CHARLES UNIVERSITY IN PRAGUE

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DEPARTMENT OF CELL BIOLOGY

OXIDATIVE DAMAGE BY ORGANIC EXTRACTS FROM URBAN AIR PARTICULATE MATTER

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DECLARATION

I hereby declare that I have written this master thesis on my own and listed all the used sources.

Prague, 30th April 2009 .....

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ABSTRACT

The aim of this master thesis was to investigate the ability of selected individual carcinogenic polycyclic aromatic hydrocarbons (c-PAHs: benzo[*a*]pyrene, B[*a*]P; dibenzo[*a,l*]pyrene, DB[*a,l*]P), an artificial mixture of c-PAHs (c-PAH mix) and extractable organic matter (EOM) from urban air particulate matter (PM) to induce oxidative damage *in vitro*. Two cell lines (human hepatoma cells, HepG2, and human diploid lung fibroblasts, HEL) were treated for 24 h and 48 h with various concentrations of compounds or mixtures. The studied oxidative stress markers included 8-oxodeoxyguanosine (8-oxodG) as a marker of oxidative DNA damage, 15-F_{2t}-isoprostane (15-F_{2t}-IsoP) as a marker of lipid peroxidation and protein carbonyl groups as a marker of oxidative damage to proteins. The response of the cell lines to the tested compounds and mixtures differed substantially. In summary the results demonstrate the ability of EOM to induce oxidative damage to DNA and lipids after 24 h of treatment and to proteins after 48 h, in HepG2 cells. The effect of c-PAHs was substantially less. The induction of oxidative damage by c-PAHs and EOM in HEL cells was weak. Since c-PAHs had lower ability to cause oxidative damage that was limited only to longer incubation periods, it is probable that other components of EOM are responsible for increased levels of oxidative markers in HepG2 cells.

KEYWORDS

Oxidative stress; carcinogenic polycyclic aromatic hydrocarbons; particulate matter; HepG2 cells; HEL cells; 8-oxodeoxyguanosine; 15-F_{2t}-isoprostane; protein carbonyls; ELISA

CONTENTS

LIST OF ABBREVIATIONS

8-oxodG	8-oxodeoxyguanosine
15-F_{2t}-IsoP	15-F_{2t}-isoprostane
A549	human lung adenocarcinoma cell line
AChe	acetylcholinesterase
AhR	aryl hydrocarbon receptor
ARNT	aryl hydrocarbon receptor nuclear translocator
ASP	authentic street particles
B[*a*]A	benz[*a*]anthracene
B[*a*]P	benzo[*a*]pyrene
B[*b*]F	benzo[*b*]fluoranthene
B[*g,h,i*]P	benzo[*g,h,i*]perylene
B[*k*]F	benzo[*k*]fluoranthene
Blk	blank
BSA	bovine serum albumin
B_0	maximum binding
c-PAH mix	an artificial mixture of carcinogenic polycyclic aromatic hydrocarbons
c-PAHs	carcinogenic polycyclic aromatic hydrocarbons
cGPx	cytosolic glutathione peroxidase
CHRY	chrysene
CI	chloroform-isoamylalcohol
CT DNA	calf thymus DNA
CYP1A1	cytochrome P450 1A1
CYP1B1	cytochrome P450 1B1
CV	coefficient of variation
dH_2O	deionised water
DB[*a,h*]A	dibenz[*a,h*]anthracene
DB[*a,l*]P	dibenzo[*a,l*]pyrene
DEP	diesel exhaust particles
DFA	deferoxamine mesylate

DNPH	dinitrophenylhydrazine
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
E-MEM	Eagle's minimal essential medium
ELISA	enzyme-linked immunosorbent assay
EH	epoxide hydrolase
EOM	extractable organic matter
FBS	foetal bovine serum
Fpg	formamidopyrimidine DNA glycosylase
giGPx	gastro-intestinal glutathione peroxidase
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	glutathione disulfide
GST	glutathione-S-transferase
HEL	human embryonic lung diploid fibroblasts
HepG2	human hepatoma cells
HPLC	high-performance liquid chromatography
IARC	International Agency for Research on Cancer
I[*1*,*2*,*3*-*c*,*d*]P	indeno[*1*,*2*,*3*-*c*,*d*]pyrene
LDH	lactate dehydrogenase
MCF-7	human mammary carcinoma cells
MDA	malondialdehyde
NSB	non-specific binding
OGG1	8-oxoguanine-DNA glycosylase
PAHs	polycyclic aromatic hydrocarbons
pGPx	plasma glutathione peroxidase
PHGPx	phospholipid-hydroperoxide glutathione peroxidase
PBS	phosphate-buffered saline
PM	particulate matter
PM_{10}	respirable particles (particles of an aerodynamic diameter < 10 μm)

$PM_{2.5}$	fine particles (particles of an aerodynamic diameter < 2.5 μm)
$PM_{0.1}$	ultrafine particles (particles of an aerodynamic diameter < 0.1 μm)
ROS	reactive oxygen species
RT	room temperature
SCGE	single-cell gel electrophoresis (the comet assay)
SD	standard deviation
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
SRM	standard reference material (standardised particulate matter)
TA	total activity
TBA	thiobarbituric acid
TMB	3,3', 5,5' – tetramethylbenzidine
Tris	tris(hydroxymethyl) aminomethane
Tween 20	polyoxyethylene sorbitan monolaurate
UFPs	ultrafine particles (also $PM_{0.1}$)
WHO	World Health Organization

1 INTRODUCTION

"Unlimited and free access to clean air of acceptable quality is a fundamental human necessity and right" (WHO 2004). Despite this statement, the quality of urban air, particularly in large cities, is less than optimal. It contains a very complex mixture of chemical compounds originating from many different sources. Among others, polycyclic aromatic hydrocarbons (PAHs) represent a group of organic pollutants of particular interest as many of them are known or suspected human mutagens and carcinogens (IARC, in preparation). In the ambient air, PAHs are mostly adsorbed on the surface of dust particles. Particulate air pollution thus poses a threat to human health. Numerous epidemiological studies have shown that particulate pollution is related to adverse health outcomes of exposed populations. However, the mechanisms by which particles influence human health are only poorly understood. Therefore, there is a need for toxicological studies, which would identify specific components or sources of the air pollution mainly responsible for the adverse health effects. One hypothesis is that airborne particles induce oxidative stress which underlies much of their adverse health effects (DONALDSON *et al.* 2003, SHI *et al.* 2003).

The aims of this master thesis were:

1. To identify the potential contribution of carcinogenic polycyclic aromatic hydrocarbons (c-PAHs) as benzo[*a*]pyrene (B[*a*]P) and dibenzo[*a,l*]pyrene DB[*a,l*]P, an artificial mixture of c-PAHs (c-PAH mix) and extractable organic matter (EOM) from urban air particulate matter (PM) to oxidative damage to macromolecules.
2. To assess the response of two different cell models to exposure to the tested compounds and mixtures: a hepatoma cell line (HepG2 cells) and human diploid lung fibroblasts (HEL cells).
3. To compare oxidative damage to DNA, lipids and proteins by the analysis of three different markers of oxidative stress including 8-oxodeoxyguanosine (8-oxodG, a marker of oxidative damage to DNA), 15-F_{2t}-isoprostane (15-F_{2t}-IsoP, a marker of lipid peroxidation) and protein carbonyl levels (a marker of protein oxidation).

To achieve these goals, the cell lines were treated with different concentrations of B[*a*]P and DB[*a,l*]P, with a mixture of c-PAHs and with EOM for 24 h and 48 h. The cell lines differing in the metabolic activity simulate target tissues for the effect of c-PAHs and

PM: HepG2 cells capable of efficient bioactivation of many xenobiotics including c-PAHs are widely used in toxicology instead of human hepatocytes. Human lung fibroblasts were chosen as a representative of lung tissue, where the deposition of PM and PM-mediated c-PAHs occurs. Since oxidative stress is a very complex process, three biomarkers were employed to have a better chance of revealing some of the mechanisms of oxidative stress induction by the selected compounds.

2 LITERATURE REVIEW

2.1 Polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons are a large group of diverse organic compounds with two or more fused aromatic rings of carbon and hydrogen atoms. They are highly lipophilic thus having a relatively low solubility in water. Physical and chemical properties of PAHs vary with molecular weight. The larger compounds are less water-soluble and less volatile. PAHs are ubiquitous pollutants formed mainly as a result of pyrolytic processes, especially the incomplete combustion of organic materials during industrial and other human activities, such as processing of coal and crude oil, asphalt production, combustion of coal, wood or natural gas, vehicle traffic, waste incineration, cooking and tobacco smoking. Natural PAHs sources include plant synthesis, volcanic eruptions and forest and prairie fires. They are also present in crude oil and coal tar (WHO 1987, STRAIF *et al.* 2005).

Distribution of PAHs depends on the material being combusted, e.g. coal burning produces a different mixture of PAHs than motor-fuel combustion or a forest fire, making the compounds potentially useful as markers of the specific source. There have been several studies concerning this problem. According to NIELSEN *et al*. (1996) and REHWAGEN *et al*. (2005) the high ratio of benzo[*g,h,i*]perylene (B[*g,h,i*]P) to benzo[*a*]pyrene (B[*a*]P) and also coronene to B[*a*]P indicates the traffic as a main source of PAHs pollution while low B[*g,h,i*]P to I[*1,2,3-c,d*]P ratio signify a higher contribution from non-traffic sources. Generally, B[*g,h,i*]P and coronene indicate traffic emissions while high level of benz[*a*]anthracene (B[*a*]A) indicates higher emissions from wood combustion and domestic heating (LI and KAMENS 1993).

When released to the environment, PAHs usually enter the air where they often bind to dust particles. Whether adsorbed on particulate matter or present in water, PAHs can undergo photodecomposition when exposed to sunlight. In the atmosphere, PAHs can also react with other pollutants such as ozone, nitrogen and sulphur oxides, yielding diones, nitro- and dinitro- PAHs, and sulphonic acids, respectively (WHO, 2000). PAHs and their derivatives are pollutants of primary concern, since some of them are known or suspected human carcinogens (IARC, in preparation).

The major sources of PAHs for general population include inhalation of polluted ambient air, smoking and consumption of PAHs in food such as sweets, cereals, bread or processed food, as well as fruit and vegetables grown in contaminated soil. Various cooking processes such as roasting, frying, grilling or smoking generate more PAHs thus increasing the amount of total PAHs in food (PHILLIPS 1999). According to the study conducted in Copenhagen between the years 1984 and 1986, food constituted the major source of PAHs for non-smokers (DE VOS *et al*. 1990). However, according to ČERNÁ *et al*. (1999) the exposure to B[*a*]P from ambient air in the Czech Republic was four times higher than the dietary intake, thus indicating that air pollution is the major source of c-PAHs exposure for the Czech population. In both studies the major contribution of PAHs exposure for smokers was attributed to cigarette smoking. Moreover, in labours working in industries such as mining, oil refining and metal working as well as in workers inhaling engine exhausts, such as mechanics, street vendors and motor vehicle drivers an occupational exposure may occur (STRAIF *et al.* 2005).

After they enter the organism, either inhaled or present in diet, PAHs are rapidly distributed to most internal organs. The process takes from minutes to hours and the highest levels of PAHs were found in liver (WHO 2000). PAHs are also deposited in mammary and other fatty tissues (MODICA *et al.* 1983). Moreover, B[*a*]P can readily cross the placental barrier (NEUBERT and TAPKEN 1988, HATCH *et al*. 1990, WITHEY *et al.* 1993), thus exhibiting its fetal toxicity. Carcinogenic PAHs themselves, as well as most chemical carcinogens in the environment, are chemically inert and require metabolic activation to exhibit their carcinogenic effects (GUENGERICH and SHIMADA 1991).

Metabolic activation of PAHs

Upon entering the organism, c-PAHs undergo metabolic transformation. They are converted to electrophilic species by cytochrome P450s enzymes. These reactive electrophilic species are capable of interacting with the nucleophilic sites of the DNA bases to form adducts. The formation and persistence of carcinogen-DNA adducts are believed to be critical events for the initiation of tumorigenesis in target cells (NESNOW *et al*. 1998b, 1998c, BINKOVÁ and ŠRÁM 2004). The bioactivation of polycyclic aromatic hydrocarbons by cytochrome P450s enzymes represents phase I reactions of the xenobiotic

metabolism. The aim of this process is to convert highly lipophilic chemicals into more water soluble products which are then more easily excreted from the organism. During the phase II reactions the polar functional groups of phase I metabolites are conjugated with glucuronic acid, sulphonates, glutathione (GSH) or with some amino acids. The products of these reactions are usually inactive and ready to be excreted from the organism in faeces or urine. However, the phase I reaction metabolites tend to be highly reactive and capable of binding covalently to cellular macromolecules. The whole mechanism has been best characterised for B[*a*]P (Figure 1.) which is one of the most often studied c-PAHs. Upon entering the cell, B[*a*]P (as well as other c-PAHs) binds to the cytosolic aryl hydrocarbon receptor (AhR). The complex then translocates to the nucleus where it forms heterodimer with AhR nuclear translocator (ARNT). Heterodimers activate transcription of genes encoding cytochromes P450 1A1 and 1B1 (also CYP1A1, CYP1B1) (SHIMADA and FUJII-KURIYAMA 2004). Cytochrome P450 enzymes are a superfamily of haem-containing monooxygenases, responsible for the phase I metabolism of a wide range of substrates (drugs, xenobiotics and steroids). They insert an atom of molecular oxygen into their substrates, resulting in increased hydrophilicity. In the case of most c-PAHs, CYP1A1 and CYP1B1 are responsible for the formation of polar hydroxy and epoxy derivatives which can be further oxidised by epoxide hydrolase (EH) to form dihydrodiols. These intermediates can be further converted to more reactive **diol-epoxides** which may covalently bind to DNA thus forming bulky adducts (SHIMADA and FUJII-KURIYAMA 2004).

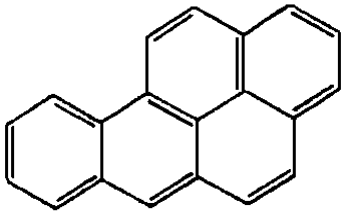


Figure 1. Benzo[*a*]pyrene (B[*a*]P)

The above-mentioned metabolic pathway of forming DNA adducts via c-PAH **diol-epoxides** was thought to be the dominant mechanism of chemical carcinogenesis of

c-PAHs until the last decade of the 20th century. However, there have been two more fundamental pathways proposed since then (XUE and WARSHAWSKY 2005):

The second pathway suggests the formation of **radical cations** of PAHs through one electron oxidation catalysed by cytochrome P450 peroxidase activity. In this pathway PAHs are oxidised independently of molecular oxygen; organic or lipid hydroperoxides are used as the oxidant source instead (ANARI *et al*. 1995). Radical cations are electrophilic and capable of interacting with nucleophilic centres in cellular macromolecules including DNA (CAVALIERI and ROGAN 1992). The radical cation mechanism involves the formation of unstable adducts primarily at guanine and adenine bases leading to DNA damage by depurination. If not repaired, these apurinic sites can lead to mutations in DNA (CAVALIERI and ROGAN 1995, XUE and WARSHAWSKY 2005).

