For example, p53 gene mutations and loss of heterozygosity in the 3P chromosome are often recognised in the bronchial epithelium, indicating a serious dysplasia in areas near the primary focus of the tumor [98]. Some other studies have ascertained chromosomal abnormalities in the normal bronchial epithelium, with pre-neoplastic cells which are dispersed throughout the lung.

Activity of Tobacco Smoke Oxidative Substances on Growth Factor Receptors

The participation of free radicals in tobacco smoke in the process of carcinogenesis is mainly due to the effect of oxidative substances on the signal transduction pathways which lead to the cell replication by transforming the signalling proteins.

Oxidative stress actually induces phosphorylation and activation of the many proteins that are involved in signal transduction to the cell nucleus. Members of the MAP kinase family [99–102], MAP kinase MEK [101], and Ras [103] are included in these proteins as well as some growth factor receptors, such as the platelet growth factor receptor (PDGF) [104] and the epidermal growth factor receptor (EGF) [105–107]. Even if the mechanism through which free radicals perform these conversions to the proteins which transfer information are not completely understood, there is, however, supporting evidence that increased phosphorylation of the epidermal growth factor receptor (EGFR) by H_2O_2 is the result of deactivation of the tyrosine phosphatases [106, 108]. This denotes that H_2O_2 exerts an inhibitive activity on the cellular mechanisms which limit the information transfer through the growth factor. The mechanism that limits signaling through EGF is a down-regulation of the intra-transfer and subsequent degradation of the activated EGF receptors. In normal conditions, when cells are stimulated by EGF, the activated receptors are recruited and covered with clathrin in specialised regions of the cell membrane, the so-called coated pits [109, 110]. Following the receptor recruitment, some hollow imprints are formed on the cytoplasmic membrane addressed to the cell cytoplasm, thus forming small vesicles, coated with clathrin. Subsequently, these small vesicles are transformed from the cell membrane to the cytoplasmic microtubules. Following disclosure, the small vesicles cast with the primary endosomes. A great part of the EGF receptors are transferred to the belated endosomes and finally to lysosomes where they are degraded [111–113]. The above down-regulation of the activated receptors is important, because cell transformation and tumor development take place because cells are unable to promote the endocytosis caused by the receptor binders [114, 115]. The oxidative stress through H_2O_2 in cigarette smoke quickly inhibits the EGF receptor endocytosis [116, 117].

p53 Gene, Pulmonary Cancer and Smoking

Transcriptional Characteristics of p53

Introduction

The p53 gene and its protein have been thoroughly studied, following the finding that in 50% of cancers this gene shows mutations which are nothing more than the loss of an allele and the production of an incorrect protein, (which is abundant in “sick” cells). In rats the null phenotype of the P53 gene shows a predisposition to cancer [118]. The p53 protein is a transcriptional factor which promotes the transcriptional rate of six to seven known genes which serve the functions of p53 in the cell. As is known, the cell performs various functions and uses certain proteins to identify DNA damage as well as various enzyme systems to repair this damage. What seems important is the fact that cellular proteins that recognize DNA damage have previously communicated with p53 and activated it. It seems that the p53 protein as well as the protein-detector of DNA damage is present at the DNA

damage and repair site, where phosphorylation and some other activation signals promote these processes. DNA helix debris is sufficient to activate p53. The entry of restriction enzyme nucleases into the nucleus stimulates the activity levels of p53. The mutated protein p53 is not capable of attaching itself to the specific base pairs of DNA. This means that the promotion of transcription for the selected transcription gene targets is impossible.

The molecular designation of the base pair order for the identification of the mutation template of the p53 gene constitutes the rule of thumb for the detection of p53 mutations. The most common p53 mutations are: GC $\rightarrow$ AT, GC $\rightarrow$ TA and AT $\rightarrow$ GC [119, 120]. These mutations are derived from aromatic amines and from N-[4-5-nitro-2-furil-2-thionyl] formamidase since both substances exist at high levels in the epithelial cells of the urinary tracts of smokers.

The loss of the protective mechanism of p53 mentioned above, allows the evolution of a clonal cell population with a selective ability to develop cancer in the future [121, 122]. Moreover, the inactivation of the p53 gene is an important step towards the evolution of pre-infiltrating anomalies [123]. The different types of alterations of the DNA base pairs in cancer tissue, indicates the participation of most of the toxic substances which are comprised in cigarette smoke. The alterations of the p53 gene mainly refer to the CpG positions when the subject is exposed to smoke constituents. These alterations at positions CpG are implicated as endogenous mutation "hot spots," derived from the methylation and deamination of cytosine through cellular enzyme processes [124]. The deamination of 5-methyl-cytosine, an endogenous mutation mechanism, is mainly responsible for the GC $\rightarrow$ AT shift of the CpG dinucleotides [125]. Also, deletions and additions can accidentally happen, due to the non-fidelity of the polymerase during replication (also an endogenous mechanism). The coding region of the p53 gene includes 39 CpG dinucleotides (i.e. 78 base pairs, which when deaminated, create potential GC $\rightarrow$ AT shift positions). Shifts at these positions constitute 24% of all p53 gene mutations. This percentage shows the wide fluctuations between the different types of cancer. Seven positions at codons 175, 196, 213, 248, 272, and 282, are related to 90% of the CpG alterations, which relate to both DNA helices.

DNA-adducts, P53 Gene and Pulmonary Cancer

Benzopyrene and other mutagenic substances in cigarette smoke are important causative factors in adduct formation at the mutated hot spots of the p53 gene, in pulmonary cancer.

Smoking is a unique and important risk factor in the development of lung cancer. Among many of the other mutagenic substances in cigarette smoke, polycyclic aromatic hydrocarbons are implicated as the main causative factors for cancer induction [126–131]. Some of these (benzo[a]pyrene, benzoanthracene, crysene) are precarcinogenic chemical substances. They become carcinogenic following a metabolic conversion to electrophilic active substances (diol-epoxides), able to react

covalently with cell macromolecules such as DNA, and to form "adducts." These oxidative reactions are catalysed by phase I enzymes of the cytochrome P450 and epoxide-hydroxylase (Fig. 16.2). The ability of the polycyclic aromatic hydrocarbon (PAH) metabolites to form special adducts with DNA gives them cancer development capabilities. Actually, some substances of the PAH family (pyrene, anthracene) which are not capable of forming adducts, are not carcinogenic [132, 133]. Benzopyrene can be found in cigarette smoke at a concentration of 20–40ng per cigarette. As mentioned above, the in vivo bioactivation of benzopyrene (B(a)P) by the cytochrome P450 and epoxide-hydroxylase, creates extremely toxic electrophiles and free radical-like intermediate products, such as benzopyrene diolic epoxides (BPDE). These products are capable of damaging DNA irreversibly, forming DNA-adducts through covalent bonding or oxidation. BPDEs connect to DNA and form covalent (+) *trans* adducts at position N2 of guanine [134]. Almost 60% of human pulmonary cancer cases include mutations of the tumor suppressive p53 gene [135, 136]. The mutation of the p53 gene includes more than 500 entries in the pulmonary cancer mutation line. There is a high percentage of G to T shifts in these tumours. Such mutations constitute a landmark in mutagenesis, in which some forms of polycyclic aromatic hydrocarbons such as BPDE, take part.

Mutational Template

These mutations can also potentially be induced by other factors such as oxidative damage to DNA [137]. The mutation distribution along the p53 gene, in pulmonary cancer, is not random. It is characterized by many mutagenic hot spots specifically in codons 157, 248 and 273 which correspond to amino acids in the attachment area of p53 to DNA. Codon 157 is a mutagenic hot spot specific to

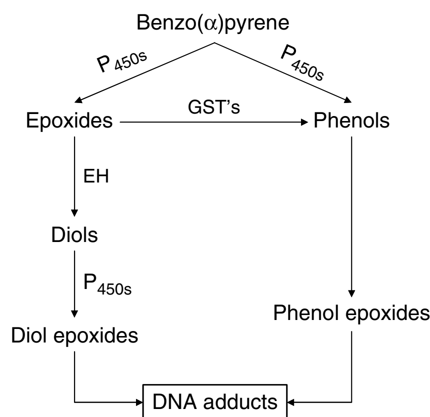

Fig. 16.2 Concise metabolic pathway of benzo(α)pyrene. EH = Epoxide hydrolase; GST = Glutathione S-transferase

lung cancer; it does not exist as a hot spot for any other type of cancer, whereas the other two codons exist in other types of cancer [23]. The majority of mutations in lung cancer at these three codon positions are G → T shifts [138] and they are the same mutations in all bronchial epithelial cells. Mutations at gene p53 are found in more than 100 different cell lines, at different positions. The mutation hot spots of the p53 gene, at codons 157, 248 and 273, act as selective positions for the attachment of mutagenic BPDE suggesting that mutations at hot spot positions of p53 are primary targets for DNA damaging factors. The mutagenic substance BPDE connects with guanine at codons 157, 248 and 273, twenty-fold more efficiently than adenine. It is certain that these mutations are caused by other mitogenic substances in cigarette smoke. It is also interesting that almost all hot spots are located in the CpG dinucleotides (a cytosine followed by a guanine). The best studied endogenous DNA damage mechanism up until now, is the phenomenon of the deamination of 5-methyl-cytosine spontaneously to uracil and thymine; if not restored, damage will result in the G:C → A:T shifts. These mutations occur frequently at the CpG dinucleotides, which are often methylated [139, 140]. CpG positions on the P53 gene are methylated in all human tissues [141, 142] and DNA methylation is the mechanism which regulates gene expression. The coexistence of mutagenic hot spots with adduct hot spots suggests that the metabolites of benzopyrene or other substances that relate to them structurally, take part in the transformation of human pulmonary tissue.

Frequency of Mutagenesis by Anti-diol-epoxide of Benzopyrene

The mutational profile which is created by a specific mutagenic pathway is expressed by the kind of upcoming mutations when proto-oncogenes (or tumour suppressive genes) are activated [135, 143–145]. Mutational profiles are discerned from a quantitative aspect (i.e. whatever affects the hot spots against the cold spots), and from a qualitative aspect (i.e. whatever affects GC → TA against GC → AT and against the GC → CG mutations). DNA base order has been recognized as one of the most important factors affecting the quantitative and qualitative profile of mutagenicity [146–154].

Some researchers have studied 4–8 codons for p53 gene mutations in pulmonary cancer [155]. They found mutations in smokers at a percentage of 21.6%, consisting of five shifts (including three C to T, one G to A and one T to C) and one horizontal modification of T to A. Comparative mutations were found on the p53 gene of long-term smokers at a percentage of 31.3%, with six horizontal modifications (four G to P, one A to T and one G to C), one shift (C to T) and two deletions of the gene. However, mutations were also found in non-smokers but in different codons and in different positions in the same codon. The authors attribute this phenomenon to exposure to different factors other than those attributed to cigarette smoke.

For an in depth understanding of the mechanism which affects mutagenicity quantitatively and qualitatively, mutagenesis by (+)-anti-B[α]P-7,8 dihydrodiolic-9,10 epoxide [(+)-anti-B[α]PDE] [156] and its adducts, were studied [157]. (+)-anti-B[α]PDE] is considered to be the most important mutagenic/carcinogenic metabolite of B[α]P.

Cellular Enzymic Protection from the Metabolic Products of B[α]P

The cell which has been exposed to cigarette smoke has the ability to neutralize the metabolic products of benzopyrene B[α]P with the enzyme glutathione-S-transferase M1 (GSTM1) (Fig. 16.2). Individuals who are genetically impoverished in GSTM1 have an increased risk of developing pulmonary cancer from smoke constituents and especially from the metabolic products of B[α]P, the diolic epoxides. However, many oncogene targets of the epoxides remain unknown [158].

The Correlation of p53 Gene Mutations with the Histological Image of Pulmonary Cancer

p53 gene mutations are present in 50% of small-cell lung cancers (SCLC) and in 47% of non-small-cell lung cancers (NSCLC), but only in 33% of adenocarcinomas [159–162]. Mutagenic profiles are important because they predominate in mutations A:T → G:C in SCLC and also predominate in deletions and additions in NSCLC. The predominance of the horizontal modifications G:C → T:A transversion in large-cell and small-cell carcinomas is the same (43–49%) while it is rarer in adenocarcinomas. This finding is compatible with the weaker correlation of adenocarcinomas with smoking [163, 164], since the G:C → T:A horizontal modifications are derived from the bulky molecules that exist in cigarette smoke.

p53 Gene Activation

In some cells, the p53 gene exists in a latent form, inactive to transcription. Under these conditions the p53 protein has to receive a stimulus or some kind of alteration in order for the p53 gene to be activated and to function. Signals that address the p53 gene are derived from stress situations. Various forms of DNA damage can activate p53, such as double bond fragments, γ-radiation and chemical damage. Such damage leads to a rapid increase in p53 levels and its activation as a transcription factor (Fig. 14.5). This increase of p53 is proportional to the extent of DNA damage. It is a truly appealing idea that proteins which recognize DNA damage communicate with p53 and activate it. It seems that DNA helix breaks are sufficient to activate p53. The entrance of restriction enzyme nucleases into the cell nucleus stimulates the levels and activity of p53.

Hypoxia can also activate the p53 protein and stimulate the p53 gene [165]. This process is another way in which p53 can act as a guardian against cancer development. Tumours initiate replication and proliferation and reach a critical mass, which

is defined by an adequate blood supply and therefore oxygen supply, which, during hypoxia, is significantly reduced and requires angiogenic factors in order to sustain the tumour. Otherwise, the resulting hypoxia can trigger activation of p53 and kill the cells.

Another signal which activates p53 is the drop in the levels of the triphosphoric ribonucleotide reservoir, which sustains DNA replication and the evolution of the cell cycle [166].

Repair or Apoptotic Function of the p53 Gene

The tumour suppressive gene p53, plays an important role as much in the DNA repair process which is a fundamental matter in gene stability, as in apoptosis. The preservation of gene stability depends on the cellular ability to detect and recognize DNA damage and either repair it or induce exit from the cell cycle through apoptosis or cellular differentiation [167]. The activated form of the p53 gene participates actively in various DNA repair and recombination processes through its ability to react with the constituents of the repair and recombination mechanisms as well as with various biochemical processes [168]. p53 seems to play an important role in DNA repair by nucleotide excision [169] and base excision [170]. Genotoxic stress activates p53 for repair through DNA base excision (BER) during the cell cycle [171]. The activation of the BER mechanism by p53 is related to its ability to react readily with AP endonuclease as well as with DNA polymerase ÿ [172].

The acknowledged role of p53 in inducing apoptosis after DNA damage on the one hand, and its role in DNA repair on the other hand, are serious indications that p53 acts as a key regulator in the choice between the two pathways. The quantity of the genotoxic factor to which the cell will be exposed, plays a role in this choice. Low dosages of the harmful factor reparatively regulate the BER mechanism which depends on p53; high dosages suppress the mechanism and induce apoptosis. When DNA damage occurs, the sensory "machine" which detects the damage, evaluates and defines the level of damage. Then, p53 is post-translationally modified and directed towards activating the control point of DNA damage according to its level.

Role of p53 in Tumour Formation

p53 mutations were found in 50–55% of all human cancer types [173]. These mutations show a different selectivity for p53 proteins which are unable to attach to DNA in a predetermined order. The cell cycle arrest function demands p53 transcriptional activity, whereas the apoptotic functions of p53 do not need gene products dependent on p53. This means that either the transcription of selected gene targets of p53 is of critical importance for the tumour suppressive function or that p53 guides the signaling pathway in lack of transcription. p53 requires binding to DNA with a specific base sequence. Individuals that are heterozygote for one allele of p53 develop cancer at a very high frequency (90–95%) and at any young age. The cellular or histological distributions of these cancers are not random and the

meaning of this is not known in relation to p53 function or in additional mutations which are required for the development of cancer in a heterozygote for the p53 cell. Obviously, the genetic substructure of the host has a significant influence on the type of cancer which is induced by p53 mutations. Besides, p53 acts with the purpose of reducing the frequency of cancers through the apoptosis of cells with activated oncogenes.

Activity Mechanisms of p53

The decision of a cell to enter cell-cycle arrest or apoptosis through p53 is influenced by various factors. In DNA damage conditions, the chances of survival of the cells are limited, while the presence of an activated oncogene pushes the cell to a replication cycle (E1A, E7, E2F or myc) or to apoptosis through p53. Therefore, cells with an unstable genome (due to DNA damage) or with activated oncogenes, undertake to enter the cells in the cell cycle or to discard them by apoptosis, as a result of the activity of p53. Two genes, which are regulated by p53, can influence the cellular decision to enter an apoptotic pathway: the genes Bax and IGF-BP3 [174, 175]. Bcl-2 overexpression can eliminate apoptosis related to p53. In this case, Bax attaches itself to Bcl-2 and antagonizes the latter's ability to eliminate apoptosis; thus, p53 dependent Bax synthesis can tip the scales towards apoptosis. Another gene product, regulated by p53 which could influence development regulation is protein-3 (BP3) which is associated with the insulin-like growth factor (IGF-BP3) [175]. The IGF-BP3 complex eliminates the signalling of the mitotic pathway of IGF since in this way it blocks the attachment of IGF to its receptor. Therefore, blocking of IGF activity promotes apoptosis or reduces cellular mitogenic response. It has also been reported that the gene fas/apo-1 is regulated by p53.

IGF activity is readily associated with many cellular proteins. Among these, the c-Abl protein has many remarkable properties. Firstly, the c-Abl protein is a nuclear protein which is activated and which displays kinase properties following DNA damage and then it is attached to p53 and promotes its transcriptional activities. c-Abl overexpression in normal cells eliminates the evolution of the cell cycle and this depends on p53 activity [176]. It seems that there is a close relationship between the c-Abl protein and p53. The protein p53 has also been proven to be associated with one basic transcriptional factor, namely RNA polymerase (TFIIH) [177].

Epilogue

Tobacco smoke contains many thousands of chemicals including a large number of carcinogens. Most of the carcinogens undergo metabolic activation in mammalian tissues via oxidation. The exposure of human tissues and organs to these carcinogens and their metabolites is an important mechanism by which smoking-related cancer is initiated.

Oxidative stress can be generated when the cell is subjected to an increased amount of endogenous and/or exogenous Reactive Oxygen Species (ROS). Exogenous oxidative stress is a key mechanism by which cigarette smoke exerts its pathological effects. The ROS can attack almost any cellular structure or molecule interacting with and modifying, through oxidation, biologically important molecules, such as lipids, proteins and DNA.

The reaction of free radicals with cellular membranes lead to the formation of lipid hydroperoxides which are degraded into a variety of toxic products; chain reactions of lipid peroxidation are important causative agents of various diseases including cancer.

Protein peroxidation by free radicals leads to oxidative alterations of aminoacid side chains and drastic cleavage of peptides by oxygen. The presence of carbonyl compounds in proteins shows that they have been subjected to oxidative damage by free radicals.

DNA damage by ROS is mainly due to oxidized DNA bases causing mis-coding. The ROS play an important role in the increase of 8-oxo-deoxyguanosine, a miscoding adduct in the DNA of smokers' lungs. Oxidative DNA damage contributes to ROS-induced carcinogenesis. The ROS may also generate a cascade causing DNA nicking and single strand breaks.

Most of the volatile organic compounds and certain compounds in tar exercise their toxic/carcinogenic effect via their metabolites.

Isoprene is metabolized to its isomeric monoepoxides by mono-oxygenase which is activated by the cytochrome P450. Monoepoxides are further metabolized into the mutagenic di-epoxides of isoprene. In cigarette smoke nitrogen monoxide is oxidized to NO_2 which reacts with isoprene and forms carbon-centered radicals that react with O_2 and form alcoxyl-radicals.

Butadiene, following oxidation, in the presence of cytochrome P450 is transversed to monoepoxide, which is further oxidized to 1,2-dihydroxy-3-butene, which is further oxidized to 3,4-epoxybutane-1,2-diole. The epoxide metabolites of butadiene are responsible for its mitogenic and carcinogenic activities.

The complex metabolism of benzene proceeds via ring oxidation and ultimately ring cleavage. Benzene metabolism takes place quickly in the liver tissue where it is converted to benzene oxide which is hydrated to dihydrodiole which is oxidized to catechol and which is further oxidized to semiquinone radical, which in turn reduces O_2 to superoxide anion. The latter is dismutated to H_2O_2, leading to DNA damage.

Acrolein is formed in vivo, either following lipid peroxidation or from the oxidative substances present in tobacco smoke.

Acrylonitrile is not directly carcinogenic/mutagenic, but it acts through its metabolites. The most interesting pathway of acrylonitrile metabolism is the direct joining with glutathion and epoxidation to form cynoethylene oxide (CEO), two reactions which are catalyzed by the cytochrome P450 (CytP450). P450 enzymes catalyse oxidation via one-electron oxidation. CEO is able to cause a variety of biological reactions, such as adducts with nucleic acids and/or with proteins.

Furan is metabolically activated by the cytochrome P450 into a cytotoxic product which stimulates cell replication.

The ROS in metal bio-toxicity participate as cancer causing agents, as well as in the multi-stage carcinogenic processes that include the activation of carcinogenic compounds, oxidative DNA damage and tumor development.

Polycyclic aromatic hydrocarbons, including diol-epoxides of benzopyrene (BPDE) are generated in the liver following enzymic processes, through oxidation by the enzymes P450 and a specific hydrolase.