The most recently discovered pathway of PAH metabolic activation involves the formation of ***o*-quinones** catalysed by dihydrodiol dehydrogenase members of the aldo-keto reductase superfamily (PENNING *et al*. 1999). These cytosolic NADP(H)-dependent oxidoreductases catalyse the oxidation of PAH *trans*-dihydrodiols (products of phase I metabolic reactions) to PAH *o*-quinones (SULLIVAN 1985, SMITHGALL *et al*. 1988, BURCZYNSKI *et al.* 1998). PAH *o*-quinones are electrophilic metabolites that enter redox cycles and generate reactive oxygen species (ROS) (PENNING *et al*. 1996) thus leading to oxidative damage of DNA and other macromolecules (BURCZYNSKI and PENNING 2000). Formation of ROS and other radicals by PAH *o*-quinones resulted in increased cytotoxicity in rat hepatocytes and the ability of PAH *o*-quinones to intercalate in DNA lead to mutagenic effect in *Salmonella typhimurium* in the study of FLOWERS-GEARY *et al*. (1996).

2.2 Particulate matter (PM)

The health effects of ambient air pollution have been widely studied over the past few decades. Many epidemiological studies have shown an association between exposure to particulate air pollution and increases in acute respiratory and cardiovascular diseases (SCHWARTZ 1993, 1999, SCHWARTZ and MORRIS 1995, POPE 2000, BRUNEKREEF and HOLGATE 2002, BROOK *et al.* 2004, BRUNEKREEF 2006) and increased death rates from lung cancer and cardiopulmonary diseases (DOCKERY *et al*. 1993, ABBEY *et al*. 1999, POPE *et al*. 2002, BOLDO *et al.* 2006). In accordance with these findings, LADEN *et al*. (2006) have found a decrease in PM-associated mortality consistent with a decrease in concentrations of fine particles in ambient air.

However, epidemiologic studies face several difficulties: a general problem is the assessment of personal exposure, because the relationship between the fixed-site measurements and personal exposure may vary from study to study (LIPFERT and WYZGA 1995). This problem can be eliminated by using a personal monitoring as demonstrated in several cohort studies (SORENSEN *et al*. 2003, RÖSSNER *et al*. 2007, 2008a, 2008b, TOPINKA *et al*. 2007). For the cohort studies the difficulty might be the choice of the adequate time frame. The major problem is to find an appropriate time between exposure to xenobiotics and detectable biological effects. The composition of the PM, which varies by the location, may influence its toxicological potency and thus results obtained from epidemiological study in one region may not necessarily be consistent with results gained elsewhere. Moreover, epidemiology cannot provide definite answers to many questions about the causality and mechanisms of the health effect of air pollution (ENGLERT 2004). To obtain these answers we need further toxicological *in vitro* studies.

Ambient air pollution consists of a variable complex mixture of different chemical compounds in the gas, liquid or solid phase. Particulate pollution, also called particulate matter (PM), is a mixture of solid and liquid constituents, including inorganic salts such as nitrates, sulphates and ammonium, elemental carbon and a large number of organic compounds including polycyclic aromatic hydrocarbons (PAHs). Thus, PM includes a variety of chemical substances emitted by different sources of air pollution. Particle size may range from a few nanometres to tens of micrometres. PM_{10} and $PM_{2.5}$ are particles

with an aerodynamic diameter of less than 10 and 2.5 μm, respectively. The PM_{10} fraction is also called "respirable particles" and the $PM_{2.5}$"fine particles". The particles between 2.5 and 10 μm are called "coarse particles". Ultrafine particles (UFPs), also called $PM_{0.1}$, are those with an aerodynamic diameter of less than 0.1 μm. (ENGLERT 2004). PM is further classified as primary (originating from many natural and anthropogenic sources and emitted directly into the atmosphere) or secondary (formed in the atmosphere by chemical and physical transformations and interactions with gases already present in the air). The gases mostly involved in the formation of secondary particles are sulphur dioxide (SO_2), nitrogen oxides (NOx), and ammonia (NH_3). Coarse particles are generally primary particles and most of them originate from larger particles by crushing or grinding. Main sources of these particles include emissions from industrial processes, traffic related emissions including tire and paving materials, and grinding of crustal, biological and combustion materials, agriculture, mining, fly ash and wind-blown dust. These particles are removed from the atmosphere by gravitational settling thus remaining in the air for shorter periods ranging from a few hours to a few days (depending on their size, atmospheric conditions and altitude). Fine particles are principally generated by combustion processes and transformation processes of precursor gases and thus are composed mainly of sulphate, nitrate, ammonium, inorganic and organic carbon compounds, and heavy metals such as lead and cadmium. This indicates that the origin of fine particles is mostly anthropogenic and that this fraction comprises mainly of the secondary particles (US-EPA 1996, WHO 2004). Furthermore, an important source of the fine particulate pollution in the urban areas is fossil fuel combustion by motor vehicles, industry and residential heating. Ultrafine particles are formed primarily by the condensation of hot vapours arising during combustion processes and by nucleation (gas molecules coming together to form a new particle). These extremely small particles coagulate to quickly form larger particles on which more vapours condensate making them grow up to the size range of 0.1-2.0 μm. These fine particles can be then transported thousands of kilometres and remain in the atmosphere for days or weeks depending also on the weather conditions. Ultrafine particles themselves remain in the atmosphere very shortly (from minutes to hours) because they are very likely to accumulate and form larger particles (US-EPA 1996).

The size of the particles not only determines their fate in the atmosphere but also their behaviour in the human respiratory tract, including how far the particles penetrate, where they deposit, and how successful is their removal from the body. Particles larger than 10 μm are filtered in the nose and throat and thus do not enter the organism but respirable particles (PM_{10}) can reach bronchi and lungs and cause health problems. These adverse effects are greater for fine and ultrafine particles because they can penetrate deeper into the respiratory tract and within hours after the exposition UFPs can even reach different compartments of the body. According to recent studies it is obvious that ultrafine particles can translocate to interstitial sites in the respiratory tract as well as to extrapulmonary sites such as liver, lymph and blood circulation, and nervous system (OBERDÖRSTER *et al.* 2004, ELDER and OBERDÖRSTER 2006, PETERS *et al*. 2006). Thus, ultrafine particles can directly affect cells in various organs inducing oxidative stress and eventually heritable mutations (CHOI *et al*. 2004, SAMET *et al.* 2004). One of the reasons why particles may induce an oxidative burst is the free radical activity of the organic pollutants carried on the particles' surface. SQUADRITO *et al*. (2001) have found out that $PM_{2.5}$ contains quinoid substances that might be responsible for ROS generation by $PM_{2.5}$ via redox cycling mechanism. The reactive oxygen species can then cause oxidative damage to lipids, nucleic acids, and proteins at deposition sites within the lungs and in secondary target organs. In addition, the particles may induce the release of inflammatory mediators and cytokines by the cells. The fine and coarse particles may be phagocytised by macrophages and dendritic cells, which can carry the particles to the lymph nodes in the lungs or to those closely associated with the lungs, whereas the uptake of ultrafine particles occurs by different, non-specific mechanisms (PETERS 2006). GEISER *et al.* (2005) have studied penetration of UFPs into several cell types *in vitro* (cultured lung macrophages and red blood cells) and *in vivo* (exposure of rats to ultrafine titan dioxide aerosol) and summarised that UFPs cross cell membranes by nonphagocytic mechanisms most likely by diffusion. OBERDÖRSTER *et al.* (2004) have shown that inhaled UFPs deposited on the olfactory epithelium can be translocated to the brain via the olfactory nerve and therefore exhibit neuropathological effects. There have been several studies concerning the problem of neurodegenerative consequence of exposure to high levels of ambient particle pollution and

a histological evidence of neurodegeneration in both canine and human brains was found (CALDERON-GARCIDUENAS *et al.* 2002, 2003, PETERS 2006).

2.3 Oxidative stress

Oxidative stress is caused by an imbalance between the production of ROS and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. ROS are either free radicals, reactive anions containing oxygen atoms, or molecules containing oxygen atoms that can either produce free radicals or are chemically activated by them. Examples are: **superoxide anion radical** ($\bullet O_2$-), **hydroxyl radical** (•OH), **hydrogen peroxide** (H_2O_2), and **alkoxy** (RO•) and **peroxy** (ROO•) **organic radicals**. In living cells these reactive molecules are formed continuously as a consequence of metabolic reactions. The main source of ROS in the organism is oxidative phosphorylation in mitochondria, though ROS are also produced by oxidative burst in leukocytes, by peroxisomal beta oxidation of fatty acids or microsomal cytochrome P450 metabolism of xenobiotic compounds (SCHOLZ *et al*. 1990). ROS can be beneficial, as they are used by the immune system as a way to attack and kill pathogens, and are also involved in cell signalling where they act as second messengers (FORMAN *et al*. 2004). However, the excessive generation of ROS can lead to the damage of lipids, proteins and nucleic acids. This damage, if unrepaired, accumulates and leads to the physiological attrition and an increased risk of several acute and chronic diseases including cancer, cardiovascular, neurodegenerative or lung diseases (MONTUSCHI *et al.* 2004, HWANG and KIM 2007).

Under normal physiological conditions, cells are able to defend themselves against ROS damage through the use of enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione-S-transferase (GST). Small molecule antioxidants such as ascorbic acid (vitamin C), tocopherol (vitamin E), uric acid, and glutathione also play important roles as cellular antioxidants.

2.3.1 Antioxidant enzyme system

Superoxide dismutase

SODs are a class of enzymes that catalyse the dismutation of two superoxide radicals into hydrogen peroxide and oxygen:

$2\ {\bullet}O_2^- \rightarrow H_2O_2 + O_2$ *(SOD)*

In humans, as well as in all other mammals, three forms of superoxide dismutase are present: SOD1 is located in the cytoplasm, consists of two subunits each containing one copper and one zinc atoms. SOD2 is located in the mitochondria, consists of four subunits and has manganese in its reactive centre. SOD3 is an extracellular superoxide dismutase isoform consisting of four subunits each containing one copper and one zinc atoms.

Catalase

Catalase is a common enzyme found in nearly all living organisms which are exposed to oxygen. It catalyses the decomposition of hydrogen peroxide to water and oxygen:

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$ (*catalase*)

Catalase, present predominantly in peroxisomes, is a tetramer containing four porphyrin haem (iron) groups in the active site. It is an enzyme with one of the highest turnover; one molecule of catalase can convert millions of molecules of hydrogen peroxide to water and oxygen per second.

Glutathione peroxidase

GPx is a selenium-containing enzyme. It reduces hydrogen peroxide to water by transferring reactive electrons from peroxide to glutathione (GSH) which is thus converted to its oxidised form, glutathione disulfide (GSSG):

$H_2O_2 + 2\ GSH \rightarrow GS–SG + 2H_2O$ (*GPx*)

There are four forms of glutathione peroxidase enzymes:

1) cytosolic glutathione peroxidase (cGPx) – ubiquitously distributed
2) phospholipid-hydroperoxide glutathione peroxidase (PHGPx) – located in plasma membranes to reduce hydroperoxides of lipids

3) plasma glutathione peroxidase (pGPx) – located in blood plasma

4) gastro-intestinal glutathione peroxidase (giGPx) – located in liver and gastrointestinal tract

cGPx, pGPX and giGPx are tetramers, while PHGPx is a monomer.

Glutathione S-transferase

The mammalian GST family consists of cytosolic dimeric isoenzymes catalysing the conjugation of reduced glutathione to electrophilic centres of a wide variety of substrates. This activity detoxifies endogenous compounds such as peroxidised lipids as well as many different xenobiotic metabolites.

2.3.2 Oxidative DNA damage

Oxidative damage to DNA is a result of interaction of DNA with ROS, in particular the hydroxyl radical. The hydroxyl radical produces a multiplicity of modifications in DNA, such as single base and sugar-phosphate damage, as well as strand breaks. One of the major types of DNA damage generated by ROS is formation of apurinic sites. In addition to this lesion, a wide spectrum of oxidative base modifications occurs by ROS activity (Figure 2.). The C_4-C_5 double bond of pyrimidine is particularly sensitive to attack by hydroxyl radical, generating a spectrum of oxidative pyrimidine damage including thymine glycol, uracil glycol, 5-oxodeoxyuridine, 5-oxodeoxycytidine and others. Similarly, interactions of hydroxyl radical with purines generate 8-oxodeoxyguanosine (8-oxodG), 8-oxodeoxyadenosine, formamidopyrimidines and other oxidative products (KOW and DARE 2000, LOFT *et al*. 2008).

Among all purine and pyrimidine bases, guanine is the one most prone to oxidation. Guanine base is oxidised in the position 8 and the result of this modification, 8-oxodG, is one of the predominant forms of free radical-induced lesions to DNA. 8-oxodG is by far the most studied oxidative DNA lesion and has gained much attention, because it is highly mutagenic in bacterial and mammalian cells (CHENG *et al*. 1992, MORIYA and GROLLMAN 1993). The oxidised guanine residue can pair both with cytosine and adenine, and if not repaired, its presence may result in G:C→T:A transversions (MORIYA

1993, LE PAGE *et al*. 1995). Thus, oxidative DNA damage may be implicated in cancer risk (RISOM *et al*. 2005).

The most frequently used methods of 8-oxodG analysis include high-performance liquid chromatography (HPLC) with electrochemical detection, single-cell gel electrophoresis (SCGE) and the competitive ELISA (CHIOU *et al*. 2003, LOFT *et al.* 2008).

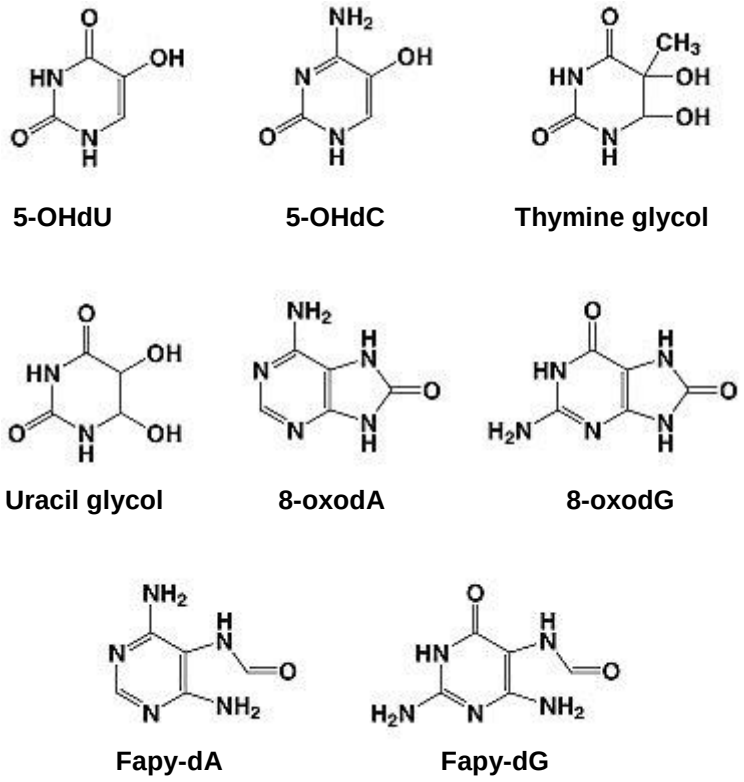


Figure 2. Chemical structures of some of the stable oxidative DNA base lesions (according to LOFT *et al.* 2008, modified)

5-OHdU = 5-hydroxydeoxyuracil; **5-OHdC** = 5-hydroxydeoxycytosine; **8-oxodA** = 8-oxodeoxyadenine; **8-oxodG** = 8-oxodeoxyguanine; **Fapy-dA** = 4,6-diamino-5-formamidopyrimidine; **Fapy-dG** = 2,6-diamino-4-oxo-5-formamidopyrimidine

2.3.3 Lipid peroxidation

After being attacked by ROS, lipids are peroxidised, forming reactive intermediates that may further react with other molecules and propagate oxidative stress (MONTUSCHI *et al*. 2004). Apart from this process, oxidised lipids also change the properties of the cellular membranes, including their fluidity and the inactivation of membrane-bound proteins. As a result, normal cellular functions are impaired. Oxidised lipids are degraded to a wide variety of products as short chain alkanes, aldehydes, alkenyls, isoprostanes and others (DE ZWART *et al*. 1999).