NNK metabolism (a tobacco-specific nitrosamine) includes a hydroxylation, pyridine-N-oxidation, carbonyl reduction to NNAL and conjugation of NNAL to the glucoronides, NNAL-Gluc (I) and NNAL-Gluc (II).

Therefore, most of the carcinogens in tobacco smoke undergo metabolic activation in mammalian tissues, via oxidation. This metabolic activation leads to the formation of DNA adducts which are carcinogen metabolites bound covalently to DNA, usually at guanine or adenine. Mutations can also be induced by certain other agents, including oxidation.

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Index

1

1,2-dihydroxy-3-butene, 148
1,2-epoxy-2-methyl-butene, 145
1,2-epoxy-3-butane, 148
1,2-epoxy-3-methylobutane, 145
1,3-butadiene, 146, 148, 150
1,4,5-triphosphate inositol (IP3), 109
1,N^6-ethino-deoxy-adenosine (EdA), 130
1,N-ethino-deoxy-guanosine (EdG), 130
11P area, 140
1-hydroxypyrene, 132

2

26S proteosome, 97
2-deoxyguanosine (dG), 197
2-hydroxy-dATP, 55
3D-structure, 191
2-methyl-1,3-butadiene, 145
2-methyl-buta-1,3-diene, 145

3

3,4-epoxybutane-1,2-diole, 149
3,N^4-ethino-deoxy-cytokine (EdC), 130
3-methyl-furan, 145
3-phosphoglyceraldehyde dehydrogenase, 86

4

4,5 phosphatidylinositol, 109
4-hydroxy-noneale, 36
4-hydroxy-nonenale, 162

5

5-deoxyribose-phosphodiesterase, 59
5-hydroxy-cytosine, 57
5-hydroxy-tryptamine, 157
5-methyl-cytosine, 186, 227, 229
5-methyl-cytosine (5-Mec), 186
5-phosphate end, 203
5-phosphate-2-deoxy-ribose (dRP), 59

7

7-(1-hydroxy-3-buten-2-yl)guanine, 150
7,8-dihydro-8-oxoguanine, 38
7-hydroxy-ethyl-guanine, 150
7-oxo-guanine, 38

8

8-hydroxy-deoxyguanosine (8-OHdG), 197, 200
8-oxo-7,8-dihydroguanine, 39, 42, 58
8-oxo-dATP, 55
8-oxodG, 55, 56, 204
8-oxo-dGTP, 55
8-oxo-dGTPases, 192
8-oxo-GTP, 211
8-oxo-guanine, 203

A

abasic site (AP), 52, 53, 59, 203
acetaldehyde, 110, 111, 124, 127, 145, 161–163, 166–168, 170, 194, 212
  dehydrogenase, 110
acetone, 78, 161, 167, 181, 212
acetonitrile, 177, 212
acetylated histone H_2A, 71
acetylcholine (ACH), 20
acetyl-coenzyme-A, 167
acid anhydrites, 167
aconitase, 25, 82, 88
acrolein, 44, 123, 124, 128, 131, 161–166, 170, 212

acrylamide, 132
acrylic fibers, 175, 177
acrylonitrile, 132, 175–177, 212
actin, 191
actinomycin-D, 164
activated receptors, 226
acute myelogenous leukaemia, 140
acyl-transferase, 123
adducts, 52, 54, 56, 84, 111, 127–132, 134, 138, 146, 150, 156, 161, 166, 167, 213, 215, 221–223, 227, 228, 230
  base, 54
  covalent, 110, 221
  DNA, 127–132, 156, 220–223
adenine, 16, 39, 42, 51, 52, 55–57, 66, 68, 74, 77, 79, 86, 215, 229
  nucleotides, 68, 77, 79, 215
adenine nucleotide sensors, 68
adenine nucleotide translocase (ANT), 66, 215
adenoma, 141, 143, 181
adenosine, 18, 79, 128, 185
  triphosphate (ATP), 18
adenosine-methionine, 185
adhesion, 97, 100, 106, 107, 191, 198
adhesion molecules, 98
ADP, 5, 20, 21, 24, 39, 64, 66–68, 70, 75, 79, 86, 96, 129, 184
  phosphorylation, 70
adrenalectomy, 163
aerobic
  cells, 80
  organisms, 8, 31
agents
  nitroso-alkylating, 138
aggregates, 32, 211
  oxidation product, 211
aggregating neutrophiles, 164
agonists, 109, 162
a-hydroxylation, 129
AIF protein, 72
air-duct epithelium, 122
airway tract, 111, 112
AKT (protein-kinase B), 129
akyl-hydroperoxidase, 120
alcohol, 13, 36, 166, 181
aldehydes, 13, 14, 36, 42, 78, 111, 124, 128, 161–163, 170, 213
  volatile, 44
  β-unsaturated, 162
aldolase, 43
aliphatic chain, 170
aliphatic nitriles, 177
alkali, 167
alkaline phosphatise, 163
alkanes, 12, 36, 216
alkylating agents, 52, 59, 137, 149, 150, 221, 223
alkylation, 137, 138, 149
alkyl-DNA-adducts, 138
alkyl-hydroperoxidase-reductase, 41
allele loss in chromosome 11, 149
alpha-tocopherol, 7
alveolar macrophages, 147
alveolar/bronchial neoplasias, 148
amines, 130, 166, 167, 212, 216, 220
  secondary, 155
amino acids, 5, 15, 34, 35, 97, 120, 122, 162, 164, 169, 221, 228
  antioxidative, 120
  hydrophilic, 35
amylase, 44
aneuploidy, 155, 184
ankyrin repeats, 97
Annexin-V, 183
ANT, 66–68, 72, 77, 79, 215
anthocyanidines, 120
anti-apoptotic pathway, 98
anti-apoptotic protein Bcl-2, 72
anti-apoptotic protein Bcl-2, 192
antioxidant, 8, 71, 76, 77, 79, 101, 104, 117–119, 122, 123, 187, 192, 199, 202
  biochemical, 101
  substance (NAC), 104
antioxidative
  enzymes, 33, 118, 120, 121
  system (intracellular), 104
antisense regulator RNA, 41
AP-1, 42, 87, 101, 103, 162, 165, 166, 189, 191, 197, 202, 213, 225
AP-1 element, 189, 191
APAF-1, 18, 69, 74, 79, 85
aplastic anemia, 155
apo-cytochrome c, 71
apoptosis, 18, 19, 22, 25, 43, 57, 60, 68–74, 77, 78, 80, 81, 84, 85, 87, 96, 97, 99, 105, 106, 108, 122, 127, 129, 156, 162–165, 178, 183, 184, 187, 191, 192, 198–202, 219, 231, 232
  inducing factor (AIF), 69, 74
apoptosome, 18, 74, 202
apoptotic bodies, 74
apoptotic index, 178
apoptotic necrosis pathway CD95, 187
a-purinic sites, 146, 150
apurinic/apyrimidinic gap (AP), 38
arabinose, 194

arachidonic acid metabolites, 129
ARE (antioxidant response element), 166
arginine, 15, 17, 34, 120, 162
aromatic acid decarboxylase, 158
aromatic amines, 130–132, 227
aromatic hydrocarbons, 153, 212, 227
arsenic, 183–186, 193, 202
arteriosclerosis, 162
ascorbate, 194, 196, 197
ascorbic acid, 8
asteroidal cells, 167
ataxia, 49, 58, 178
atoms
  nitrogen, 137
  oxygen, 137
ATP, 5, 9, 16, 18, 19, 21, 24, 25, 31, 38, 56, 64–68, 70, 71, 74, 77, 78, 80–84, 86–88, 96, 124, 184, 215
ATPase
  complex V, 23
  Na^+-K^+, 157
autocatalysis, 75
autoimmune disease, 70, 186, 187
autoimmunity, 187
autolytic, 14
auto-oxidation, 34, 35
autoreacting lymphocytes, 187

B

B[a]P, 224
bacterial mutagens, 146
base, 31, 37–40, 51–59, 80, 128, 131, 137, 143, 149, 166, 192, 203, 216, 221, 224, 227, 231
  excision repair (BER), 51, 53
  excision repair (BER), 52
  modification, 37, 58
  oxidized, 39, 53, 81
base excision, 51–53, 57, 192, 231
base transitions, 203
Bcl-2, 19, 22, 68, 71, 72, 74, 75, 79, 80, 85–87, 202, 232
Bcl-xl, 80, 85
benzene, 132, 133, 147, 153–156, 159, 181, 212
  metabolite, 132, 154, 156
benzene oxide, 153, 154
benzo[a]pyrene, 227
benzoanthracene, 227
benzopyrene
  bioactivation of, 228
BH_3 domains, 72
bicarboxylate compounds, 131
Bid, 72
bio-amines, 168
biogenic amines, 157
biomarkers, 130–133
biomolecules, 14, 43, 204, 213
biotransformation, 148, 185, 186, 215
bladder, 184, 222
bonds, 1, 2, 16, 34, 35, 39, 76, 203
  hydrophobic, 34
bone marrow, 153–155, 181
brain, 10, 17, 96, 153, 157, 158, 169, 175
  white matter of, 157
brain synaptosomes, 157
breast, 128, 146, 148–150, 221
breast tissue, 146
bronchial epithelium, 110, 141, 147, 166, 211, 219, 220, 225
bronchogenic cancer, 167, 168
butadiene, 145–150, 212, 215, 220
  monoxide (BMO), 148
butylated hydroxytoluene, 187

C

c-phosphoglyceric end, 203
C5 (C5a), 167
C5a receptors, 110
C^8-Gua-AF, 130
Ca^{2+}, 10, 11, 17, 41, 65–69, 76–79, 81–83, 106, 109, 190, 191, 202, 214
Ca^{2+} channels, 109
caderin disruption, 187
cadmium, 133, 187–193
calcium, 5, 10, 11, 14, 16, 18, 25, 38, 41, 65–68, 76, 81, 83, 103, 104, 106–108, 164, 183, 190, 191, 201, 202, 213
  ATPase, 83
  concentration, 38, 83
  homeostasis, 10, 25
  influx, 103
  ionophores, 104
  ions, 41, 65, 76, 106, 191
  release, 213
  transport, 11, 65, 66
  uniporter, 65
calcium-related genes, 201
calmodulin, 11, 17, 191
cancer, 17, 18, 26, 42, 43, 49, 50, 58, 70, 80, 88, 96, 120, 127, 129–132, 139–142, 146, 149, 156, 167, 169, 170, 172, 173, 177, 183–185, 187, 190, 198, 201, 204,

211, 213, 214, 216, 218–220, 222–231, 235, 236, 238
breast, 149, 221
pathogenesis of lung, 223
cancer development, 58, 80, 130, 131, 169, 177, 184, 198, 214, 216, 219, 222–225, 228, 230
car exhaust gases, 147
carbamidic esters, 131
carbon atoms, 129
carbon dioxide, 23, 167, 212
carbonyls, 14, 15, 39, 44, 162, 170
a,b,-unsaturated, 131
carboxy-terminal, 55
carcinogenesis, 42, 50, 63, 88, 106, 108, 127, 128, 130, 134, 137–139, 146, 155, 168, 175, 184, 185, 187, 188, 191, 197, 199, 204, 211, 215, 216, 218, 221, 222, 224, 225
chemical, 188, 216, 221
multi-staged, 149
carcinogenic, 50, 128–132, 137, 145–147, 149, 150, 153, 155, 159, 166, 175, 181–184, 188, 192, 194, 197, 199, 204, 211, 212, 214–216, 220, 221, 223–225, 227, 230, 237, 238
carcinogens, 127, 129, 131–133, 141, 142, 149, 153, 218–225
tobacco smoke, 225
carcinoma, 141, 181, 219
granular cell, 148
lobular-cell, 148
micro-cell-lung, 130
cardiovascular system, 222
casein kinase-II, 191
caspase-3, 22, 73, 79, 85, 87, 187, 199
caspase-9, 74, 79, 191
caspases, 18, 22, 69, 73, 85, 87, 202
activation, 18, 73, 74, 85–87
catalases, 7, 16, 33, 121
catecholamines, 120
catechols, 120, 219
catenins, 191
cations, 15, 34, 65, 74
divalent, 74
ceanorhabditis elegans, 22
ced-3 protein, 75
ced-9 protein, 71
cell
metamorphosis, 189
transformation, 177, 186, 190, 224, 226
cell cycle, 49, 50, 52, 53, 57, 60, 85, 127, 141, 165, 184, 186, 190, 198, 201, 202, 215, 231, 232
arrest, 85, 231
control, 186, 201, 202
G_0 phase, 141
G_1 phase, 52, 58
G_1/S phases, 201
M-phase, 183
multiple, 143
regulation, 156, 201
S-phase, 58, 141, 165
cell death, 5, 15, 19, 22, 25, 38, 43, 57, 69–71, 74, 78–80, 83–85, 102, 162, 184, 199, 201
cell hyperplasia, 141
cell proliferation, 97, 108, 122, 129, 155, 162–165, 170, 178, 198, 201, 213, 223
cell rest, 165
cells
B, 186
bone marrow, 155, 181
mammalian, 50, 52, 58, 59, 70, 75, 121, 155, 167, 193
myeloid, 155
neoplasmic, 141, 199
olfactory epithelial, 163, 170
ovary, 181
precancerous, 141
progenic, 220
pulmonary, 163, 214
red blood, 132, 169, 177
retina, 158
ceramide, 80, 85, 87
cerebrospinal fluid, 10
CEVAL [N-(2-cynoethyl)-valine], 175
c-fos, 105, 162, 188, 189
chaperones, 188
chemotactic factors, 97
chloroacrylonitrile, 177
choline phospholipids, 109
chromatin, 16, 18, 40, 70, 71, 74, 139, 141–143, 204, 211, 225
aggregation, 70
structure, 142, 143, 225
chromatin-bound iron, 204
chromium, 183, 194, 195, 199, 201, 202
chromosomal
deletion, 169, 177, 181
deviation, 184
chromosomes, 50, 140, 150, 155, 219
11, 150, 156
17, 150

2P, 140
3P, 141, 225
cigarette smoke, 20, 21, 43, 44, 50, 77, 97, 98, 100, 110, 111, 120, 123, 127–129, 131, 138, 142, 146, 151, 157, 161, 163, 166–170, 175, 183, 187, 192, 204, 211–216, 220–222, 224–227, 229, 230
gas phase, 20
solid phase of (tar), 141, 212
cilia, 161, 163, 166, 167, 169
ciliary beat, 110, 166
ciliary proteins, 110
ciliotoxic, 161
cis-2-butadiene-1,4-diale, 181
cis-2-pentanonitrile, 177
citric acid cycle, 83
c-jun, 188–191
clathrin, 226
clonal cell population, 227
clone index, 220
clotting time, 168
c-myc, 188–191, 197
coated pits, 226
cobalt, 193
co-carcinogenic, 120
co-carcinogens, 223
codon 13, 149
codon hot spot, 42
co-enzyme NADH, 166
coenzyme Q, 75
co-factor, 169
cofactors, 106, 108
cold spots, 229
collagen, 164, 167
color recognition, 158
complex, 22, 24, 31, 33, 35, 53–55, 66–68, 72, 73, 77, 79, 82, 84, 98, 100, 103–105, 139, 147, 186, 187, 194, 196, 199, 203, 212–214, 216, 225, 232
cycline D/CDK4 kinase, 149
Jun/fos, 225
complexes
I, III, IV and V, 215
proteosomic, 34, 35
co-polymers, 177
copper, 8, 10, 32, 40, 82, 86, 118, 183
corticosteroids, 79
coumenic hydroxy-peroxide, 130
coupling
of DNA-DNA, 37
of DNA-protein, 169
with adenine, 38
with cytocine, 38
covalent bonds, 131, 196
covalent joining, 169
CpG, 51, 138–143, 185, 186, 224, 225, 227, 229
dinucleotides, 227, 229
islands, 185, 186
islets, 138–140, 142, 143, 224, 225
regions, 139, 140, 143, 225
C-Phosphogyceric end
creatine kinase, 24, 25, 81, 82, 88, 123
mitochondrial (MCK), 23
creatinine, 156
CREB factor (c-AMP-response element binding protein), 190
critical mass, 230
cross-linking, 146, 167, 169, 170
crotonaldehyde, 44, 124, 128, 131, 161, 162, 212
crysene, 227
C-terminal subunit, 106
cyanamide, 110, 167
cyanate, 44
cyanide, 44, 176–178
ions, 44
cyanoethylene oxide (CEO), 175
cyanohydrin, 176
cyclic dien-ether, 181
cycline-dependent kinase genes, 201
cyclines, 186
cyclosporine, 67, 87
CYP2E1, 148, 157
cysteine, 16, 18, 33, 34, 73, 76, 86, 98, 105, 107, 121, 122, 161, 164, 166, 176, 194, 195, 215
nucleophilic, 164
cytochrome
oxidase, 7, 19, 31, 63, 82, 84, 88
P450, 9, 127, 145, 148, 154, 175, 181, 224, 228
phase I enzymes of P450, 228
cytochrome c, 69, 71, 72, 75, 78, 80–82, 84, 85, 87, 202
cytochrome-c, 25, 73
cytokines, 17, 100, 110, 123
cytoplasm, 11, 18, 24, 34, 35, 37, 56, 70, 74, 79, 98, 99, 101, 102, 104, 108, 112, 121, 124, 149, 164, 202, 226
cytoplasmic microtubules, 226
cytosine, 16, 37, 42, 55, 57, 139, 186, 193, 202, 225, 227, 229
deamination, 225
cytoskeleton, 107, 108

cytosol, 64, 68, 71, 75, 79, 80, 84, 85, 87, 97
cytotoxicity, 11, 17, 39, 40, 84, 147, 148

D

deamination, 51, 52, 139, 227, 229
  hydrolytic, 52
death-inducing signaling complex (DISC), 73
decarboxylation (reaction), 23
dehydrogenase, 11, 25, 75, 82, 86, 167, 169, 194, 213
  pyruvate, 23
  succinic, 22
deletions, 131, 227, 229, 230
de-methylated, 185
depolarization, 19, 68, 69, 83, 202
dGTP oxidation, 192
diacylglycerol (DAG), 106
dichromate, 194
dienes, 147
  conjugated, 12
  coupled, 36
dienine, 166
diepoxibutane, 146
di-epoxides, 145
diepoxybutane, 149
diesterases, 39
diethylmaleic salt (DEM), 164
differentiation, 102, 106, 108, 198, 201, 202, 231
dihydrodiole, 153
dihydroquinols, 121
dihydroquinones, 121
dihydroxy-metabolites, 153
dimerization, 97
dimers, 97, 213
dinein, 110
diolephin, 145
diol-epoxides, 216, 224, 227
diolic epoxides (BPDE), 228
diols, 194
diphenyl propane, 120
diploid fibroblasts, 183
dismutation, 16, 32, 33
disulfide
  bonds, 7
  bridges, 100
D-mannitol, 187
DMSO, 6
DNA, 5, 7–12, 15–17, 21, 22, 32, 36–43, 45, 49–62, 69, 73, 74, 79–81, 85, 86, 88, 96–98, 101, 103, 104, 111, 120, 123, 127–132, 134, 137–144, 146, 149, 154–156, 163–170, 175, 177, 181, 183–187, 189, 190, 192–194, 197, 200–204, 211–216, 219–223, 225–232
  adenine adducts, 146
  base order, 229
  binding, 97, 98, 101, 103, 105, 197
  circular, 88
  cytosine methylation, 190
  damage, 5, 16, 37–40, 42, 49–55, 57, 58, 73, 80, 81, 85, 86, 111, 123, 127, 130, 154, 156, 165, 175, 181, 183, 184, 192, 197, 200, 201, 213, 214, 222, 223, 226, 229–232
  division, 201
  exocyclic adducts, 39
  fragmentation, 69, 74, 79, 85, 187, 194
  genomic, 50, 183, 202
  helixes, 38, 40, 163
  hydroperoxides, 36
  hypermethylation, 138, 140, 186
  methylases, 38
  methylation, 138–142, 185, 193, 225, 229
  methyl-transferase (DNA-MTase), 138
  non-methylated, 138
  oxidation, 39, 55, 58
  oxidized, 38
  polymerases, 38, 39, 50
  proteins, 163, 170
  purines, 130
  repair, 8, 49, 52, 56, 81, 130, 187, 189, 192, 193, 214, 222, 231
  repair mechanisms, 49, 52, 81, 214
  repairing, 8, 130, 222
  replication, 16, 38, 39, 42, 51, 53, 58, 60, 138, 139, 155, 184, 185, 221, 231
  semi-methylated, 138
  sub-methylation, 139
  genome, 49
D-nitrosothiols, 18
dopamine, 122, 157, 158
dopaminergic mechanisms, 158
double bond fragments, 230
double carbon-carbon bond, 161
double helix, 38, 54, 56
DT diaphorase, 34
DT-diaphorase, 33, 121, 122, 214
dyschromatopsia, 158
dysplasia, 127, 141, 219, 225