To monitor lipid peroxidation *in vivo*, several biomarkers have been proposed. Traditionally, measurement of malondialdehyde (MDA) using a reaction with thiobarbituric acid (TBA) has been widely used as a marker of lipid peroxidation (GUTTERIDGE and HALLIWELL 1990). Unfortunately, the TBA assay was proved to be non-specific for MDA as TBA is also reactive with other compounds that may be present in biological samples (HALLIWELL *et al*. 2000, KORCHAZHKINA *et al*. 2003). Since isoprostanes were described to be formed *in vivo* in humans almost two decades ago (MORROW *et al.* 1990), they have become the most reliable marker of lipid peroxidation that was proved to be associated with oxidative damage in a number of human diseases (ROBERTS and MORROW 2000). Isoprostanes are prostaglandin-like compounds formed by a non-enzymatic peroxidation of arachidonic acid. They include several groups, but F_2-isoprostanes, particularly the 15-F_{2t}-isoprostane isomer (15-F_{2t}-IsoP, also called 8-isoprostane), are the best characterised and most often studied compounds (NOUROOZ-ZADEH 2008).

2.3.4 Protein oxidation

Because proteins are major components of most biological systems, they are an important target for ROS, scavenging 50-75% of reactive intermediates (DALLE-DONNE *et al.* 2006a). Protein oxidation leads to the formation of a number of products, with protein carbonyls being the best characterised (HWANG and KIM 2007). Protein carbonylation is an irreversible, non-enzymatic process that results in the introduction of carbonyl groups into protein molecules in various ways. Proteins can react either directly

with ROS or indirectly via a reaction with other oxidised macromolecules (lipids or sugars). Direct oxidation results in the formation of oxidation products on the side chains of the amino acids lysine, arginine, proline and threonine, in the oxidation of glutamyl residues, or in the cleavage of peptide bonds (DALLE-DONNE *et al.* 2006a). Indirect oxidation includes the reaction of a protein molecule with oxidised lipids (such as MDA or 4-hydroxynonenal) or with the oxidation products of sugars. The oxidation of proteins results in changes in their conformation leading to their partial or total inactivation (STADTMAN and LEVINE 2003), their failure to fold correctly (DALLE-DONNE *et al*. 2006a), or, in the case of heavy oxidation, to proteolysis (GRUNE *et al*. 2004). Protein oxidation has been linked with the progression of various diseases, although in many cases it remains unclear whether protein oxidation is the cause or the consequence of a particular illness (DALLE-DONNE *et al*. 2006b). At present, the analysis of protein carbonyl levels by ELISA is considered the most sensitive measurement of protein oxidation (SHACTER 2000).

2.4 PAHs and oxidative stress

Several studies were concerning the influence of PAHs, especially B[*a*]P, on the induction of oxidative stress *in vivo* and *in vitro*.

KIM and LEE (1997) investigated the induction of oxidative damage to DNA and proteins and activities of antioxidant enzymes SOD and catalase in target organs of rats following oral exposure to B[a]P. Levels of 8-oxodG, carbonyl content of proteins and activity of SOD and catalase were determined in liver, kidney and lung tissues at different time points. The results showed that B[*a*]P was able to cause oxidative damage to DNA and proteins and to decrease the activity of antioxidant enzymes SOD and catalase.

Similarly, GARCON *et al*. (2001a) determined oxidative stress induced in the lungs of rats exposed to hematite (Fe_2O_3), B[*a*]P or B[*a*]P coated onto Fe_2O_3 particles. As markers of oxidative stress, levels of MDA and 8-oxodG and activities of antioxidant enzymes SOD, GPx and GR were determined. The results showed increase in MDA production in all exposed animals and increased levels of 8-oxodG in lung tissue biopsies of rats treated with B[*a*]P or B[*a*]P coated onto Fe_2O_3 particles. SOD activity decreased

following the treatment in all exposed animals while other enzyme activities remained unaltered. The main finding of this work was that B[*a*]P coated onto Fe_2O_3 particles caused approximately ten times higher oxidative damage to the lungs than B[*a*]P alone.

A similar study was conducted by the same authors (GARCON *et al.* 2001b) but *in vitro* on human lung adenocarcinoma cell line (A549). The cells were incubated with Fe_2O_3, B[*a*]P, pyrene, B[*a*]P coated onto Fe_2O_3 particles or pyrene coated onto Fe_2O_3 particles. However, exposure to B[*a*]P did not result in increases in lipid peroxidation measured as the MDA production. This effect was restricted only to cells treated with B[*a*]P coated onto Fe_2O_3 particles or pyrene coated onto Fe_2O_3 particles.

PARK *et al*. (2006) investigated the potential of B[*a*]P to induce oxidative stress-related DNA damage and p53 modification in HepG2 cells. Exposure to B[*a*]P induced a decrease in cell viability, but increased the antioxidant enzyme activity as well as lipid peroxidation and non-specific DNA damage.

In the study of GÁBELOVÁ *et al*. (2007) the induction of oxidative DNA damage by B[*a*]P was determined in HepG2 cells by a modified SCGE with the use of a repair-specific DNA endonuclease (formamidopyrimidine-DNA glycosylase, Fpg). However, the results of this study do not show any increase in oxidative base modifications following exposure to B[*a*]P.

Generally, the results of above-mentioned studies do not show any clear conclusion about the oxidative stress induction by B[*a*]P.

2.5 Particulate matter and oxidative stress

To investigate the mechanism of oxidative stress induction by particulate matter, numerous *in vivo* as well as *in vitro* studies have been conducted in recent years. In these projects different types of particles were used: from diesel exhaust particles (the most often used ones in *in vivo* studies) to urban air particles of different size and composition. It is also important to mention that in *in vitro* studies the solvents, in which the particles are suspended, vary. For *in vivo* studies, modes of particle administration vary with inhalation and intratracheal instillation being used most often. Other differences involve various markers of oxidative stress and different methods of their analysis. Despite these

differences, the results in general indicate that the particles induce oxidative stress (RISOM *et al.* 2005).

2.5.1 Particulate matter and oxidative DNA damage *in vivo*

2.5.1.1 Model studies in animals and humans

Respiratory tract toxicity of air pollution particles has been extensively studied over the years but the focus on the DNA oxidation in the lungs is only since the mid- 1990s (MOLLER *et al*. 2008). Though a large variety of PM is available, most of the *in vivo* studies use diesel exhaust particles (DEP). DEP are present in urban air pollution, however the composition of DEP and total urban PM can be fairly distinct.

NAGASHIMA *et al*. (1995) investigated the formation of 8-oxodG in the lung DNA of mice after an intratracheal instillation of DEP. The influence of high dietary fat and beta-carotene on the oxidative DNA damage formation was also examined. The results showed a bell-shaped induction of 8-oxodG in lung tissue with the peak effect twelve hours after the administration and the dose of 0.2-0.3 mg having the highest effect. High dietary fat enhanced the formation of 8-oxodG while the intake of beta-carotene had the opposite effect.

Similar results were obtained by ICHINOSE *et al*. (1997), who examined the relationship between the formation of 8-oxodG in lung tissue of mice and lung tumour response. Mice were intratracheally injected with different concentrations of DEP once a week for ten weeks. After ten weeks, the inflammatory reaction was observed in the respiratory tract and after twelve months, the lung tumour incidence was increased in treated mice. The 8-oxodG levels in treated mice showed a dose-dependent increase. The formation of 8-oxodG was enhanced by high dietary fat and was also significantly correlated with the lung tumour incidence. Beta-carotene partially reduced the 8-oxodG formation and the development of benign and malignant tumours induced by the combination of high dietary fat and exposure to DEP.

TSURUDOME *et al*. (1999) studied the levels of 8-oxodG and the induction of the specific repair enzyme transcription (OGG1) in the lungs of rats after a single instillation of DEP. The highest levels of 8-oxodG were observed 2 hours after exposure to

4 mg DEP and were decreasing gradually until they reached control levels the fifth day after the treatment. *OGG1* gene transcription was strongly induced in rats treated with 4 mg of DEP five to seven days after the administration.

Another study of rats exposed to DEP by inhalation was conducted by IWAI *et al*. (2000). The results of this study showed a gradual increase in 8-oxodG following the duration of repeated exposures to DEP, and the development of lung cancer in treated animals.

Similar results obtained MOLLER *et al*. (2003) in their study on guinea pigs. The animals were exposed to DEP by intratracheal instillation. Five days later, increased levels of oxidised amino acid marker (gamma-glutamyl semialdehyde), DNA strand breaks, and 8-oxodG in treated guinea pigs were observed. Moreover, the activity of glutathione reductase (GR) was increased while activities of other antioxidant enzymes GPx and SOD remained unaltered.

BRÄUNER *et al*. (2007) investigated oxidative DNA damage in peripheral blood mononuclear cells in humans during controlled exposure to ultrafine urban air particles. The levels of strand breaks and oxidised bases were measured by the SCGE with Fpg. In this assay, Fpg removes oxidised purine bases (primarily guanine) and introduces breaks in the DNA. The results of the study show that UFPs, especially the fraction with a median diameter of 57 nm, was associated with oxidative stress.

A conclusion that can be made from the data obtained from *in vivo* studies is that exposures to DEP or urban UFPs are associated with elevated levels of oxidative DNA damage and with increased cancer risk (RISOM *et al*. 2005, MOLLER *et al*. 2008).

2.5.1.2 Human biomonitoring studies

As oxidative stress is believed to be one of the major sources of PM-mediated adverse health effects, there have been also several human biomonitoring studies conducted over the last decade, which were concerning PM and oxidative stress. In these studies positive correlations between the PM exposure and oxidative stress markers were observed.

LOFT *et al*. (1999) have found increased excretion of urinary 8-oxodG in a group with higher exposure to ambient air pollution (bus drivers from central Copenhagen) as

compared with a group with lower exposure (drivers from rural/suburban greater Copenhagen).

Similarly, CHUANG *et al*. (2003) have seen significantly elevated marker of oxidative DNA damage (urinary 8-oxodG) in an exposed group of city taxi drivers in Taipei compared to a reference group of men from the residential community with the lowest levels of air pollution in the city of Taipei.

SORENSEN *et al*. (2003) studied the influence of personal $PM_{2.5}$ exposure on the levels of DNA damage in 50 students living and studying in Copenhagen. The results of this study indicate that personal exposure to $PM_{2.5}$ was a predictor of 8-oxodG levels in lymphocyte DNA.

In accordance with these findings, RÖSSNER *et al*. (2007) have observed significantly increased levels of oxidative stress markers (8-oxodG, protein carbonyls and 15-F_{2t}-IsoP) in 50 bus drivers from Prague when compared to controls (a group of administrative workers).

The results of above-mentioned studies suggest that exposure to ambient air pollution causes oxidative damage to humans. However, most of these studies encompass only particle size and concentration. They do not elucidate the importance of different chemical fractions of PM, nor do they clarify the mechanism of the oxidative stress induction by ambient air pollution.

2.5.2 Particulate matter and oxidative stress *in vitro*

SHI *et al*. (2003) determined the induction of 8-oxodG by PM in aqueous suspension in a cell-free system (calf thymus DNA) and in A549 cells. The results showed that PM was able to induce 8-oxodG in CT DNA as well as in A549 cells.

Similarly PRAHALAD *et al*. (2001) tested the induction of 8-oxodG by many different types of ambient particles in cell free systems – free deoxyguanosine and CT DNA – and in human epithelial cells. The levels of induced 8-oxodG depended on the type and composition of particles: generally, damage was affected by the concentration of water-soluble contents of particles.

Another *in vitro* study was conducted by GARCON *et al*. (2006) determining oxidative stress caused by Dunkerque city air pollution in human epithelial lung cells (L132) in culture. The results show concentration and time-dependent increases in MDA and 8-oxodG levels following PM exposure.

The study of GÁBELOVÁ *et al.* (2007) was aimed at determining the impact of the EOM from airborne particles on oxidative damage to DNA in HepG2 cells. The levels of 8-oxodG were analysed using a modified SCGE with Fpg. From the eight EOM tested, only one induced significant increase in oxidative DNA lesions in three from total six concentrations applied.

Contrary to the study of GÁBELOVÁ *et al.* (2007), LAZAROVÁ and SLAMEŇOVÁ (2004) found a significant increase in oxidative DNA damage in HepG2 cells after the treatment with all tested concentrations of two different EOM samples using also a modified SCGE.

Though using different fractions of PM originating from different sources and using distinct methods of oxidative stress detection, the results of *in vitro* studies in general show some positive effect of PM on oxidative stress induction.

2.6 Cell lines

Two cell lines were used in the study: the human hepatoma cells (HepG2) and human diploid lung fibroblasts (HEL). The HepG2 cells retain many characteristic enzyme pathways of human hepatocytes, they are easily available, and it is possible to passage them more than hundred times (SILVERS *et al.* 1994). In contrast, primary human hepatocytes, regarded as a "gold standard" in toxicology, are not easily available because of the shortage of human livers needed for hepatocyte isolation. HepG2 cells contain PAH-inducible CYP1A1 enzyme (BURCZYNSKI *et al.* 1999, VAKHARIA *et al*. 2001, WU *et al*. 2003) and their overall xenobiotic metabolising capacity is comparable to that of human hepatocytes in primary culture (IBA e*t al*. 2002). HepG2 cells are able to activate PAHs to DNA reactive metabolites (DIAMOND *et al*. 1980). They are considered a good model for *in vitro* testing of individual PAHs, their artificial mixtures as well as organic extracts from ambient air particles (SEVASTYANOVA *et al*. 2007). However, toxicogenomic studies

(HARRIS *et al*. 2004) indicated that though expressing similar genes to human liver and primary cultured hepatocytes, HepG2 cells also express a significant number of other genes, not found in human hepatocytes and probably related to a transformed phenotype. When compared to HepG2 cells, human diploid lung fibroblasts at confluency are closer to *in vivo* conditions, since tumour cell lines may contain many mutations which may affect the DNA damage response (DI LEONARDO *et al*. 1994, VENKATACHALAM *et al.* 1997).

According to SEVASTYANOVA *et al.* (2007), HepG2 cells represent a metabolically competent system for activating c-PAHs as well as EOM in a wide range of concentrations while confluent cultures of HEL cells represent a relevant *in vitro* model of lung tissue as a major target for air pollutants, suitable for testing individual PAHs but with reduced capacity of metabolising the artificial c-PAH mixtures and real EOM.