E

early and immediate response genes (IERGs), 188
E-cadherin, 191

edema
  cellular, 14
elastomers, 177
electrochemical density gradient, 14
electron, 1, 3, 4, 6–12, 15–17, 21–23, 25, 31, 38, 63, 65, 72, 74, 77, 80–82, 84, 86, 88, 98, 117, 155, 216
  acceptor, 11, 63, 86
  donors, 23, 98
  flux, 38
  non-conjugated, 3
  single, 9, 12
  transport chain, 7, 17, 25, 31, 63, 82, 84
  uncoupled, 31, 39, 137
  unpaired, 3, 10
electrophile aldehyde, 162
electrophile response element (ERE), 166
electrophilic, 122, 161, 166, 213, 215, 221, 227
  compounds, 122
embryogenesis, 138
emphysema, 162, 164, 167, 192
endonuclease, 41, 53, 54, 59, 231
  APE, 53
  hydrolytic AP, 53
  III, 38
endonucleolytic activity, 38
endoplasmic reticulum, 9, 18, 41, 67, 72, 79, 80, 83, 87, 216
endosomes, 226
endothelial cells, 11, 20, 123, 162
energy, 3, 5, 10, 11, 16, 18, 19, 23, 25, 38, 63–65, 67–70, 78, 79, 87, 117, 145
environmental stress, 188
enzymes, 1, 8–10, 16, 17, 22, 33, 34, 36, 40–44, 50, 53, 58, 59, 81, 83, 97, 101, 120, 121, 123, 130, 147, 155, 158, 161, 163, 164, 169, 186, 194, 202, 213–216, 223, 224
  antioxidative, 33, 118, 120, 121
  bound to metal, 120
  cell, 221
  cell membrane, 42
  glycolytic, 43
  nucleasic, 40
  oxidizing, 9
  proteolytic, 34, 186
  reconstitution, 59
  repairing, 203
  suicide, 52
epithelium
  lung, 122, 220
epoxidation, 130, 175, 176
epoxide-hydroxylase, 228
epoxides, 13, 42, 128, 145, 146, 150, 213, 216, 230
epoxybutene, 149, 150
epoxy-metabolites, 3, 150
ERK (extracellular signal-regulated kinase), 98
Escherichia Coli, 138
eterocyclic organic compounds, 181
eterodimer, 55, 165
ethanol, 9, 166, 168
ether, 181
ethino-adducts, 130
ethino-C, 51
ethyl-adenine, 132, 150
ethylating agent, 132
ethylation, 132
ethylene oxide, 132, 150, 176, 177
ethyl-thymidine, 132
eukaryotics, 51
exfoliative cancer, 129
exfoliative epithelia, 178
exfoliative neoplasms, 148
exhaust-gases, 162
exocytosis, 41
exon 3, 150
exonuclease III, 38
exonuclease IV, 41
experimental animal, 130, 131, 146, 148, 150, 155, 157, 166, 175, 177

F

F2-isoprenes, 133
Fas-associated via death domain (FADD), 73
fat tissue, 153
fatty acids, 8, 12
  polyunsaturated, 5, 10, 12
  unsaturated, 13, 107, 109
Fenton reaction, 10, 16, 76, 197, 203, 204, 211
Fenton-metal, 191
ferric (Fe^{2+}), 204
ferritin, 8, 10, 204
ferroredoxin oxidoreductase, 194
ferrous (Fe^{3+}), 204
fibroblasts (hamster), 81
fibronectin, 164, 167
flavoenzymes, 194, 196
flavones, 120
flavonoids, 119, 120
flavonons, 120
flavo-phenoxyl radicals, 119
flavoproteins, 63
flow cytometry, 184
fluorescent in situ hybridism, 220

formaldehyde, 124, 127, 145, 161, 163, 168–170, 173
formaldehyde-dehydrogenase, 169
formic acid, 169
free iron, 204
free radical-like intermediate products, 228
fructose, 194
fumarase, 41
furan, 129, 181, 212

G

G_2/M, 201
gastrointestinal system, 153
gene, 41, 42, 55, 58, 70, 71, 75, 81, 88, 98–100, 102, 103, 105, 123, 130, 138–143, 146, 147, 149, 156, 162–167, 169, 177, 181, 183–188, 190, 193, 198–202, 213–215, 220, 221, 223–232
  ERE, 166
  expression, 41, 42, 81, 88, 98, 99, 102, 139–141, 147, 163, 184, 186, 188, 190, 201, 202, 214, 229
  H-ras, 42
  instability, 130
  mutation, 42, 130, 143, 167, 169, 177, 181, 224, 225, 227, 229, 230
  p15, 140
  p16, 140
  p53, 42
  promoter, 138, 140, 142, 166, 224
  regulation, 165, 200
  specific, 105, 122, 140, 164, 185
  stress, 162
  suppressive, 140, 229
  suppressor, 142, 185
  transcription, 105, 123, 138, 139, 156, 183, 185, 225
  tumor suppressor, 127, 130, 140, 149, 185, 189, 222–225
genetic deletion, 33, 121
genetic polymorphism, 222
genome
  unstable, 232
genome instability, 59, 156
genotoxic factors, 57, 137
genotoxicity, 131, 137, 187, 189, 190, 192, 194
glial fibrinoid acid protein (GFAP), 158
glucocorticosteroids, 158, 164
glucosaminoglycans, 11
glucose, 41, 71, 85, 86
glucose-6-phosphate-dehydrogenase, 41
glucuronides (NNAL-Glucs), 133
glutamine
  L, 122
glutathione, 7, 8, 11, 19, 24, 25, 33, 36, 37, 41, 76, 81, 83, 86, 101, 104, 108, 118, 120–124, 128, 148, 153, 162–164, 166, 169, 175–177, 186, 188, 192, 194–196, 201, 213, 214, 224, 230
  peroxidase, 33, 36, 41, 76, 81, 83, 101, 118, 120–122, 188, 192, 201
  reductase, 33, 76, 101, 120, 122, 124, 188, 194, 196, 214
  synthesis, 104, 188
  system, 8, 104
Glutathione transporting pumps (GSSG), 124
glutathione-S-transferase (GSTase), 123
glutathione-S-transferase M1 (GSTM1), 230
glutathione-S-transferases (GST-a and GST-p), 189
glyceraldehyde triphosphate dehydrogenase, 123
glycine, 122, 169
glycolaldehyde, 162
glycol-thymine, 38
glycolysis, 18, 19, 25, 71, 79, 86, 162
glycosylases, 51–53, 56
  3-alkyl-N-purino, 53
  8-oxo-DNA (hOGG1), 53
  bifunctional, 53
  DNA, 51, 52, 54
  mBD4, 51
  monofunctional, 53
  nNg, 51
  SMUG1, 51
  uracil-DNA (UDG), 53
glycuronides, 153
glyoxal methylate, 131
glyoxyale, 162
G-protein, 17, 201
granulocytes, 21, 33, 155
  polymorphonuclear, 21
gray matter, 157
group, 3, 4, 32, 35, 49, 67, 76, 81, 84, 105, 107, 137, 146, 156, 163, 184–186, 212
  amino, 110, 111, 137
  carbonyl, 15, 34, 166, 170
  carboxylic, 161
  hydroxyl, 119
  methoxy, 119
  methyl, 52, 57, 139, 145, 146, 185, 186
  methylene, 129
  N-nitroso, 129
  prosthetic transitional metal, 32
  sulfhydryl, 5, 11, 15, 34, 49, 155
  sulphydryl, 122

thiolic, 188
growth factor signals, 202
growth factors, 105, 110, 155, 165, 185, 211
growth hormone, 97, 109
GSH, 21, 33, 76, 98, 104, 105, 121–124, 132, 161, 162, 164, 169, 176, 188, 194, 196, 213
GSH-S-transferase, 176
GS-SG, 33, 121
guanine, 38, 39, 52, 56, 57, 59, 80, 131, 137, 149, 150, 197, 200, 203, 221, 223, 228, 229
methylation, 39
guanine-O^6, 137
guanyl cyclase, 17, 21, 83
soluble, 17

H

H_2S, 167
Haber-Weiss reaction, 7, 203
halogen, 33
chlorine, 33
halogens, 161
HCN, 167
hearing loss, 158
heart, 148, 153
heat-stress proteins (HSPs), 188
hemangiosarcomas, 148
heme, 8, 21, 23, 72, 82, 88, 162, 184
a_3, 88
a3, 82
heme oxygenase, 184
heme-oxygenase, 162
hemoglobin, 132, 137, 146, 151, 175, 204, 212
hemoglobin adduct, 146, 151
hemoglobin valine, 146
hemoglobin-carcinogen adduct, 132
hemopoiesis
regulation of, 155
hepatocellular adenoma, 181
hepatocellular neoplasms, 148
heterocycle amines, 131
heterodimeric complexes, 100
heterozygosity
loss of, 141, 150, 156, 225
hexanal, 12
hexokinase, 43
hippocampus, 158
hippuric acid, 157
histidine, 15, 34, 70, 137, 162
histones, 81, 88, 143, 168
H1e, 143
homeostasis, 42, 68–70, 124
cellular, 21, 105, 106, 108
Glutathione-reducing, 123
homodimer, 165
homodimeric complexes, 100
homologous recombination, 52, 57
hormonal homeostasis, 158
hormones, 105, 110
hot spot, 58, 225, 227–229
hprt region, 150
human pulmonary cancer, 228
hydrocarbons, 1, 32, 145, 212, 216, 220, 228
gaseous, 12
hydrogen, 3, 7–12, 19, 31, 36, 41, 42, 44, 63–65, 76, 78, 81, 83, 100, 101, 104, 119, 123, 138, 192, 195–197, 203, 215, 216
bonds, 138
ions, 63–65
hydrogen peroxide, 3, 7–11, 19, 31, 41, 42, 76, 81, 83, 100, 101, 104, 123, 192, 195–197, 203, 215
dismutase, 76, 83
hydrogenation, 161
hydrolysis, 15, 68, 78, 79, 106, 109
enzymic, 79
hydroperoxide, 3, 13, 36, 124
route, 13
hydroquinones, 120
hydroxyalkanes, 12
hydroxy-alkenales, 130
hydroxylation, 16, 129, 161, 197
hypermethylation, 140–143, 185
hyperphosphorylation, 155, 156
hyperplasia, 127, 143, 219
hypomethylation, 140, 185, 186
hypophysectomy, 163
hypothalamus-pituitary-suprarenal axis, 158
hypoxia, 11, 80, 231