3 MATERIALS AND METHODS

3.1 Chemicals, biochemicals and solutions

3.1.1 Cultivation media and cell treatment solutions

William's medium E without L-glutamine - (Gibco, Invitrogen, USA)

Eagle's minimal essential medium (E-MEM) - (Sevapharma, Czech Republic)

Foetal bovine serum (FBS) - (Biochrom AG, Germany)

Glutamine – (Sevapharma, Czech Republic)

Penicillin – (Biotika, Slovakia)

Sodium Bicarbonate – (7.5% solution, Biochrom AG, Germany)

Trypsin - (0.25% solution with 1mM EDTA, without calcium and magnesium, MP Biomedicals, USA)

Phosphate-buffered saline (PBS)

for 500 ml:

4 g NaCl, 0.58 g Na_2HPO_4, 0.1 g KH_2PO_4, 0.1 g KCl

volume adjusted to 500 ml with dH_2O

3.1.2 Chemicals and solutions

c-PAHs: **benz[*a*]anthracene** (B[*a*]A), **benzo[*a*]pyrene** (B[*a*]P), **benzo[*b*]fluoranthene** (B[*b*]F), **benzo[*k*]fluoranthene** (B[*k*]F), **benzo[*g,h,i*]perylene** (B[*g,h,i*]P), **chrysene** (CHRY), **dibenz[*a,h*]anthracene** (DB[*a,h*]A), **dibenzo[*a,l*]pyrene** (DB[*a,l*]P), **indeno[*1,2,3-c,d*]pyrene** (I[*1,2,3-c,d*]P) – (Sigma-Aldrich, USA)

Dimethyl sulfoxide - (Sigma-Aldrich, USA)

CelLytic reagent - (Sigma-Aldrich, USA)

Bicinchoninic acid - (Sigma-Aldrich, USA)

Copper (II) sulphate solution ($CuSO_4 \cdot 5\ H_2O$**)** - (Sigma-Aldrich, USA), 4% solution of Copper (II) sulphate pentahydrate

Tween 20 (Polyoxyethylene sorbitan monolaurate) - (Sigma-Aldrich, USA)

Bovine serum albumin (BSA) - (Sigma-Aldrich, USA)

Ribonuclease A - (Sigma-Aldrich, USA)

Ribonuclease T1 - (5000 U/ml, Sigma-Aldrich, USA)

Proteinase K - (Sigma-Aldrich, USA)

Other chemicals not listed in this section were obtained from Sigma-Aldrich, USA.

Solutions for DNA isolation:

Deferoxamine mesylate (DFA) 0.1 mM

for 100 ml:

6.6 mg DFA in 100 ml dH_2O

50 mM Tris

1.21 g Trizma-base in 200 ml 0.1 mM DFA

pH adjusted to 7.4 with HCl

Extraction buffer

for 1 l:

2.42 g Trizma-base, 3.72 g EDTA, 10.0 g Sodiumdodecylsulphate (SDS), 66 mg DFA, 1000 ml dH_2O

RNAse mix

10 mg Ribonuclease A, 1 ml 50 mM Tris

Mixed and incubated 10 minutes at 80 °C, then cooled down, pipetted into a vial of Ribonuclease T1 (5000 U/ml), mixed gently and stored at –20 °C.

Proteinase K (concentration 40 U/mg)

10 mg/ml 0.1mM DFA

5M NaCl + DFA

29.23 g NaCl in 100 ml 0.1mM DFA

CI + antioxidant

240 ml chloroform, 10 ml 3-methyl-1-butanol, 80 ml of 50 mM Tris

Solutions for ELISA

- **8-oxodG ELISA**

PBS pH 7.4

for 1000 ml:

8g NaCl, 0.2 g KCl, 1.42 g Na_2HPO_4, 0.24 g KH_2PO_4

volume adjusted to 1000 ml with dH_2O

pH adjusted with HCl

Wash buffer

for 1000 ml:

2 ml 10% NaN_3, 0.5 ml Tween 20 in 997.5 ml PBS pH 7.4

Blocking buffer - 1% FBS in PBS/Tween

for 100ml:

1 ml FBS, 50 µl Tween 20 in 99 ml PBS

8-oxodG standard – (Sigma-Aldrich, USA)

Primary antibody N45.1 – (JaICA, Japan)

Secondary antibody – anti-mouse IgG, alkaline phosphatase conjugated – (Sigma-Aldrich, USA)

Phosphatase substrate – 5 mg/tablet (Sigma-Aldrich, USA)

- **15-F_{2t}-isoprostane ELISA**

8-Isoprostane EIA Kit – (Cayman Chemical Company, USA)

EIA Buffer, Wash Buffer, 8-Isoprostane Standard (50 ng/ml),

8-Isoprostane AChE Tracer, 8-Isoprostane Antiserum,
Ellman's Reagent (substrate) – acetylthiocholine and 5,5' – dithio – *bis*-(2-nitrobenzoic acid)

Isoprostane Affinity Sorbent – (Cayman Chemical Company, USA)
Potassium hydroxide (KOH) - (Sigma-Aldrich, USA)
Elution solution – 95% ethanol in dH_2O (Sigma-Aldrich, USA)

1M potassium phosphate:
for 1000 ml:
136.09 g KH_2PO_4 volume adjusted to 1000 ml with dH_2O

Column buffer
for 1000 ml:
13.6 g KH_2PO_4, 29.23 g NaCl, 0.5 g NaN_3
volume adjusted to 1000 ml with dH_2O

- **Carbonyl ELISA**

Coating buffer – 10mM sodium phosphate buffer in 140 mM NaCl (PBS) – pH 7.0
for 1000 ml:
1.42 g Na_2HPO_4, 8.19 g NaCl in 1000 ml dH_2O
pH adjusted to 7.0 with HCl

Blocking buffer - 0.1% BSA in PBS
100 mg BSA in 100 ml Coating buffer

0.1% BSA, 0.1% Tween 20 in PBS
100 mg BSA, 100 µl Tween 20 in 100 ml Coating buffer

Derivatisation solution – 10 mM 2,4- Dinitrophenylhydrazine (DNPH), 6.0M Guanidine Hydrochloride, 0.5M Potassium Phosphate, pH 2.5

for 100 ml:

198 mg 2,4-DNPH in 3.33 ml concentrated phosphoric acid (85%)

57.32 g guanidine hydrochloride in 25 ml dH_2O

pH adjusted to 2.5 with 10M KOH

volume adjusted to 100 ml with dH_2O

PBS pH 7.4

for 1000 ml:

8 g NaCl, 0.2 g KCl, 1.42 g Na_2HPO_4, 0.24 g KH_2PO_4

pH adjusted to 7.4 with HCl

volume adjusted to 1000 ml with dH_2O

Wash buffer

for 1000 ml:

2 ml 10% NaN_3, 0.5 ml Tween 20 in 997.5 ml PBS pH 7.4

2M Tris base

for 100 ml:

24.2 g in 100 ml dH_2O

2.5M sulphuric acid

for 100ml:

2.68 ml H_2SO_4 (98%) in 97.32 ml dH_2O

primary antibody – biotinylated anti-dinitrophenyl-keyhole limpet hemocyanin conjugate (anti-DNP-KLH) – (Molecular Probes, USA)

streptavidin-biotinylated horseradish peroxidase conjugate – (GE Healthcare, UK)

3,3', 5,5' - tetramethylbenzidine substrate (TMB) – (Sigma-Aldrich, USA)

LDH - cytotoxicity assay kit II – (BioVision, USA)

WST Substrate Mix, LDH Assay Buffer, Cell lysis solution, LDH (0.1 µg/ml)

3.2 Air sampling, organic matter extraction and chemical analysis

Airborne particles of an aerodynamic diameter < 10 μm (PM_{10}) were collected in the centre of Prague (Smíchov) 24 hours a day in three different periods: in summer 2000 (June 15th 2000 – September 15th 2000), in winter 2001 (December 4th 2000 – March 3rd 2001) and in winter 2005 (November 7th 2005 – December 22nd 2005) using HiVol (high volume) air samplers (Anderson, USA) with 20x30 cm Pallflex filters (T60A20). EOM was obtained from the filters by dichloromethane extraction. Extracts were divided into two equal parts. The first was used for chemical analysis and the second for *in vitro* testing. Quantitative chemical analysis of PAHs was performed in the certified laboratory ALS Czech Republic (ISO 17025).

The concentrations of eight PAHs classified by International Agency for Research on Cancer (IARC, in preparation) as c-PAHs, including B[*a*]A, CHRY, B[*b*]F, B[*k*]F, B[*a*]P, DB[*a,h*]A, B[*g,h,i*]P, and I[*1,2,3-c,d*]P were analysed in each EOM sample. Individual c-PAH content in EOM from the three periods is summarised in Table 1. For the *in vitro* experiments, the EOM samples were evaporated to dryness under a stream of nitrogen and re-dissolved in dimethyl sulfoxide (DMSO) to a concentration of 50 mg/ml.

Table 1. c-PAHs in the EOM extracted from respirable air particles collected in Prague during three different periods (according to SEVASTYNOVA *et al*. 2008, modified)

Compound	Summer 2000 ng/mg EOM	Winter 2001 ng/mg EOM	Winter 2005 ng/mg EOM
Benz[*a*]anthracene	34	348	478
Chrysene	39	315	687
Benzo[*b*]fluoranthene	74	308	299
Benzo[*k*]fluoranthene	36	134	224
Benzo[*a*]pyrene	50	234	493
Dibenz[*a,h*]anthracene	10	19	40
Benzo[*g,h,i*]perylene	132	127	343
Indeno[*1,2,3-c,d*]pyrene	87	167	343
Total c-PAHs	**462**	**1654**	**2906**

c-PAHs – carcinogenic polycyclic aromatic hydrocarbons
EOM – organic extracts from respirable air particles

3.3 Artificial mixture of c-PAHs (c-PAH mix)

The artificial mixture of c-PAHs was prepared in the Department of Genetic Ecotoxicology, Institute of Experimental Medicine, v.v.i., Prague, according to the chemical analysis of c-PAHs in EOM obtained in Prague during the sampling period of winter 2001. The concentration of B[a]P was used as a basis and the concentrations of other components were calculated according to the relative abundance of other c-PAHs as detected in real EOM (Table 2.).

Table 2. Proportion of individual c-PAHs in the c-PAH mix

Compound	%
Benz[*a*]anthracene	21.1
Chrysene	19.0
Benzo[*b*]fluoranthene	18.6
Benzo[*k*]fluoranthene	8.1
Benzo[*a*]pyrene	14.2
Dibenzo[*a,h*]anthracene	1.2
Benzo[*g,h,i*]perylene	7.7
Indeno[*1,2,3-c,d*]pyrene	10.1

c-PAHs –carcinogenic polycyclic aromatic hydrocarbons
c-PAH mix – an artificial mixture of c-PAHs

3.4 Cell culture conditions and treatment

3.4.1 HepG2 cells

The human HepG2 cell line, derived from a primary hepatoblastoma isolated from an 11-year-old Argentinean male (ADEN *et al.* 1979), was kindly provided by A. Gábelová (Cancer Research Institute, Bratislava, Slovakia).

The HepG2 cells were cultivated in 75 cm^2 flasks in William's medium containing 10% FBS, 2 mmol/l glutamine and 50 U/ml penicillin, at 37 °C and 5% CO_2. The cells were seeded in the plastic cell culture flasks (75 cm^2) at an initial concentration of ~ 25,000 cells/cm^2. The macrophotograph of the growing cells is shown in Figure 3. (magnification 200x).

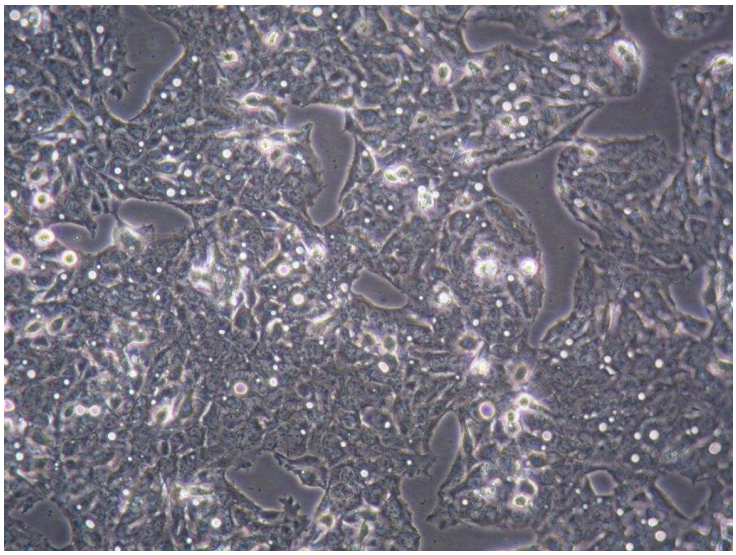


Figure 3. HepG2 cells in culture.

3.4.2 HEL cells

Human embryonic lung diploid fibroblasts (HEL Sevapharma, Czech Republic) were grown in minimal essential medium E-MEM supplemented with 10% FBS, 0.2% sodium bicarbonate and 100 U/ml penicillin (Figure 4., magnification 200x). Cells were seeded in the plastic cell culture flasks (75 cm^2) at an initial concentration of ~ 17,000 cells/cm^2 and incubated at 37 °C in 5% CO_2.

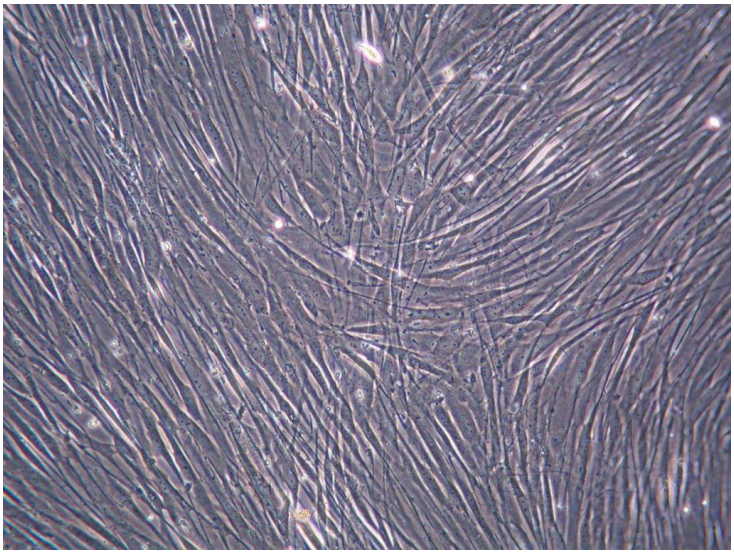


Figure 4. HEL cells in culture.

3.4.3 Cell passaging

Both cell lines are adherent. When the cells reached about 70% confluency, usually three or four days after passaging for HepG2 and seven days for HEL cells, the medium was removed, the cells were washed with PBS. Then 1.5 ml of trypsin solution was added to detach the cells from the bottom of the flask. After 5 minutes of incubating at 37 ºC the enzymatic reaction was stopped by adding 8.5 ml of medium, containing 10% FBS. The cells were resuspended in the medium and counted in the Bürker chamber. 2-3 millions of HepG2 and 1.25-1.5 millions of HEL cells were seeded per flask and supplied with fresh medium.

3.4.4 Cell treatment with c-PAHs and EOM

The cells were cultivated in the medium with 10% FBS until confluence. Before the cell treatment, the medium was replaced with fresh medium supplemented with 1% bovine serum. Individual c-PAHs (B[*a*]P, DB[*a,l*]P), the c-PAH mix and EOM were diluted in DMSO. The confluent cells were exposed for 24 or 48 hours to different concentrations of the compounds and mixtures. All chemicals and concentrations used for the treatment are summarised in Table 3. Cells incubated with individual c-PAHs, their artificial mixture and EOM were analysed in triplicates in at least two independent experiments. Every experiment included negative controls with DMSO only. After exposure, the medium was removed and the cells washed twice with PBS. Then the cells were harvested by scraping. Each flask of HepG2 or HEL cells was harvested into a 15 ml centrifugation tube with 4 ml of PBS and centrifuged for 5 min at 800g. Cell pellets were washed twice with 13 ml of PBS.

Finally the cells were resuspended in 5 ml of PBS. 1 ml of suspension from each sample was transferred to a 1.5 ml tube, supernatant was aspirated and cells stored at -80 ºC for future preparation of the cell lysates. The remaining cell suspension (4 ml) was processed and frozen separately and used for DNA isolation.

Table 3. Tested compounds

Compound	Concentration	Unit
Benzo[*a*]pyrene	1, 10, 100	µM
Dibenzo[*a,l*]pyrene	0.1, 0.5	µM
c-PAH mix*	5, 50	µM B[a]P**
EOM Summer 2000	10, 25, 50	µg/ml
EOM Winter 2001	10, 25, 50	µg/ml
EOM Winter 2005	10, 25	µg/ml

B[a]P - benzo[*a*]pyrene; c-PAHs – carcinogenic polycyclic aromatic hydrocarbons
EOM – organic extracts from respirable air particles
* c-PAH mixture was prepared on the basis of chemical analysis of c-PAHs in EOM extracted from respirable air particles collected in Prague in the winter period 2001 as described in the chapter 3.3
** the abundance of each individual c-PAH in the mixture was calculated in relation to the B[*a*]P content and is shown in Table 2.

3.4.5 Cytotoxicity assay

Cytotoxicity of the tested compounds was scored using the LDH-cytotoxicity test based on a lactate dehydrogenase (LDH) release from cells following damage to their plasma membranes. LDH released from damaged cells oxidises lactate to generate NADH, which then reacts with the substrate mix (WST-1) to generate yellow colour. The intensity of the generated colour correlates directly with the number of cells lysed.

The suspensions of HepG2 and HEL cells in fresh medium with 1% FBS were seeded in a 96-well flat-bottomed tissue culture plate (Corning, USA). 100 µl of the cell suspension (containing 6 x 10^4 of HepG2 cells or 4 x 10^4 of HEL cells) was seeded per well in triplicates. Then the tested compounds were added and the plates were incubated for an adequate time (24 or 48 hours) in an incubator at 37°C and 5% CO_2. 100 µl of the culture medium with no cells in triplicate was used as a blank and 100 µl of the cell suspension without any compounds in triplicate was used as a low control. The high control was established by adding 10 µl of cell lysis solution to the triplicate wells with the cells only (no tested compounds) 30 minutes before the end of the incubation. At the end of the incubation, the plate was gently shaken and centrifuged at 250 g for 10 minutes. Subsequently the clear media (10 µl per well) were transferred into the corresponding wells of another 96-well plate and 100 µl of LDH reaction mix was added (200 µl of WST substrate mix in 5 ml of LDH assay buffer), the plate was shaken and incubated for 30 minutes at RT. One µl of LDH was used as a positive control to test whether all reagents were working properly. Finally the absorbance was read at 450 nm and the cytotoxicity was calculated using the following formula:

$$\text{Cytotoxicity (\%)} = ((A_{\text{sample}} - A_{\text{low control}}) / (A_{\text{high control}} - A_{\text{low control}})) \times 100$$

For the calculation, absorbance values were used (blank subtracted).

3.5 Oxidative damage by Enzyme-Linked ImmunoSorbent Assay (ELISA)

3.5.1 Principle of ELISA

ELISA is a method used for measuring the concentration of a specific analyte with the use of a highly specific antibody. As there are three different assay formats used in this work, the principles of each of them are mentioned in respective chapters.

3.5.2 Determination of oxidative DNA damage

3.5.2.1 DNA extraction and sample preparation

Tubes with frozen cell pellets were removed from –80 °C and thawed at room temperature. 1 ml of extraction buffer and 20 µl of RNAse mix were added, tubes were vortexed and incubated 1 hour at 37 °C. Following the incubation, 20 µl of proteinase K was added and samples were incubated for another 1.5 h at 37 °C. The supernatants were transferred to 15 ml tubes containing Phase Lock Gel, 1 ml of CI with antioxidant was added, the samples were shaken vigorously and centrifuged 5 min, 1800 g. DNA precipitation followed by removing the upper phase and mixing it well with 100 µl of 5M sodium chloride and 2.2 ml of ice-cold absolute ethanol. Precipitated DNA was then removed with a pipette tip, transferred into a new 1.5 ml tube, washed with 70% ethanol and spun 5 minutes, 7,250 g. DNA pellet was vacuum-dried and stored at –80 °C.

To dissolve DNA, PBS with 1mM DFA was added and DNA pellet was homogenized using the Pellet Pestle Motor. Subsequently the samples were incubated 40 minutes at 55 °C. Dissolved DNA samples were then denatured 5 minutes at 100 °C and fragmented using a syringe and a 22G needle. Finally the absorbance at 260 nm was read to determine the DNA concentration, which was then adjusted by PBS with 1mM DFA to 0.75 - 1.25 mg/ml.

3.5.2.2 8-oxodG levels in DNA by ELISA

Principle of the assay

The 8-oxodG ELISA is a competitive assay based on the published protocol (YIN *et al*. 1995), with some modifications. The modifications include changes in incubation steps, the use of a different primary antibody (RÖSSNER *et al*. 2007) and changes in DNA extraction as well as DNA samples processing before the ELISA assay.

ELISA plates used in the assay are first coated with the 8-oxoG-BSA conjugate. Then, analysed samples along with the primary antibody are added. 8-oxodG in the samples competes with the 8-oxoG-BSA conjugate adsorbed to the plate for binding to the antibody. The unbound material is washed and the secondary antibody conjugated with alkaline phosphatase is pipetted to the wells. 8-oxodG in samples is detected by adding the substrate to alkaline phosphatase and reading the absorbance at 405 nm. The absorbance is inversely proportional to the amount of 8-oxodG in analysed samples.

8-oxoG-BSA conjugate preparation

8-oxoG-BSA conjugate was previously prepared by Pavel Rössner, Jr. according to the protocol described by YIN *et al*. (1995). 8-oxoG was treated with sodium periodate to form the electrophilic dialdehyde species that are subsequently conjugated to the amino groups of the carrier protein (BSA). Reduction of the Schiff's base with sodium cyanoborohydride forms a stable conjugate.

Three mg of 8-oxoG (Cayman Chemical Company, USA) were dissolved in 100 µl of 0.1 M sodium periodate and incubated on ice for 15 min. BSA (5 mg) was dissolved in 400 µl of 0.15 M NaCl and slowly mixed with 8-oxoG. pH of the solution was adjusted to 8.5-9.0 using 0.15 M Na_2CO_3 (pH 12.0); the solution was stirred on ice for 20 min. Finally, 150 µl of 0.25% sodium cyanoborohydride was added, pH was adjusted to 7.0 with 1M acetic acid and the solution was stirred on ice for 40 min. The solution was dialysed against PBS in the cold room overnight and the modification level of BSA (n) was

estimated by measuring absorbances at 260 nm and 280 nm and using the following formula:

$$n = [\varepsilon_{c(280)} * (A_{260}/A_{280}) - \varepsilon_{c(260)}]/ [\varepsilon_{N(260)} - \varepsilon_{N(280)} * (A_{260}/A_{280})]$$

ε_c – molar extinction coefficient of the carrier protein;
BSA: $\varepsilon_{c(280)} = 4.3x10^4$, $\varepsilon_{c(260)} = 2.6x10^4$
ε_N – molar extinction coefficient of the nucleotide;
8-oxoG: $\varepsilon_{N(280)} = 1.16x10^4$, $\varepsilon_{N(260)} = 8x10^3$

The conjugate was stored in small aliquots (5 µl) at -20 °C. It can be repeatedly thawed and frozen without changing its properties.

8-oxodG ELISA assay

For coating the plate (Corning 3690, Half Area Plates, Corning, USA), 8-oxoG-BSA conjugate was used (5 ng in 25 µl PBS per well) and the plate was incubated at 37 °C overnight.

The next day, the plate was washed with washing buffer and blocked with 100 µl of blocking buffer per well for 1 h at RT. The standard curve was prepared by diluting the 8-oxodG standard (concentration of stock solution: 1 mg/ml) in PBS to the following concentrations: 40, 20, 10, 5, 2.5 and 1.25 ng/ml. After the incubation, the blocking solution was removed and the samples and standards were added to the plate (25 µl per well). To determine total binding, three wells were left without competitor, which was replaced by PBS only (25 µl per well). The primary antibody N45.1 was diluted 1:500 in blocking buffer to the final concentration 0.2 µg/ml and added (25 µl per well) to all wells except blank wells, which were supplied with 25 µl of PBS and 25 µl of blocking buffer. The plate was incubated 90 minutes at RT then it was washed and 50 µl of the secondary antibody (anti-mouse, alkaline phosphatase conjugated, diluted 1:750) was added to all wells. After 90 minutes of incubation with 50 µl of the secondary antibody at RT, the plate was washed, rinsed once with 0.01M diethanolamine solution and 50 µl of substrate per well was added (p-nitrophenyl phosphate, 1 mg/ml, 1 tablet in 5 ml of 1M diethanolamine, Sigma-Aldrich). Plate was incubated 20-60 minutes at RT and then absorbance was read at 405 nm. Results were calculated using the standard curve.

3.5.3 Determination of oxidative damage to proteins and lipids

3.5.3.1 Preparation of cell lysates for protein carbonyl and 15-F_{2t}-isoprostane analysis

The samples (cell pellets) were mixed with 100 µl of CelLytic reagent, incubated for 15 minutes at RT on a shaker and vortexed every 5 minutes. Then the samples were centrifuged 15 minutes at 17,750 g at 4 ºC. The supernatant (cell lysate) was transferred to a new tube and stored at -80 ºC.

The lysate (5 µl) was diluted 5x in dH_2O and used for the determination of total protein concentration.

3.5.3.2 Determination of total protein concentration

Using 30 mg BSA and dissolving it in 1 ml of dH_2O, the stock solution of BSA was made. Then the standard curve was prepared by diluting the BSA stock solution in dH_2O to the following concentrations: 3.0, 2.5, 2.0, 1.5, 1.0 and 0.5 mg/ml. Diluted BSA standards and cell lysates (10 µl) were added to a 96-well plate in duplicates. Bicinchoninic acid (20 ml) was mixed with 400 µl of copper sulphate solution and 200 µl/well of the solution was added to the samples in the 96-well plate. The plate was incubated 30 minutes at 37 ºC and then the absorbance at 562 nm was read. Total protein concentration was calculated using the standard curve and the dilution factor 5.

3.5.3.3 15-F_{2t}-isoprostane concentration in cell lysates by ELISA

Principle of the assay

The 15-F_{2t}-isoprostane ELISA is a competitive assay. It is based on a competition between 15-F_{2t}-IsoP in analysed samples and a 15-F_{2t}-IsoP-acetylcholinesterase (AChe) conjugate (15-F_{2t}-IsoP AChe Tracer) for a limited number of 15-F_{2t}-IsoP-specific rabbit antiserum binding sites. Because the concentration of the 15-F_{2t}-IsoP tracer is constant while the concentration of 15-F_{2t}-IsoP in the samples varies, the amount of 15-F_{2t}-IsoP tracer that is able to bind to the rabbit antiserum is inversely proportional to the

concentration of 15-F_{2t}-IsoP in the sample. The rabbit antiserum-15-F_{2t}-IsoP complex binds to the anti-rabbit IgG mouse monoclonal antibody that was previously attached to wells of the 96-well ELISA plate. The unbound reagents are removed from the plate by washing and the Ellman's Reagent, which contains the substrate to AChe, is added. The absorbance of the product of the enzymatic reaction is inversely proportional to the amount of 15-F_{2t}-IsoP in analysed samples.

Before running the ELISA assay, the membrane-bound 15-F_{2t}-IsoP must be hydrolysed and the samples purified.

Sample purification

Cell lysates samples containing 50 µg of proteins were transferred into 1.5 ml tubes and diluted with dH_2O to 100 µl. Then 100 µl of 15% KOH was added, the samples were vortexed and incubated 60 minutes at 40 °C. The pH of the samples was adjusted by adding 300 µl of 1 M KH_2PO_4 (resulting pH around 7.3). Consecutively, 100 µl of column buffer was added and the samples were mixed. As a next step, 50 µl of Isoprostane Affinity Sorbent was added and the samples were incubated 60 minutes at RT on a shaker. After the incubation, the samples were centrifuged 1 minute at 5,000 g and the supernatant was decanted. The washing step followed by adding 1 ml of dH_2O, vortexing, centrifuging 1 minute at 5,000 g and finally by decanting the supernatant. The procedure of purification of the 15-F_{2t}-IsoP continued by its elution from the sorbent: the pellets were resuspended in 0.5 ml of Elution solution (95% ethanol), vortexed and centrifuged 1 minute at 5,000 g. The supernatant (ethanol wash) was removed and stored in a 1.5 ml tube and the elution step was repeated. Both supernatants were combined and stored at –80 °C until analysis.

15-F_{2t}-isoprostane ELISA assay

The samples were vacuum-dried, resuspended in 110 µl of EIA buffer and immediately used for the assay. The ELISA itself was performed according to the manufacturer's recommendations:

The 8-Isoprostane standard (50 ng/ml) was diluted ten times to prepare "the bulk standard" (5 ng/ml). This bulk standard was then diluted to eight different concentrations to

prepare the standard curve: 500, 200, 80, 32, 12.8, 5.1, 2.0 and 0.8 pg/ml. Each concentration of the standard was then added to the plate in duplicate (50 µl per well). The prepared samples were analysed in duplicates as well (50 µl per well). The 96-well plate also contained two blanks (Blk), two non-specific binding wells (NSB), three maximum binding wells (B_0) and one total activity well (TA, total enzymatic activity of the AChE-linked tracer). To NSB wells 100 µl of EIA buffer was added, for B_0 wells the volume of buffer was 50 µl. Consecutively, 50 µl of AChE Tracer was added to each well except the TA and Blk wells. Finally, 50 µl of 15-F_{2t}-IsoP Antiserum was added to each well except the TA, the NSB, and the Blk wells and the plate was covered with the plastic film and incubated for 18 hours at 4 °C. When ready to develop the plate, the wells were rinsed five times with the Wash Buffer (250 µl per well) and the substrate was added (200 µl per well, the Ellman's Reagent – one vial dissolved in 20 ml of dH_2O). Lastly, 5 µl of Tracer was added to the TA well, the plate was covered with the plastic film and developed in the dark for 60-90 minutes. Later on, the plate was read at 405 nm and the results were calculated using the standard curve.

3.5.3.4 Protein carbonyl concentration in cell lysates by ELISA

Principle of the assay

The carbonyl assay is an indirect ELISA method described by BUSS *et al*. 1997. The original method was developed for the analysis of protein carbonyls in blood plasma; however, it was modified in our laboratory to make it suitable for the analysis of cell lysates. Before running the assay, oxidised BSA was prepared and the concentration of carbonyl groups was measured using a colorimetric method. Oxidised BSA with known levels of proteins oxidation was used to make a standard curve during the ELISA assay. Oxidised BSA may be kept at -80 °C for a long time (several years) without any change of level of oxidation.

The principle of the detection of carbonyl groups in analysed samples is based on the incubation of the samples with dinitrophenylhydrazine (DNPH). During the process called derivatisation, DNPH binds to carbonyl groups and changes to

dinitrophenylhydrazon. A 96-well ELISA plate is coated with derivatised samples and modified carbonyl groups are detected using a biotinylated primary antibody recognising dinitrophenyl and a streptavidin-horseradish peroxidase conjugate. Finally the enzymatic reaction is started by adding the peroxidase substrate. The more carbonyl groups are present in the sample, the stronger enzymatic reaction occurs.