I

ICAM-1 (intracellular adhesion molecule-1), 100
idiopathic pulmonary fibrosis, 122
IkB, 97, 102–104
family of proteins, 97
immune
cell activation, 103
function, 21, 42, 103
immune response, 21, 97, 155
immunoactivation, 186
immunofluorescence, 107
immunotoxic agent, 186

inflammation, 11, 21, 99, 100, 105, 122, 123, 155, 162, 164, 185
inflammation cytokines, 185
inflammation messengers, 155
inflammatory reactions, 147
inorganic mercury (Hg^{2+}), 187
inositol phospholipid hydrolysis, 109, 110
inositol-1,4,5-triphosphate, 106
insulin-like growth factor (IGF-BP3), 232
interleukin
  1 (IL-1), 100
  2 (IL-2), 99
  8 (IL-8), 147
interleukin-1β converting enzyme (ICE), 75
intermittent methylation, 224
International Agency for Research on Cancer (IARC), 145, 152, 175
intestine, 5, 10, 140
invasive metastatic cancer, 219
iodine propide, 183
ion channel regulation, 198
ion transport, 78, 106
ionization of hydrogen, 63
ions
  metal, 7, 10, 82, 86, 120
iron, 8–10, 16, 17, 21, 32, 40, 63, 78, 82, 86, 118, 119, 130, 155, 183, 203, 204, 216
  sulfur centers, 82
  sulfur complexes, 86
ischemic, 5, 11, 18
isoprene, 20, 145–147, 151, 212, 215
isoprene DNA adducts, 146
isothiocyanate compounds, 167

J

JNK, 85, 184, 191, 197
  phosphatase, 184
Jun-B, 201

K

ketones, 167, 212
kidneys, 127, 146, 156, 169, 187
kinase cascades, 42, 197, 201
kinase-engines, 186
kinases, 18, 68, 102, 105, 190, 197
  c-Jun N-terminal, 102
  MAP, 18, 98, 102, 201
  p38, 98
  pyruvate, 43
  tyrosine, 102, 191, 199
K-ras, 42, 112, 140, 149, 189, 223–225
Krebs cycle, 23, 167
  citric acid, 167

L

lactic dehydrogenase, 44
large bowel, 222
laryngeal malignant tumors, 166
lecithin cholesterol, 123
leukemia, 155
  monocytic, 181
leukemogenesis, 155
ligands, 7, 66, 73, 204
  CD2, CD58, 103
lipases, 8
lipid bilayer, 157
lipid epoxides, 13, 36
lipid hydroperoxides, 39, 42
lipid peroxy-radical, 12, 36
lipids, 8, 10–12, 17, 31, 32, 37, 42, 44, 75, 76, 78, 87, 108, 118, 120, 157, 193, 212
  endogenous, 3
  membrane, 75, 78
  peroxidation, 5, 7, 9, 12, 14, 26, 36, 39, 42, 43, 51, 77, 78, 81, 119, 128, 130, 162, 175, 189, 192, 193, 203, 213
  poly-unsaturated, 78
  unsaturated, 42, 78
lipofuscin, 37
lipoic acid, 194
lipophilic antioxidant, 118
lipophilic factors, 108
lipopolysaccharides (LPS), 104
lipoxidation, 162
liver, 9, 10, 21, 128, 129, 139, 146, 148, 149, 153, 154, 157, 163, 167, 169, 176–178, 187, 192, 194, 195, 216, 222
long-chain fatty acids, 121
loss in heterozygosity (LOH), 149
loss of alleles, 220
low molecular weight complexes of Fe^{2+}, 75
lower oxidation states, 201
lung
  interstitium, 214
  lipoid tissue of, 213
lung adenocarcinoma, 140, 223, 224
lung cancer, 111, 129, 140, 143, 146, 149, 150, 175, 183, 186, 188, 189, 211, 219, 220, 223, 224, 227, 229, 230, 236
lung fibrosis, 193

lung tissue, 122, 127, 129, 131, 141, 146, 156, 161, 162, 177, 193, 220, 222, 223
lungs, 10, 20, 110, 153, 157, 183, 187, 193, 211, 220
lyase, 52, 59, 215
lymphocyte
B, 155
T, 103, 104, 140, 149, 150, 155, 186
lymphocytes, 85, 131, 155, 177, 183, 184, 187
lymphoma, 147, 149, 181, 197
lymphocytic, 148, 149
lysine, 15, 34, 162
lysophosphatidylcholine, 110
lysosomes, 226

M

macromolecules, 7, 10, 137, 228
macrophages, 21, 103, 220
magnesium, 203
major histocompatibility complex (MHC), 186
malignant transformation, 192, 221
malondealdehyde, 12, 36, 39, 111, 128, 131, 154, 162, 213
mammals, 23, 33, 75, 77, 121, 127, 138, 181
manganese, 8, 118
MAPK pathway, 87, 202
markers, 15, 69, 74, 131, 154, 156
membrane-bound vesicles, 70
menadione, 16
mercapturic acid, 132, 176
messengers
intracellular, 100, 104
secondary, 12, 190
metabolism, 4, 5, 14, 25, 39, 43, 78, 79, 88, 123, 128, 132, 145, 148, 153, 154, 157, 159, 175–178, 192, 198, 214, 222
metabolites, 4, 5, 50, 127, 128, 131–133, 145, 146, 148–150, 153–157, 175, 176, 218, 224, 228, 229
metal, 7, 10, 16, 23, 32, 82, 84, 86, 120, 183, 187, 189, 192, 194, 202, 212
transition, 6, 7, 10, 23, 32, 84, 118–120
metal transcription factor (MTF1), 189
metallothionein, 16, 188, 192, 201
metallothionein IIA, 201
metaplasia, 178, 219
metastasis, 17, 106
methacrolein, 145, 212
methacrylonitrile, 177
toxicity, 178
methane-diazo-hydroxide, 129
methionine, 34, 36, 39, 49, 52, 76, 139, 169, 212
oxidation, 76
sulfoxide, 34
sulfoxyl-reductase, 49
methyl cytosine, 139
methyl-adenine, 40
methyl-arsenic acid, 185
methylate substances
N-, O- and S-, 169
methylated cytosines, 139, 185
methylated double helix, 185
methylating agents, 39
methylation, 38, 39, 84, 137–143, 185, 186, 221, 225, 227
de novo, 138
methyl-guanine, 39, 52, 57, 221
methyl-transferase, 52, 57, 141, 185, 225
Mg^{++}-ATPase, 157
mice, 16, 56, 128, 138–140, 145–150, 155–158, 166, 176, 181
microsatellites, 220
microsomal membranes, 130
microsomal system, 148
microsomes, 149, 157, 176, 194, 216
hepatic, 148
microspots, 53
microtubules, 155
miscoding, 50, 56, 127
mismatch repair (MMR), 51
mismatch repair (MMR), 55
mis-repairing, 221
mis-replication, 146
mitochondria, 3, 8, 9, 16, 18, 22–24, 31, 34, 38, 41, 43, 51, 56, 63, 65, 67–69, 71, 72, 74–78, 80, 81, 83–85, 87–89, 96, 102, 123, 191, 192, 194, 202, 215
aconitase, 25, 82, 88
calcium, 65, 79, 82
catalase, 76
depolarization, 19
DNA bases, 80
electric potential, 63
glutathione peroxidase, 33, 36, 41, 76, 81, 83, 101, 118, 120–122, 188, 192, 201
inner matrix, 63
matrix, 24, 25, 64, 66, 68–70, 83, 88, 202
matrix Ca^{2+}, 66, 83
mega-channels, 69, 74
membrane potential, 66, 68, 71, 74, 78, 83–85, 87, 88
optical density, 69
permeability transition (MPT), 18
phospholipids, 18, 85
phosphorylation, 64

programmed cell death, 22, 24, 70, 71, 75, 79, 98
pyrimidine nucleotide, 76
respiration, 18, 22, 23, 74, 76, 78, 81, 83, 85, 86, 88
RNAs, 81
swelling, 69, 84
calcium, 14, 67
mitochondrial
genetic code, 88
genome, 88, 96, 215
lipids, 87
mitochondrial biogenesis, 215
mitochondrial gene, 81, 88, 215
mitochondrial permeability transition pores, 214
mitogen oxides, 147
mitogenesis, 102, 106
mitogenics, 148
mitogens of the MAPK family, 191
mitosis, 184, 201
mitotic spindle, 155, 183
MMR proteins, 55
monocytes, 103, 155
monoepoxides, 145, 146
mono-oxygenase, 9, 145, 154
monophosphoric deoxynucleotides, 80
morphology, 74, 198, 202
MRP transporting pumps, 124
MTSI region of chromosome 4, 149
mucociliary catharsis, 110
mucocoagulating properties, 161
mucostasis, 169
mucous, 122, 168
muscle, 96, 153, 169
Mut proteins, 55
mutagenesis, 40, 42, 51, 58–60, 130, 131, 134, 137, 167, 215, 221, 228–230
mutagenic, 42, 50, 52, 55–57, 59, 120, 130, 131, 137, 145, 149, 150, 168, 175, 185, 188, 189, 192, 193, 203, 213, 214, 221, 223, 225, 227–230
mutagenic substances, 227
mutation, 16, 38, 39, 56, 80, 88, 121, 130, 150, 190, 201, 220, 221, 223, 225, 227, 228
lethal, 33
Muts eterodimer, 55
myc-gene, 225
myconic acid, 153, 154, 156
myeloperoxidase, 33, 43, 154, 155, 216
myoglobin, 204

N

N-[4-5-nitro-2-furil-2-thionyl] formamidase, 227
N^7-(2-hydroxy-3-butene-1-yl) guanine, 149
N^7-guanine, 149
N7-Gua-adducts, 146
Na^+/H^+ antiporter, 65
N-acetyl-cysteine (NAC), 165, 199
N-acetyl-S-(2-cyanoethyl) cysteine, 175
NAD, 5, 9, 11, 16, 21, 24, 38, 63, 65, 76, 83, 86, 167, 169, 184, 194
NAD(P) transhydrogenase, 76
NAD(P)H, 9, 83, 194
NADPH, 17, 24, 33, 76, 77, 100, 121, 194–196, 198, 216
NADPH oxidase, 33, 100
necrosis, 5, 18, 19, 22, 43, 70, 73, 74, 79, 84, 86
NEIL 1, 2, 3, 53, 57
nervous system, 157
central, 157
neurons, 158
neurotransmitters, 105, 110, 158
neutrophilic granulocytes, 193
NFkB, 97–105
NH_3, 167
nickel, 142, 193
nicotinamide, 11, 21, 24
nicotinamide-adenino-nucleotide, 11
nicotinamide-nucleotide-transhydrogenase, 24
nicotinic-cholinergic, 129
nitration, 23, 82, 84, 212
nitric oxide, 20, 21, 27, 97, 99, 101, 184, 192
constitutive, 17
endogenous inhibitor, 21
endothelial, 18
inducible, 17
neuronic, 18
synthase (NOS), 17
nitriles, 175, 177
nitrile-triacetate, 9
nitrogen, 3, 7, 20, 23, 82, 120, 137, 147, 166, 184, 211, 212
nitrogen dioxide, 18–20, 33, 83, 87, 120, 146, 212
nitrosamines, 40, 128, 129, 131, 155, 212
nitroso-compounds, 167
nitrosylation, 84, 86, 87, 101
nitrothiols, 83
NO, 6, 17–23, 33, 78–88, 97, 101, 102, 120, 124, 146, 155, 184, 211
synthase inhibitors, 184

non-small-cell lung cancers (NSCLC), 230
norepinephrine, 157
normocytic, 178
nuclear membrane, 79
nucleophilia, 137
nucleophilic
  atoms, 221
  centers, 137
  molecules, 166
  properties, 166, 170
  proteins, 111
  substances, 120, 137, 162, 213
  substitution, 161
nucleotide excision, 50, 54, 192
nucleotide reservoirs, 198
nucleotides, 16, 50, 54, 55, 58, 60, 66, 68, 77, 79, 128, 138, 214, 221
  excision repair (NER), 50
  excision repair (NER), 54
  hydroxylated, 38
  pool sanitation, 55–57, 68
nucleus condensation, 187
null phenotype, 226

O

O^4-alkyl adducts, 138
O^4-alkyl-T-G bases, 138
O^4-Medt adducts, 138
O^4-methyl-deoxythymine (O^4-Medt), 138
O^6-alkyl-guanine-DNA-alkyl-transferase, 59
O^6-methyl-guanine-DNA-methyltransferase (MGMT), 223
O6-methyl-guanine, 40, 52, 57, 130, 221, 223
O6-methyl-guanine-methyl-transferase, 52, 130
octahedral, 196
olfactory ciliated epithelium, 168
olfactory mucosa, 169, 170
oligonucleotide, 54
oncogenes, 140, 142, 190, 197, 201, 202, 211, 222, 223, 225, 232, 236
  primary, 188
  Ras, 102, 103, 106, 109, 185, 225, 226, 236
  suppressive, 140
oncogenesis, 56, 138, 141, 143, 189
one-electron reduction, 198
open ring pathway, 153
optic nerve fibers, 158
orbital, 3, 31
ortho-trihydroxyl group, 120
ovary, 148, 181
oxidants, 23, 36, 50, 71, 74, 76, 77, 79–81, 85, 101, 102, 118, 123, 162, 165, 201, 202
  cigarette smoke, 50, 97, 100
oxidase complex Rac/NADPH, 100
oxidation, 1, 3, 7, 8, 11, 14–16, 18–22, 24, 31, 32, 34, 35, 37–40, 44, 52, 56–58, 75–81, 83, 87, 98, 100, 103, 105, 107, 108, 117, 118, 120, 121, 128, 145, 147, 148, 163, 167, 169, 183, 184, 203, 211, 212, 214, 216, 228
oxidative
  damage, 9–12, 15, 24, 25, 33, 35, 37–40, 42, 43, 45, 49, 51, 54, 58, 60, 78, 81, 108, 118, 122, 130, 183, 193, 201, 211, 213, 215, 228
  demethylation, 38
  phosphorylation, 56, 65, 77, 78, 84, 86, 88, 215
  phosphorylation factors, 77
  respiration, 42, 58
  stress, 15, 19, 23, 24, 31–34, 36–38, 40–42, 53, 60, 67, 75–78, 81, 82, 85, 98, 100–102, 104, 108, 120, 123, 124, 130, 162, 184, 185, 187–189, 193, 197, 198, 201, 203, 213–215, 226
  substances, 5, 18, 20, 22, 41, 101, 105, 107, 108, 162, 170, 215, 225
oxidative load, 214, 215
OxoR-regulon, 41
OxoRS, 41
oxygen, 1, 3–5, 7–12, 15–18, 21, 31–33, 36, 38, 39, 44, 57, 58, 63, 65, 75, 77, 78, 82, 83, 86, 88, 101–103, 110, 111, 117, 123, 137, 147, 183, 184, 192, 203, 204, 211, 212, 214, 215, 231
  diatomic, 1
  free radicals, 5, 7, 8, 39, 44, 102, 211
  molecular, 3, 10, 11, 23, 31, 78, 86, 194, 201
  univalent, 75
oxygen oxidoreductase, 31
OxyR, 41, 120
ozone, 145, 147

P

p38 MAP kinase, 87
p53
  mutation, 222, 227, 231
p53 allele, 156
PAH-DNA adducts, 220
PAHs, 212
pancreas, 128, 129, 222
paramagnetic center, 3
paraquat (PQ), 16
PARP degradation, 187
patch-clamp techniques, 69

pathways, 13, 18, 24, 49, 50, 52, 71, 85, 87, 88, 97, 99–101, 104, 120, 141, 153, 162, 165, 166, 175, 176, 199, 202, 213, 216, 231
P-ATPase transport pump, 203
peripheral blood, 10, 132, 157, 178, 184, 204, 222
permeability transition pores, 214
peroxidases, 7, 16, 33, 36, 44, 76, 121, 216
peroxidation, 5, 12–16, 34, 36–38, 42, 51, 77, 78, 81, 85, 87, 130, 162, 177, 193, 213, 218
  ω-3 polyunsaturated fatty acids, 14
  ω-6 polyunsaturated fatty acids, 14
peroxide dismutase, 76, 83, 110
peroxides, 3, 12, 77, 108, 123, 214
peroxisomes, 121
peroxisomes bodies, 121
peroxynitrite, 18, 21–24, 78, 79, 82, 120, 215
peroxynitrous acid, 23, 25
phagocytes, 33, 123
phagocytosis, 5
phenols, 120, 167
phorbol
  esters, 104–106, 109
phosphatase, 44, 53
phosphate, 38, 39, 53, 59
phosphatidylcholine, 106, 109, 110
phosphatidylinositol-4,5-diphosphate, 106
phosphatidyl-serine, 18, 183, 187
phosphocreatine, 24
phosphodiesteric backbone, 38
phosphoinositide pathway, 110
phospholipases, 15, 37, 68, 102
  A_2, 109
  C, 109
  D, 109
phospholipids, 15, 37, 42, 85, 105, 106, 109, 158
  anionic, 106
  methylation, 157
  mobilization, 103
phosphoric glyceraldehyde dehydrogenase, 213
phosphorylation, 56, 64, 65, 70, 77, 78, 84, 86, 88, 97–99, 102–105, 191, 197, 198, 202, 215, 226, 227
  Bax, 86
  cascade, 99, 105
  growth factor receptor, 102
  IkBa, 104
  oxidative, 56, 65, 77, 78, 84, 86, 88, 215
  protein, 84, 103
photochemical cloud, 33
photochemical degradation, 145
photo-chemical reaction products, 147
photosynthesis, 145
pictines, 170
pituitary gland, 158
pituitary-adrenal system, 164
PKC, 87, 102, 103, 105–111
plants, 120, 187
plasma, 10, 11, 44, 123, 204, 213
plasma paraoxanase, 123, 213
plasmid genome, 130
plastics, 175, 177
platelet growth factor receptor (PDGF), 226
platelets, 17, 20
  aggregation, 17, 70, 132, 211
poly-(ADP-ribose)-polymerase, 16, 18, 38, 184
poly-(ADP-ribose)-synthase, 21
polycycle aromatic hydrocarbons, 131
polymerase, 5, 39, 50, 55, 57, 59, 60, 75, 130, 184, 222, 227, 231
polymerases δ and ε, 55
polymers, 21, 38, 147
polymorphonuclear neutrophils, 33
polyphenolic compounds, 211
polyphenols of tea, 120
polysaccharides, 170
polyunsaturated fatty acids, 5, 10, 12
pre-apoptotic protein Bax, 192
precarcinogenic chemical substances, 227
pre-neoplastic cells, 225
pro-caspase, 18, 79
pro-caspase-9, 69
procollagen, 193
programmed cell death (PCD), 70, 71, 80
prolactin, 158
proliferating cell nuclear antigen (PCNA), 55
pro-mercapturic acid, 153
promoter, 100, 129, 138, 141, 143, 164, 165, 184–186, 190, 224, 225
promoter gene, 224
promoter hypermethylation, 129
promoter region, 185, 186, 190, 224
promoters, 106, 112, 140
pro-oxidant agents, 117
pro-oxidants, 36, 74, 77, 117, 118
propanol, 124, 161
propenal base, 203
propionaldehyde, 170, 212
propionitrile, 177
prostaglandin, 14, 193
prostate, 177, 187

protease family ICE, 75
proteases, 8, 22, 72, 73
protein, 5, 10, 15, 16, 32, 34, 35, 37, 41, 44, 49, 52–60, 64, 67, 71–75, 79, 81, 83–85, 87, 97, 98, 100, 102, 103, 105, 107–109, 111, 120, 123, 124, 129, 131, 155, 162, 163, 165, 167, 169, 188, 189, 191, 193, 197, 200–202, 204, 212–214, 226, 230, 232
  -3 (BP3), 232
  acute phase, 98
  adducts, 111, 131, 168
  blood coagulation, 168
  c-Abl, 232
  chelating, 10
  G-coupled, 98
  kinase C (PKC), 103, 111
  membrane-associated, 223
  multi-drug resistance (MRP), 124
  p50, 101
  pro-apoptotic, 72, 79, 87
  synthesis, 34, 163, 189
proteoglycan, 193
proteolysis, 5, 11, 15, 34, 75, 106
proto-oncogenes
  c-fos, c-myc, c-jun, 106
proton, 2, 4, 24, 31, 38, 70, 76, 77, 84, 88, 123, 166
  electrochemical gradient, 77
  transport, 31
proto-oncogenes, 105, 106, 156, 188, 189, 229
protoplasm, 8, 14
pumps, 124
  calcium, 18
  sodium, 18
purines, 10, 16, 38, 42, 51, 52, 57, 58, 118, 130, 169
  substrates, 11
puromycin, 164
pyrene, 228
pyrimidines, 16, 37, 38, 42, 58
  oxidized, 39, 57
pyrrolidine dithiocarbamate (PDTC), 100
pyruvate kinase, 43

Q

quinines, 120
quinol, 31
quinone, 31, 34, 75, 120–122, 212–214, 219
  reductase, 34, 121
quinone complex, 213, 214, 219
quinone-hydroquinone-semiquinone complex, 212