Preparation of the oxidised standards

- **Oxidation of BSA**

Oxidised BSA stock standard was previously prepared by Pavel Rössner, Jr. by the following procedure:

$FeSO_4.H_2O$ was dissolved in PBS to a final concentration 0.42 mM Fe^{2+}, H_2O_2 was added to a final concentration 0.73 M H_2O_2. BSA (50 mg/ml) was dissolved in the solution and incubated for 1h at 37 °C in a water bath. Oxidation was stopped with butylated hydroxytoluene (a final concentration 40 μM).

- **Carbonyl and protein concentration in oxidised and unoxidised (native) BSA**

Native (unoxidised) BSA was dissolved in PBS (final concentration 50 mg/ml). Both oxidised and native BSA (0.25 ml) were added to 3.6 ml tubes, two tubes for each sample. DNPH (1 ml; stock solution 10 mM in 2.5M HCl) was added to one of the tubes with oxidised or native BSA; the solution served as a sample. 2.5M HCl (1 ml) was added to the other tube with oxidised or native BSA; it served as a control without DNPH. Solutions were incubated for 1 h at RT in the dark, vortexed every 15 min. Then 1 ml of 20% trichloroacetic acid was added and samples were incubated on ice for 10 min. The tubes were centrifuged at 1800 g for 5 min, the supernatant was discarded and the sediment washed three times with 1 ml of the ethanol-ethyl acetate mixture (1:1, v/v). Finally, the supernatant was dissolved in 0.5 ml of 6M guanidine hydrochloride and incubated at 37 °C for 10 min. Tubes were centrifuged again (1800 g, 5 min) to remove any insoluble material. The concentration of proteins in samples was measured by reading the absorbance of the samples at 280 nm and comparing with the standard curve

constructed by diluting native BSA (stock solution 50 mg/ml) in guanidine hydrochloride to final concentrations 2.0, 1.5, 1.0, 0.5 and 0.25 mg/ml. To estimate the level of carbonyl groups (nmol/ml), the absorbance of the samples at 375 nm was measured and the result was multiplied by a constant 45.45. Then the value obtained for controls without DNPH was subtracted from the value obtained for samples (containing oxidised or native BSA). The carbonyl concentration normalized per mg of protein (nmol/mg protein) was further calculated by dividing the carbonyl levels expressed in nmol/ml by the concentration of proteins in control samples. To make a standard for the carbonyl ELISA, native and oxidised BSA were mixed in various ratios and the dilution that gives the final carbonyl concentration 1.9-2.0 nmol/mg protein was determined. In this solution, the protein concentration is 50 mg/ml. To reduce the protein concentration to 4 mg/ml, the mixture of oxidised and native BSA was diluted 12.5x with PBS. The final solution containing 1.9-2.0 carbonyl groups/mg protein, the protein concentration 4 mg/ml, was stored in aliquots at -80 °C and used as a standard for the carbonyl ELISA assay.

Preparation of samples and standards - derivatisation

The standard curve was made by diluting the stock standard BSA with PBS to the following concentrations of carbonyl: 0.612, 0.510, 0.408, 0.306, 0.204 and 0.102 nmol/mg protein. The analysed samples were diluted with PBS to a final protein concentration of 2 mg/ml. 10 µl of the samples and standards were mixed with 10 µl of derivatisation solution to give a final protein concentration of 1 mg/ml. The samples were incubated at room temperature in the dark for 45 min and vortexed every 10 to 15 min. The derivatisation process was stopped by adding 30 µl of 2M Tris; then the samples were mixed on a shaker and briefly centrifuged. 6.25 µl of the derivatised samples were added to 1.25 ml of the coating buffer, mixed and used for coating the ELISA plates.

Carbonyl ELISA assay

Samples and standards were added to the plate in triplicates (200 µl per well) to coat the plate overnight at 4 ºC in humidified atmosphere. Derivatised PBS was used as a blank. The next day, the plate was washed with 350 µl of wash buffer per well and blocked with

0.1% BSA in PBS (250 µl per well) at RT for 1.5 hour. After blocking, the plate was washed again and 200 µl of primary antibody was added (biotinylated anti-DNP antibody diluted 1:1500 in 0.1% BSA, 0.1% Tween 20 in blocking buffer containing 0.1% Tween 20). After the incubation with the antibody (1 h at 37 ºC), the plate was washed again and 200 µl of conjugate (streptavidin-biotinylated horseradish peroxidase conjugate diluted 1:4000 in blocking buffer containing 0.1% Tween 20) was added and incubated at RT for 1 hour. After the incubation with the conjugate the plate was washed, and incubated with TMB substrate (200 µl per well) at RT for 15 – 25 minutes. Finally, the reaction was stopped by adding 2.5M sulphuric acid (H_2SO_4, 100 µl per well) and the absorbance was read at 450 nm. Subtracting the blank absorbance value and using the standard curve, the carbonyl concentration in samples was calculated.

3.6 Equipment used

Incubator IG 150 – (Jouan, France)
Microscope CKX41 – (Olympus, Japan)
Biohazard hood LC2S.18 – (Jouan, France)
Pellet Pestle Motor - (Sigma Aldrich, USA)
Nanodrop spectrophotometer ND-1000 – (Thermo Scientific, USA)
Centrifuge 5810 R – (Eppendorf, Germany)
Centrifuge MR 22i - (Jouan, France)
Centrifuge Speedfuge 24D – (Labnet International, USA)
Water bath 1086 – (GFL, Germany)
pH meter 50 – (Beckman Coulter, USA)
Minishaker MS1 – (IKA, Germany)
Analytical balance RC 210D – (Sartorius, Germany)
Microplate strip washer ELx 50 – (BIO-TEK, USA)
Spectrophotometer SpectraMax M5e – (Molecular Devices, USA)
Pipettes Eppendorf Research – adjustable single channel and 8-channel – (Eppendorf, Germany)

3.7 Statistical analysis

To ensure maximum reliability of the data and to control for inter-experiment variability, each tested compound was analysed in a triplicate and the whole experiment was repeated at least twice. The data from two or more experiments were pooled and analysed together. As described further in the Results chapter, the biological inter-experiment variability was quite high. For that reason, the results of every experiment were normalised to the basal level of oxidative damage in the control sample, and these relative numbers were pooled and used for the statistical analysis.

The data obtained from the experiments were calculated and processed using Microsoft Excel 2007. The graphs, also created in Microsoft Excel, show mean values of relative oxidative damage levels ± standard deviation.

Statistical differences between the levels of oxidative damage determined in treated cells and controls were calculated by Student's t-test using SPSS programme (version 16.0). The null hypothesis (H0) tested was that the data obtained from the treated samples do not significantly differ from the data obtained from the control samples. The null hypothesis was rejected on the 5% significance level ($p<0.05$).

4 RESULTS

4.1 Cytotoxicity of the tested chemicals

The cytotoxicity of individual c-PAHs (B[*a*]P, DB[*a,l*]P), the artificial mixture of c-PAHs and EOM measured by the LDH kit differed between the two cell lines. Generally, the amount of LDH released from HepG2 cells was higher than that released from HEL cells, suggesting that the hepatoma cell line was more sensitive to the tested compounds.

For EOM, the cytotoxic effect on both cell lines was quite similar: EOM from summer 2000 was cytotoxic at a concentration of 50 µg/ml for both treatment periods in HepG2 cells, while in HEL cells this concentration was cytotoxic only after 48 h incubation. No cytotoxicity of EOM from either winter period (2001 or 2005) was observed for either cell line.

However, the cytotoxic responses of the cell lines to exposure to B[*a*]P and DB[*a,l*]P was different. While exposure to the higher concentrations of B[*a*]P and DB[*a,l*]P resulted in increased LDH release from HepG2 cells already after 24 h incubation, the only compound increasing the cytotoxicity in HEL cells was DB[*a,l*]P at the higher tested concentration and after 48 h treatment.

Even though the levels of cytotoxicity in treated cells were higher when compared to the control samples, the amount of released LDH was generally low and the percentage of cytotoxicity rarely exceeded 20%. The concentrations of the tested chemicals resulting in cytotoxicity > 20% are indicated by the letter “C” in Graphs 1-12.

4.2 Oxidative damage to macromolecules

The results of oxidative damage to macromolecules are shown separately for oxidative damage to DNA, lipids and proteins. Each chapter presents the results for all tested compounds and mixtures, both cell lines and both treatment periods. As described in the Materials and methods chapter, oxidative damage is expressed as a relative number, rather than as the absolute value of the respective marker of oxidative stress (Graphs 1-12). The reason why the data were analysed in this way is the variability of the basal levels of oxidative damage between individual experiments and also the fact that measurements from at least two independent experiments were pooled and statistically analysed together.

The mean values of the oxidative stress markers, the standard deviations and the coefficient of variation (CV, calculated as the ratio of the standard deviation and the mean, expressed in %) illustrating the variability between the experiments are shown in Table 4. These values were obtained from control cells incubated in culture media with DMSO only. The CV, ranging between 27.3% and 68.2 %, cannot be explained by the inter-assay variability of the ELISA assay, which typically has a maximum CV of 15%. This variability is rather a result of the biological variability in the basal levels of oxidative damage between individual experiments. Despite the observed variability, significant differences between the two cell lines were found for all markers of oxidative stress: HEL cells had higher levels of basal oxidative damage than did HepG2 cells.

Table 4. Variability in the basal levels of oxidative markers and a comparison of the HepG2 and HEL cell lines.

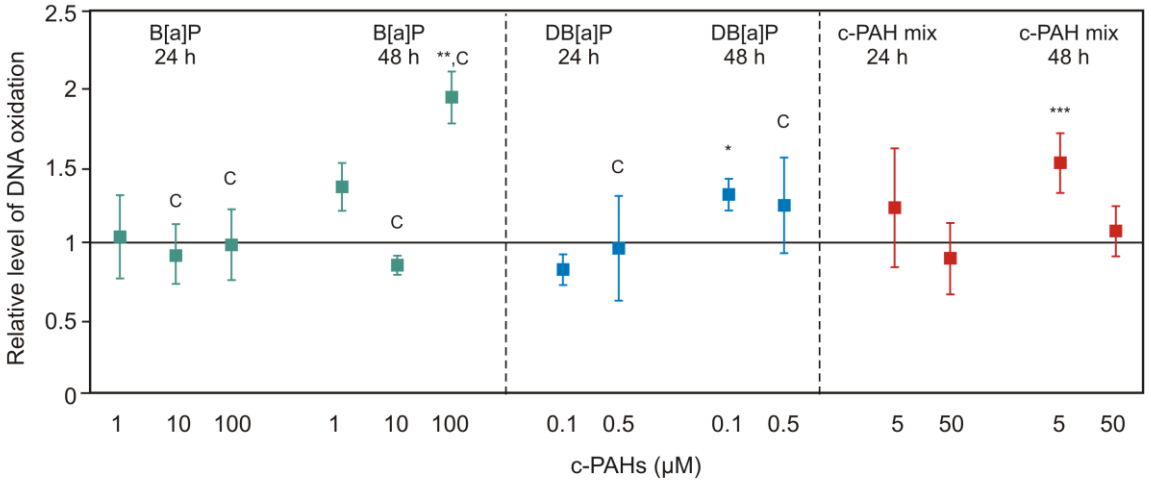
Oxidative stress marker	**8-oxodG (per 10^5 dG)**		**15-F_{2t}-IsoP (pg/ml)**		**Carbonyl (nmol/mg)**	
Cell line	**HepG2**	**HEL**	**HepG2**	**HEL**	**HepG2**	**HEL**
Mean	1.50	4.28	23.84	41.96	0.92	1.14
SD	1.03	2.54	8.23	13.24	0.36	0.31
CV (%)	68.17	59.32	34.51	31.54	39.07	27.26
Comparison HepG2 vs. HEL	p<0.001		p<0.001		p=0.001	

SD – standard deviation; CV – coefficient of variation
Mean and SD were calculated from at least 35 individual values of the respective marker of oxidative stress.

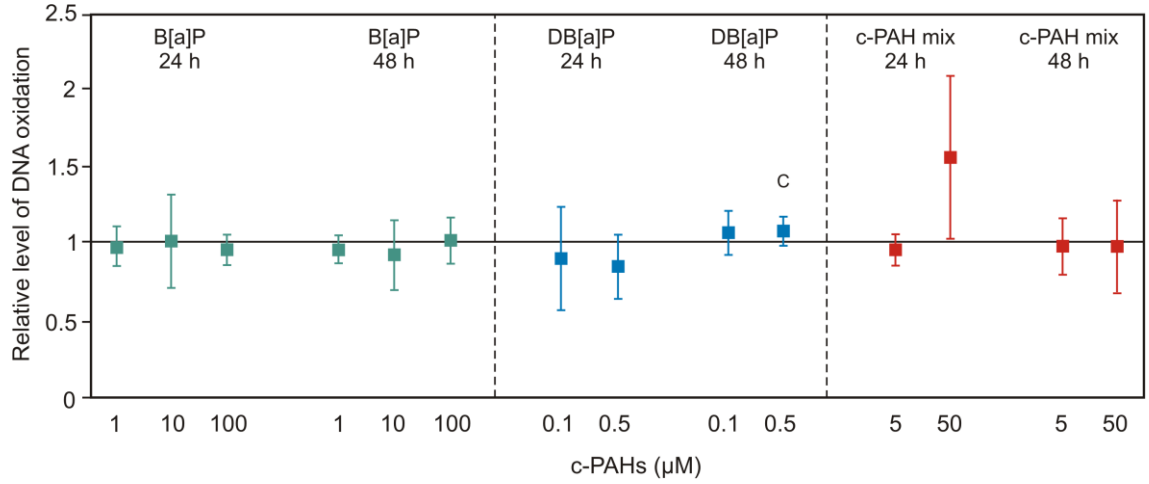
4.2.1 Oxidative DNA damage

Graphs 1 and 2 summarise the results of analyses of 8-oxodG obtained for HepG2 and HEL cells following the treatment with B[*a*]P, DB[*a,l*]P and the c-PAH mix for 24 and 48 h. The effect of the tested compounds was weak. In HepG2 cells some increase in 8-oxodG levels was detected after 48 h of treatment with B[*a*]P, DB[*a,l*]P and the c-PAH mix, but in HEL cells no significant increase in 8-oxodG compared to the control occurred.

Oxidative DNA damage induced by EOM is shown in Graph 3 (HepG2) and Graph 4 (HEL). In HepG2 cells, the levels of 8-oxodG significantly increased above control levels for all EOM and most concentrations and treatment periods tested. However, in HEL cells no significant increase above control levels was observed. With the exception of the summer 2000 EOM after the 24 h treatment in HepG2 cells, no apparent dose-response was detected. Differences between the 24 h and 48 h treatment periods in HepG2 cells were not consistent: a significant decrease in the levels of 8-oxodG was observed for all concentrations of the winter 2001 EOM ($p<0.05$) and for the lower concentration of the winter 2005 EOM ($p<0.01$). For the summer 2000 EOM, no significant differences between 24 h and 48 h incubations were found.