R

radical
  carbon-centered, 36, 147
  carbon-centered (R·), 147
  hydroxyl, 3, 7, 9, 10, 15, 16, 23, 31, 32, 40, 42, 52, 76, 192, 194–196, 204, 213
  oxy, 3
  oxygen free, 7, 8, 39, 44, 102, 211
  oxygen frees, 5
  oxygen-centric, 7
  peroxyl, 147
  phenoxy, 155
  semiquinone, 122
  superoxide, 3, 7–11, 40, 75, 123, 192, 198, 201, 203
radical (free radicals), 3–9, 11, 12, 17, 23, 31, 33, 36, 38, 58, 71, 75, 111, 119, 147, 199, 203, 218
radicals
  glutathionyl (GS·), 194
  hydroxyl, 7, 9, 15, 16, 23, 31, 32, 40, 42, 52, 76, 192, 194–196, 204, 213
  nitrogen monoxide (NO), 211
  peroxy, 14, 20, 39, 124, 203, 211
  quinone complex, 214
  stable, 212, 219
  sulfur (RS·), 194
  superoxide, 7, 11, 40, 75, 123, 192, 198, 203
  thiol (GS·), 195
raf, 201
reactive nitrogen species, 82
reactive oxygen species, 3, 4, 9, 43, 57, 75, 100, 101, 108, 110, 123, 183, 193, 203, 211, 212, 215, 219
reactive protein-1, 123
receptor binders, 226
receptors, 11, 14, 17, 42, 73, 84, 98, 102, 104, 110, 111, 129, 158, 163, 211, 213, 225, 226
  cell membrane, 102, 158
  D2 brain, 158
  epidermal growth factor (EGFR), 226
  macrophage, 162
  methyl group, 52
  molecules, 98, 102
  T-cell, 103
recombination mechanisms, 51
recombination processes, 231

reconstitution mechanisms, 50, 59
redox cycline, 34
redox pathways, 104
redox state, 84, 98, 100, 101, 108, 214
redox substances, 103
redox system, 107, 191
redoxo-endonucleases, 38
reductant, 117
reduction, 1–3, 7, 8, 10, 23, 24, 31–33, 35, 49, 71, 74, 75, 77, 83, 87, 105, 117, 120–124, 139, 150, 162, 165, 168, 183, 192, 194–196, 198–201, 203, 214
  monovalent, 31, 32
  tetravalent, 31
rejoining pathways, 51
Rel, 97, 104
  family proteins, 97
  Homology Domain (RHD), 97
renal cortex, 167, 187
renin-angiotensin system (RAS), 168
repair
  by base excision, 192
  by nucleotide excision, 192, 231
  in erroneous base pairing, 192
  recombination, 52, 192
replication, 38, 50, 51, 53–58, 60, 80, 128, 181, 215, 221, 225, 227, 230, 232
  cycle, 232
  factor C (RFC), 55
  protein A (RPA), 55
respiratory chain, 7, 65, 71, 74–77, 82, 83, 88
retinoblastoma tumor suppressor gene, 149
retroviruses, 147
ribose, 5, 20, 21, 39, 53, 59, 75, 184, 194
ribosylation, 84, 86, 184
ring hydroxylation, 153
risk factor, 130, 142, 222, 227
RNA, 32, 155, 190, 216, 221, 232
RNA polymerase (TFIIH), 232
rodents, 129, 139, 142
ROS, 3, 9, 10, 15, 16, 19, 32, 33, 39, 42, 43, 50, 52, 57, 58, 71, 75–81, 83, 84, 100, 101, 103, 104, 108, 118–120, 123, 165, 183, 188, 190, 192–194, 196, 197, 199–204, 211–214, 216, 219

S

S-adenosyl-methionine (SAM), 52, 185
saliva, 43, 44
SAM/MTase-cytosine, 185
second messengers, 100, 104
  universal, 103
selenium, 118, 120
semiquinone, 31, 34, 83, 120, 122, 212
sensory nerves, 147
series of Alu, 143
serine, 98, 105, 111, 169, 197
shock, 21, 41, 87, 188, 190
signal transduction, 42, 43, 49, 100, 122, 186, 198, 202, 223, 225
  pathway, 42, 100, 202, 223, 225
signalling cascade, 102, 104, 185, 202
  intracellular, 103
  T-cell, 103
signalling molecules, 104
single strand breaks, 169, 213
skin, 153, 156, 218
small-cell lung carcinomas, 223
smokers, 15, 43, 122, 131, 132, 141, 153, 156, 181, 187, 192, 214, 219, 220, 222–225, 227, 229
smoking, 80, 130, 135, 156, 161, 168, 170, 183, 184, 193, 211, 220, 222, 223, 226, 230, 235
S-nitration, 82
S-nitrosothiols, 18, 87
sodium cyanate, 177
Sp-1 positions, 143
spasms, 178
S-phenyl-mercapturic acid (S-PMA), 154
sphingomyelinase, 85
spleen, 153
spot, 53, 140, 150, 228
  hydrophobic, 35
squamous cell carcinoma, 219
Src family tyrosine kinases, 199
stereoisomers of BMO (R and S), 148
stomach, 148, 175, 178, 222
strand breaks, 37, 49, 51, 53, 57, 197, 213
stress, 16, 18, 31, 33, 34, 41, 42, 53, 60, 67, 70, 71, 73, 75–79, 81, 83, 85, 87, 98, 101, 102, 108, 123, 124, 126, 130, 162, 171, 175, 184–186, 188, 191, 199, 201, 202, 213–215, 226, 230, 231, 234
  oxidative, 15, 19, 23, 24, 31–34, 36–38, 40–42, 53, 60, 67, 75–78, 81, 82, 85, 98, 100–102, 104, 108, 120, 123, 124, 130, 162, 184, 185, 187–189, 193, 197, 198, 201, 203, 213–215, 226
stress-activated protein kinase, 191
succinic dehydrogenase, 22
sugar, 38, 39, 53, 203
sugar ring, 203
sulfhydryl (groups), 5, 11, 15, 34, 49, 155
sulfuric conjugations, 153
sun radiation, 145

supermethylation, 138
superoxide, 1, 3, 7–11, 14, 16, 18, 19, 21, 25, 31–33, 40–42, 74–76, 84, 101, 108, 120, 122, 123, 184, 189, 192, 198, 201, 203, 213, 214
 anion, 7, 10, 21, 31, 42
 dismutase, 7, 8, 16, 25, 76, 101, 120, 122, 123, 189, 192
suppressor gene p53, 149, 186
synaptosomes, 157
synthetic polynucleotides, 138
synthetic rubber, 147

T

t,t-MA, 154, 156
t,t-myconic acid, 132
talin, 107
tar particles, 214, 222
t-butyl-hydroperoxide, 16
Tcell
 activation, 103, 104
 receptor complex (TCR), 104
T-cell tumors, 140
T-cell, 103, 104, 140, 149, 150, 186
testicles, 146, 187
tetrahedral structure, 196
tetra-peroxy chromate, 196
thiocyanate, 43, 178
thioethers, 137
thioglycolic acid, 176
thiol, 7, 10, 22, 24, 67, 68, 74, 76, 77, 81, 82, 84, 86, 100, 107, 137, 162, 164, 165, 176, 186, 188, 195, 202, 213
 groups, 10, 67, 76, 81, 84, 100, 107, 188
 peroxidases, 76
 sensors, 68
thiol-redox, 162, 165
thioredoxin, 98, 101
 reductase mRNA, 98
threonine, 105, 197
thymidine, 16, 37, 38, 169
thymine, 16, 39, 51, 57, 138, 192, 221, 229
 glycol, 16, 57
thymine dimmers, 192
thymus, 79, 148, 155, 156
 cells, 79
 lymphomas, 156
thymus T-cells, 155
thyocyanide, 178
thyrotropin-releasing hormone (TRH), 107
tissue
 connective, 193
 hemopoietic, 140, 149, 175
 lipoid, 187, 212
 lymphatic, 175
TNF receptors, 100
 TNF-α
 synthesis, 202
TNF-α, 73, 80, 123, 202
TNF-related apoptosis-inducing ligand (TRAIL), 73
tobacco
 carcinogens, 222
 leaves, 128, 145, 187, 193
tobacco smoke, 43, 44, 50, 122, 123, 127, 130, 132, 140, 146, 147, 162, 168–170, 181, 193, 216, 219, 221–225
 carcinogens, 140, 223
tobacco-specific nitrosamine, 129
tocopherol, 101
toluene, 157, 158, 212
toxic demyelination, 158
toxic metals, 183
trans-4-hydroxy-2-nonenale, 130
transcription, 38, 40–42, 50–55, 85, 97–101, 103, 105, 122, 123, 139, 140, 142, 143, 156, 162–165, 186, 188–190, 197–199, 202, 211–213, 215, 220, 224, 225, 227, 230, 231
 CHOP, 18
transcription factor, 42, 53, 55, 97, 99–101, 103, 105, 123, 139, 143, 156, 162–165, 186, 188–190, 197, 198, 202, 212, 213, 225, 230
 AP-1, 42, 87, 101, 103, 162, 165, 166, 189, 191, 197, 202, 213, 225
 ERK, 98, 105, 184, 191, 202
 NF-kB, 42, 86, 87, 123, 162, 164, 165, 189, 197, 202, 213
 redox-sensitive, 164
 subunits, 99
transferrin, 6, 10, 204
transformation, 17, 34, 35, 64, 67, 82, 99, 105, 106, 129, 147, 149, 162, 186, 229
transhydrogenase, 24, 76
transition metals, 6, 7, 10, 23, 32, 84, 118–120
translational factors, 189
translocase, 66, 68, 215
trans-myconaldehyde, 153
transplantation (organ), 10
trans-trans-myconaldehyde, 155
transversions, 38, 42, 50, 55, 58, 138, 223–225
triglycerides, 170

trivalent chromium, 183
tubulin, 110, 155, 166, 168
tubulin amino groups, 166
tumor, 17, 73, 88, 127, 130, 139–143, 149, 162, 175, 181, 183, 185, 189, 191, 197, 218, 222–226, 228
  development, 17, 88, 162, 175, 181, 183, 225, 226
  necrosis factor (TNF), 73
  solid, 140
tumor promoters, 223
TUNEL assay, 184
tyrosine, 15, 23, 82, 98, 102, 111, 163, 191, 197, 199, 202, 212, 226
  kinase, 102, 191, 199
  nitration, 82
  phosphatase, 102, 226
tyrosine aminotransferase, 163
tyrosine-phosphorylated proteins, 202

U

ubiquinol, 23, 25
ubiquinone, 83
ubi-semiquinone, 83
uracil, 51, 53, 57, 59, 80, 229
uric acid, 8, 118
urinary tracts, 227
urine metabolites, 132
uterus
  neck of, 222

V

valine, 132
VDAC-ANT-CyP-D complex, 79
vegetables, 120
vertebrates, 138, 139
vesicles, 70, 74, 226
vessel
  large, 10
  tone, 11, 17
vibrational movement, 72
vicious cycle, 78
vinculin, 107
viral proteins, 72
viruses, 130, 147
vitamine C, 76
vitamine E, 76
voltage sensors, 68

W

white blood cells, 43, 128, 155

X

xanthine dehydrogenase, 11
xanthine oxidase, 4, 6, 9, 11, 81
xenobiotics, 5, 9

Z

zinc, 8, 107, 118, 120, 183, 188
zinc fingers, 107
Zn, 8, 33, 42, 81, 188, 192, 205