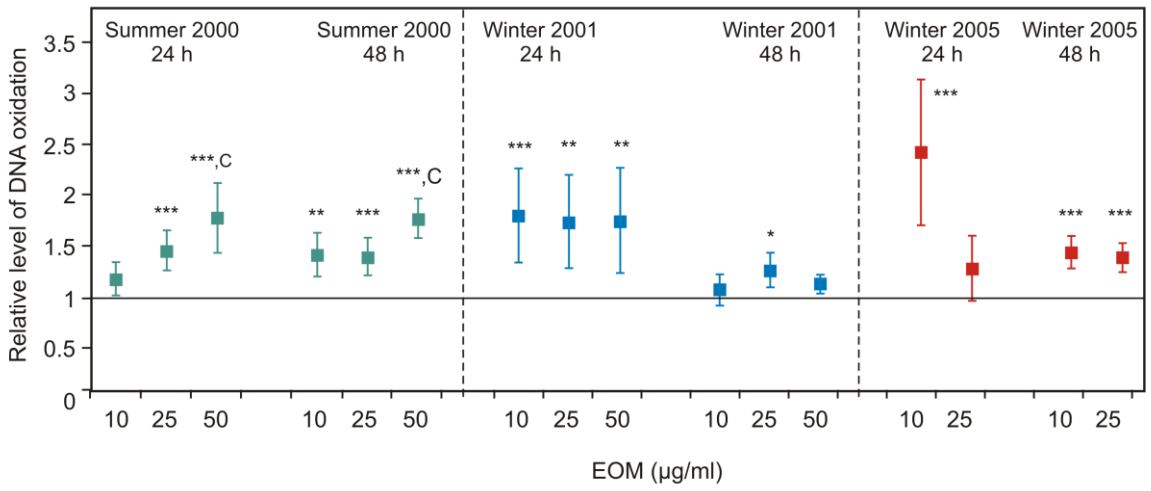


Graph 1. 8-oxodG levels in HepG2 cells after the treatment with B[*a*]P, DB[*a,l*]P or the c-PAH mix for 24 or 48 h

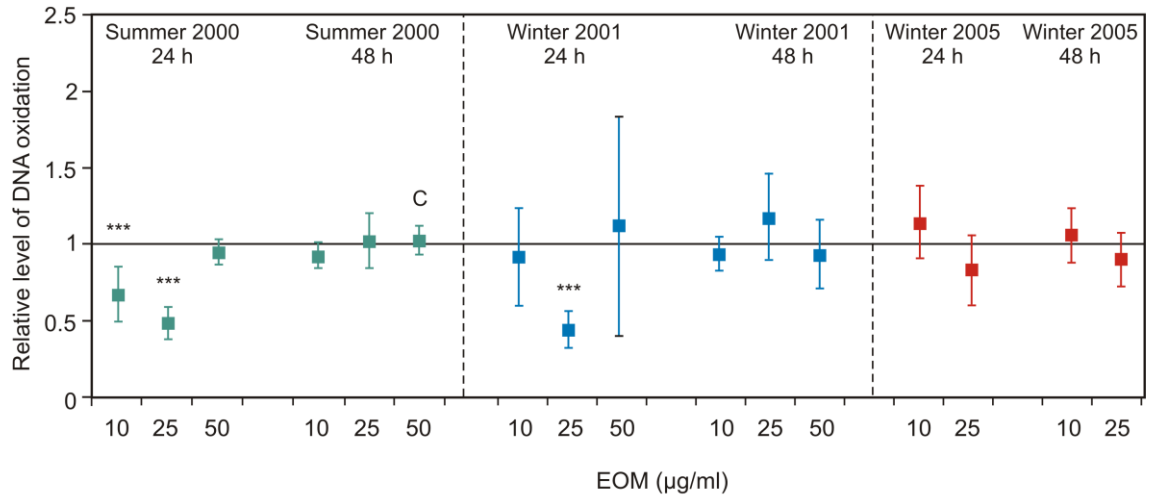


Graph 2. 8-oxodG levels in HEL cells after treatment with B[*a*]P, DB[*a,l*]P or the c-PAH mix for 24 or 48 h

Mean relative levels of DNA oxidation ± standard deviation; the solid line indicates the basal level of DNA oxidation in the controls.
c-PAH mix: values on the x-axis indicate the concentrations of B[*a*]P in the mixture; the concentrations of the other c-PAHs were calculated in relation to B[*a*]P content as described in the Materials and methods chapter.
The significant differences between oxidative DNA damage in the experimental samples compared to the control are indicated by asterisks; significance level: $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).
"C" denotes a dose of a compound that increases the release of lactate dehydrogenase by more than 20% compared to the control sample.
B[*a*]P – benzo[*a*]pyrene; DB[*a,l*]P – dibenzo[*a,l*]pyrene; c-PAH mix – an artificial mixture of c-PAHs
c-PAHs – carcinogenic polycyclic aromatic hydrocarbons.



Graph 3. 8-oxodG levels in HepG2 cells after treatment with EOM for 24 or 48 h



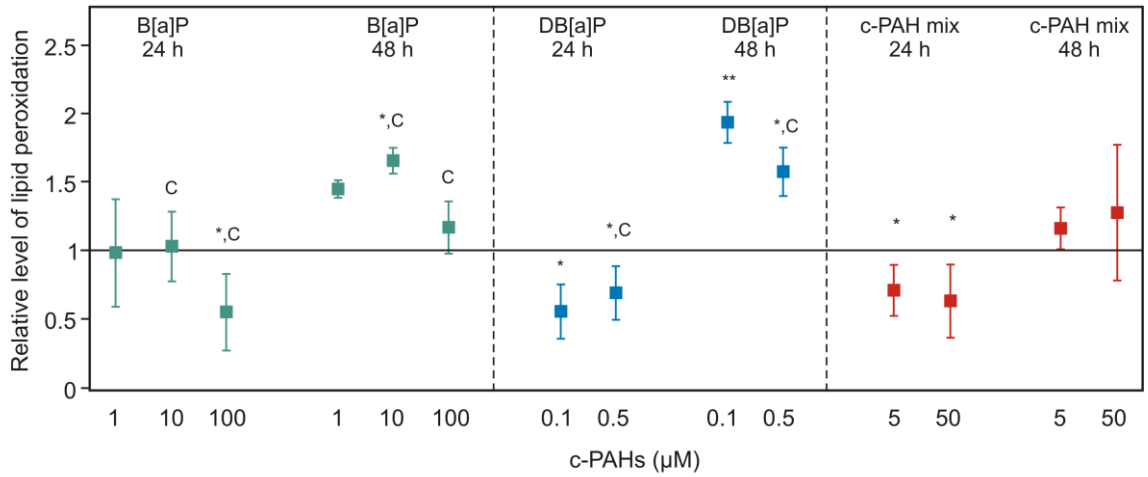
Graph 4. 8-oxodG levels in HEL cells after treatment with EOM for 24 or 48 h

Mean relative levels of DNA oxidation ± standard deviation; the solid line indicates the basal level of DNA oxidation in the controls.
The significant differences between oxidative DNA damage in the experimental samples compared to the control are indicated by asterisks; significance level: $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).
"C" denotes a dose of a compound that increases the release of lactate dehydrogenase by more than 20% compared to the control sample.
EOM – organic extract from respirable air particles.

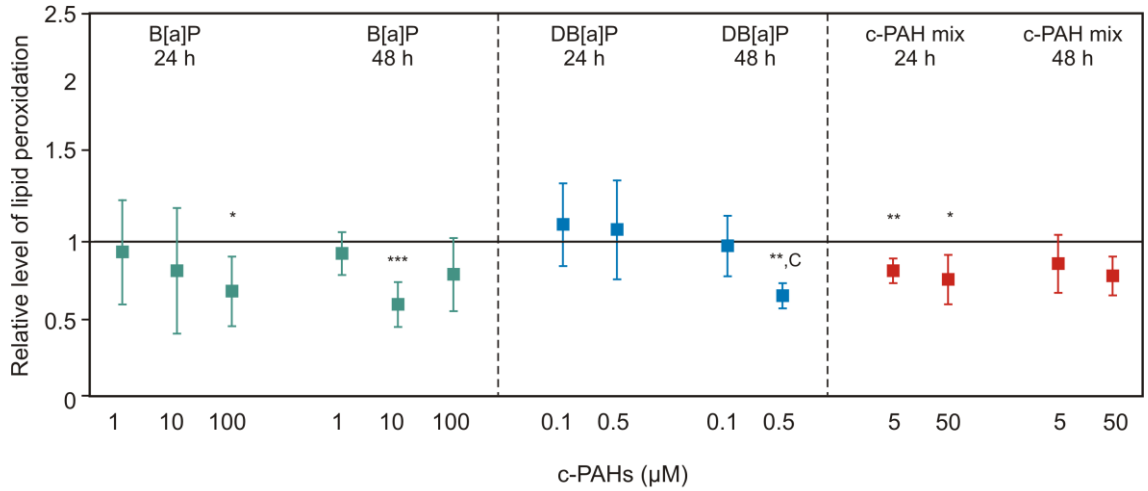
4.2.2 Lipid peroxidation

Individual c-PAHs as well as the c-PAH mix either had no significant effect on the levels of lipid peroxidation in HepG2 cells, or else they decreased 15-F_{2t}-IsoP concentrations below control levels after 24 h incubation, but increased them above control levels after 48 h treatment (Graph 5). The effect of c-PAHs and the c-PAH mix on lipid peroxidation in HEL cells differed from that observed in HepG2 cells. The levels of 15-F_{2t}-IsoP remained at control levels or even decreased for most concentrations in both treatment times (Graph 6).

This unexpected trend in HEL cells was even more pronounced after the treatment with EOM: the decrease was significant for all concentrations and both incubation periods with the only exception of the winter 2001 EOM, concentration 10 µg/ml and 48 h, where the decrease was not significant due to high variability of the data (Graph 8). On the contrary, in HepG2 cells exposure to EOM lead to similar results for lipid peroxidation as for DNA oxidation. As Graph 7 indicates, 15-F_{2t}-IsoP levels were significantly increased compared to the control for most concentrations of EOM. The 48 h treatment did not result in increase of 15-F_{2t}-IsoP above the levels observed after 24 h. In several cases, however, the levels significantly decreased (EOM winter 2001, 25 µg/ml, $p<0.05$; EOM winter 2005, 10 µg/ml, $p<0.05$ and 25 µg/ml, $p<0.01$).

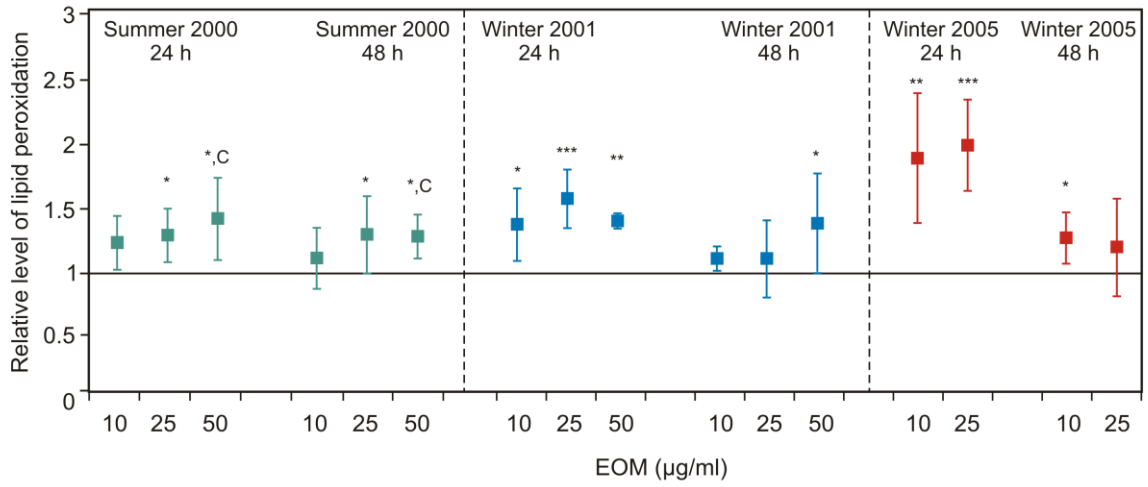


Graph 5. 15-F_{2t}-IsoP levels in HepG2 cells after treatment with B[*a*]P, DB[*a,l*]P or the c-PAH mix for 24 or 48 h

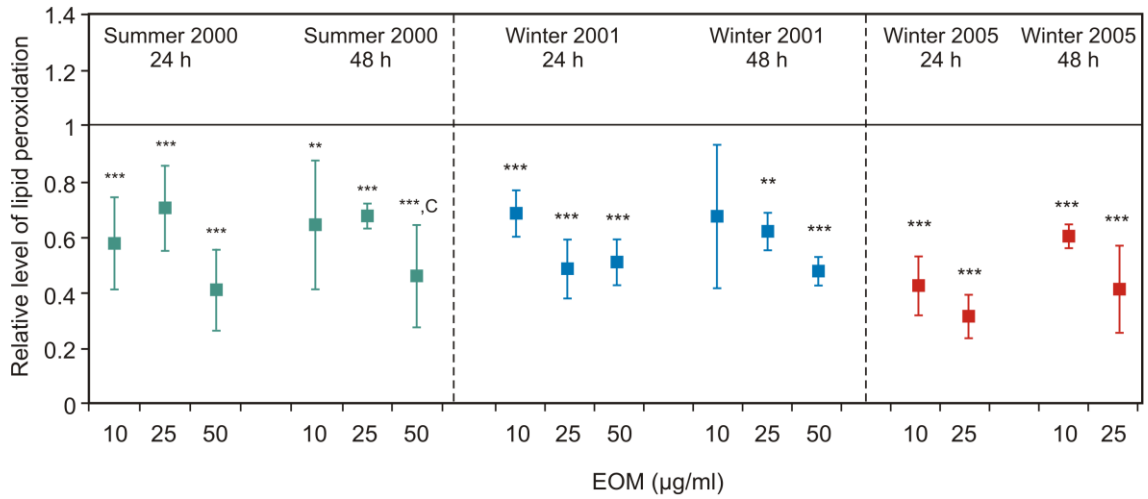


Graph 6. 15-F_{2t}-IsoP levels in HEL cells after treatment with B[*a*]P, DB[*a,l*]P or the c-PAH mix for 24 or 48 h

Mean relative levels of lipid peroxidation ± standard deviation; the solid line indicates the basal level of lipid peroxidation in the controls.
c-PAH mix: values on the x-axis indicate the concentrations of B[*a*]P in the mixture; the concentrations of other c-PAHs were calculated in relation to B[*a*]P content as described in the Materials and methods chapter.
The significant differences between lipid peroxidation in the experimental samples compared to the control are indicated by asterisks; significance level: $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).
"C" denotes a dose of a compound that increases the release of lactate dehyrogenase by more than 20% compared to the control sample.
B[*a*]P – benzo[*a*]pyrene; DB[*a,l*]P – dibenzo[*a,l*]pyrene; c-PAH mix – an artificial mixture of c-PAHs
c-PAHs – carcinogenic polycyclic aromatic hydrocarbons.



Graph 7. 15-F_{2t}-IsoP levels in HepG2 cells after treatment with EOM for 24 or 48 h



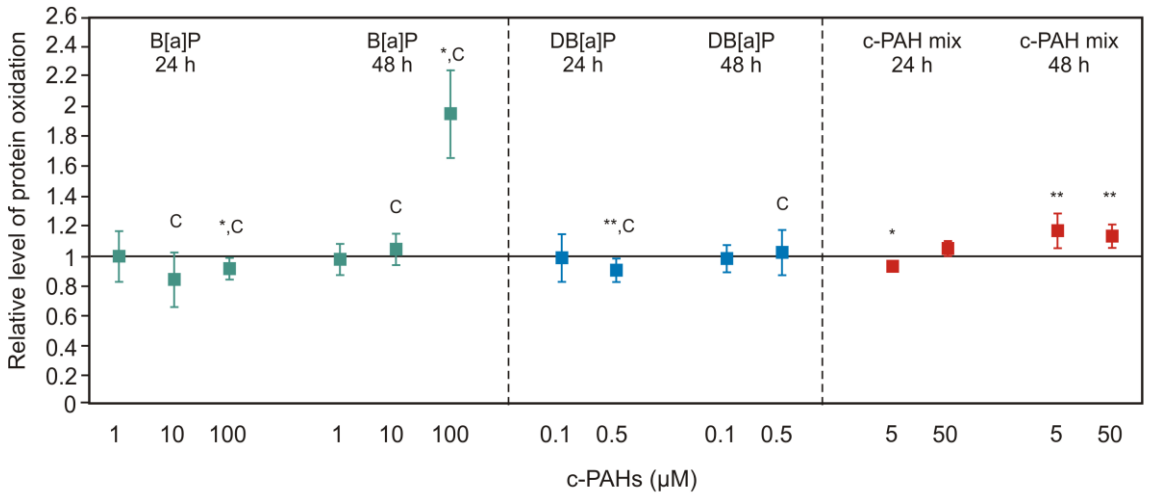
Graph 8. 15-F_{2t}-IsoP levels in HEL cells after treatment with EOM for 24 or 48 h

Mean relative levels of lipid peroxidation ± standard deviation; the solid line indicates the basal level of lipid peroxidation in the control.
The significant differences between lipid peroxidation in the experimental samples compared to the control are indicated by asterisks; significance level: $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).
"C" denotes a dose of a compound that increases the release of lactate dehydrogenase by more than 20% compared to the control sample.
EOM – organic extract from respirable air particles.

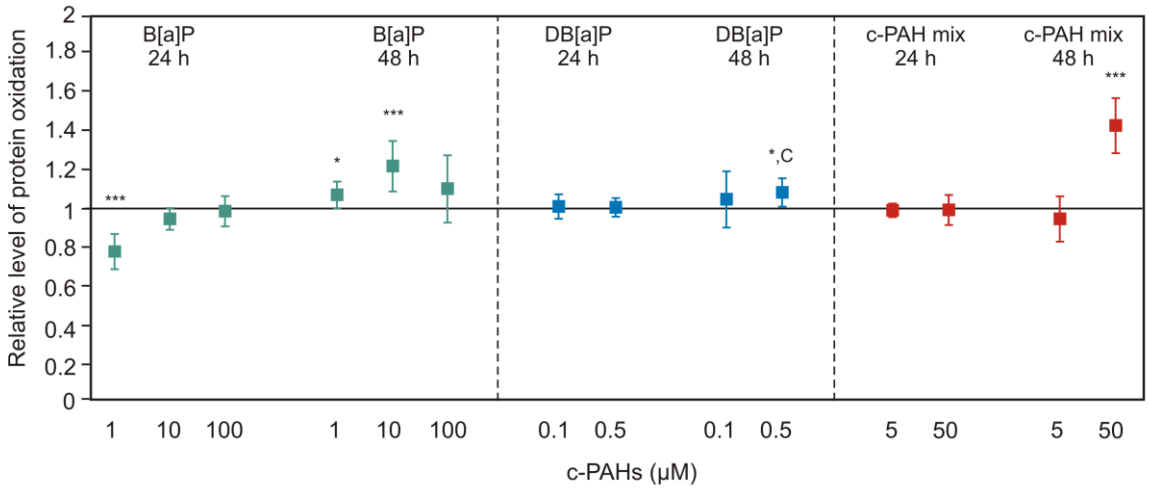
4.2.3 Protein oxidation

The effect of individual c-PAHs on protein oxidation in HepG2 cells was not consistent. Generally, there was no effect after 24 h incubation. Slightly decreased levels of protein oxidation were observed for B[*a*]P and DB[*a,l*]P at cytotoxic concentrations (Graph 9) after 24 h treatment. Increased protein carbonyl levels were found for the highest concentration of B[*a*]P after 48 h of incubation (Graph 9). An increase in protein oxidation was also observed for both concentrations of the c-PAH mix after 48 h of incubation. The results obtained for HEL cells slightly differed from those seen in HepG2 cells. The 24 h incubation period had generally no effect on protein oxidation. A decrease of the carbonyl levels was observed only with the lowest concentration of B[*a*]P (Graph 10). Protein oxidation in HEL cells was induced after 48 h treatment with B[*a*]P and DB[*a,l*]P and also by the higher tested dose of c-PAH mix (Graph 10).

None of the tested EOM significantly increased carbonyl levels above the controls in either cell line after 24 h of incubation (Graphs 11 and 12). In HepG2 cells, oxidative damage to proteins mostly occurred after 48 h treatment with the higher tested concentrations of EOM. The difference between the two incubation periods, however, was not significant except for the highest tested concentrations of EOM from summer 2000 ($p<0.01$) and winter 2001 ($p<0.05$). For HEL cells, the only significant increase in carbonyl levels following the treatment with EOM was detected for the winter 2005 EOM, the higher tested concentration (25 μg/ml).

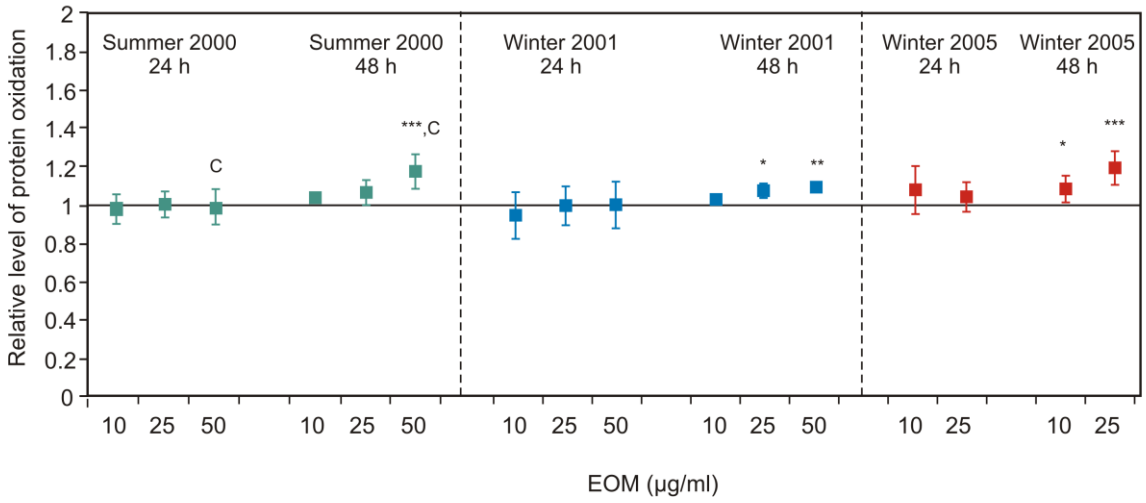


Graph 9. Protein carbonyl levels in HepG2 cells after treatment with B[*a*]P, DB[*a*,*l*]P or the c-PAH mix for 24 or 48 h

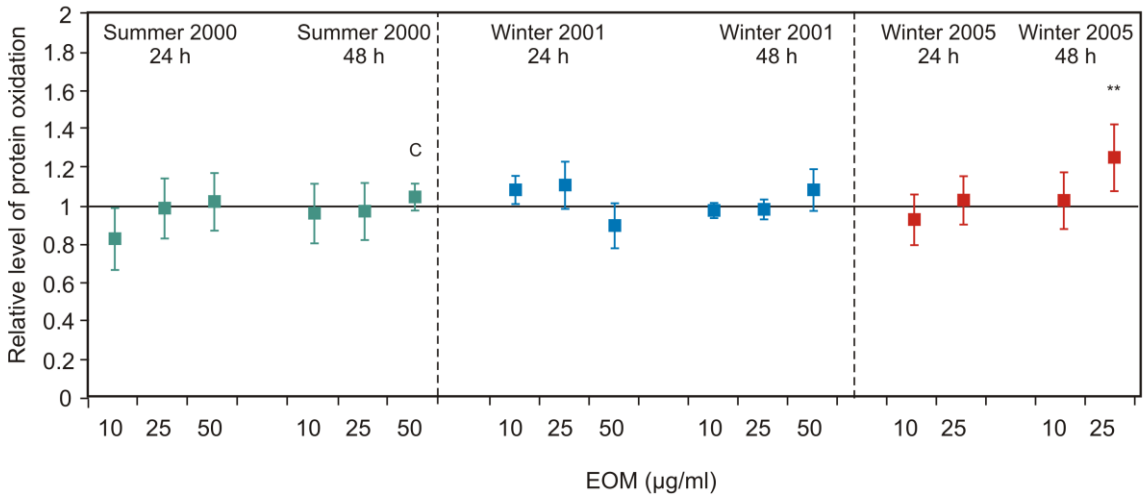


Graph 10. Protein carbonyl levels in HEL cells after treatment with B[*a*]P, DB[*a*,*l*]P or the c-PAH mix for 24 or 48 h

Mean relative levels of protein oxidation ± standard deviation; the solid line indicates the basal level of protein oxidation in the control.
c-PAH mix: values on the x-axis indicate concentrations of B[*a*]P in the mixture; the concentrations of the other c-PAHs were calculated in relation to B[*a*]P content as described in the Materials and methods chapter.
The significant differences between protein oxidation in the experimental samples compared to the control are indicated by asterisks; significance level: $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).
"C" denotes a dose of a compound that increases the release of lactate dehydrogenase by more than 20% compared to the control sample.
B[*a*]P – benzo[*a*]pyrene; DB[*a*,*l*]P – dibenzo[*a*,*l*]pyrene; c-PAH mix – an artificial mixture of c-PAHs
c-PAHs – carcinogenic polycyclic aromatic hydrocarbons.



Graph 11. Protein carbonyl levels in HepG2 cells after treatment with EOM for 24 or 48 h



Graph 12. Protein carbonyl levels in HEL cells after treatment with EOM for 24 or 48 h

Mean relative levels of protein oxidation ± standard deviation; the solid line indicates the basal level of protein oxidation in the control.
The significant differences between protein oxidation in the experimental samples compared to the control are indicated by asterisks; significance level: $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).
"C" denotes a dose of a compound that increases the release of lactate dehydrogenase by more than 20% compared to the control sample.
EOM – organic extract from respirable air particles.

5 DISCUSSION

B[*a*]P as well as DB[*a,l*]P are known carcinogens (IARC, in preparation). However, the exact mechanism of their carcinogenic potency is still under investigation. The major pathway responsible for carcinogenicity of these two compounds was thought to be via their bioactivation to the diol-epoxide metabolic intermediates and DNA adducts formation (THAKKER *et al.* 1976, YANG *et al.* 1976, RALSTON *et al.* 1994, 1995, NESNOW *et al.* 1997, 1998a, XUE and WARSHAWSKY 2005). However, there is growing evidence that during metabolic activation, c-PAHs can also produce various quinone derivatives and subsequently cause the ROS formation (PENNING *et al*. 1996, BURCZYNSKI *et al.* 1998). The formation of ROS and consecutive oxidative damage to DNA is also thought to play an important role in the process of c-PAH-mediated carcinogenesis (TRUSH and KENSLER 1991, WITZ 1991, GUYTON and KENSLER 1993, KLAUNIG and KAMENDULIS 2004).

Exposure to higher levels of PM_{10} or $PM_{2.5}$ is associated with higher risk of lung cancer and mortality from cardiopulmonary diseases (ABBEY *et al.* 1999, POPE *et al*. 2002, BOLDO *et al*. 2006). One hypothesis is that the free radical generating activity of respirable air particles underlies much of their adverse health effects (DONALDSON *et al.* 2003, SHI *et al.* 2003). However, the identification of components which are responsible for oxidative stress induction by PM is the matter of ongoing investigation.

The principal aim of the present master thesis was to determine whether c-PAHs (B[*a*]P, DB[*a,l*]P) alone are able to induce oxidative damage to macromolecules and how this potential ability is affected by the interactions between c-PAHs in the artificial mixture, or by other compounds in EOM prepared by organic extraction from PM. To achieve this goal two individual c-PAHs were selected: B[*a*]P, probably the best characterised and most often studied c-PAH, and DB[*a,l*]P, the most potent c-PAH ever tested (NESNOW *et al*. 1997, 1998a, BINKOVÁ and ŠRÁM 2004). An artificial c-PAH mixture and three different EOM were also employed. The content of c-PAHs (excluding DB[*a,l*]P, which is normally present in the air in non-detectable concentrations) in all tested EOM was analysed in the study of SEVASTYANOVA *et al*. (2008) and is summarised in Materials and methods chapter in Table 1.

The cell lines selected for the experiments are able to activate c-PAHs and form bulky DNA adducts, though the ability to induce DNA adducts was 10 to 100-fold higher in HepG2 than in HEL cells in the study of SEVASTYANOVA *et al*. (2007). Moreover, BURCZYNSKI and PENNING (2000) showed that the activity of CYP enzymes in HepG2 cells was feedback stimulated by *o*-quinones (formed by the action of CYPs and epoxid hydrolase) that act as bifunctional inducers affecting both phase II detoxification and phase I activation enzymes.

This work is probably the first in which the biomarkers of oxidative damage to DNA, lipids and proteins were used along with the combination of different c-PAHs and EOM, two cell lines and two treatment periods. As oxidative stress is a complex process, the simultaneous use of the three biomarkers as well as the employment of different cell lines and treatment periods was proposed to have a better chance of revealing potential differences in the mechanisms of oxidative stress induction by individual c-PAHs, their mixture and EOM.

5.1 c-PAHs

Several *in vitro* studies concerning the induction of oxidative damage by B[*a*]P have been published and their results are summarised below:

GÁBELOVÁ *et al*. (2007) did not find any significant increase of oxidative base lesions (measured by a modified SCGE with Fpg) following exposure of HepG2 cells to B[*a*]P in the concentration range 1-7.5 μM for different treatment periods (2, 24 or 48 h). In the study conducted by PARK *et al*. (2006), B[*a*]P at concentrations 0.1, 1, 4 and 10 μM induced nonspecific DNA damage (measured by the SCGE) after 24 h treatment of HepG2 cells. B[*a*]P-treated cells showed increased MDA formation, but the statistically significant difference was found for 1 μM concentration only. On the other hand all tested concentrations resulted in the increased catalase activity in treated cells, but without any concentration-dependent response. In the A549 cell line, GARCON *et al*. (2001b) tested the effect of B[*a*]P treatment on lipid peroxidation (MDA), antioxidant enzyme activities (SOD, GPx and GR), glutathione status (GSSG/GSH) and alpha-tocopherol consumption. They exposed the cells to 0.05 μM B[*a*]P for 72 h but found no increase of MDA levels. However, exposure to B[*a*]P was accompanied by decreases in the levels of reduced

glutathione (GSH) and alpha-tocopherol. Thus, the authors concluded that the non-enzymatic defences might be active enough to prevent a free radical-induced injury in cells exposed to B[*a*]P. Finally, MAUTHE *et al*. (1995) treated Syrian hamster embryo and human mammary carcinoma (MCF-7) cell lines for 24 h with different concentrations of B[*a*]P up to 20 μM and then either exposed them to fluorescent light or kept them in the dark. Oxidative damage to DNA measured as levels of 8-oxodG by HPLC with electrochemical detection was increased only in cells exposed to fluorescent light. With the exception of increased MDA levels in the study of PARK *et al.* (2006), the results of these *in vitro* studies generally indicate that B[*a*]P did not increase the levels of oxidative damage in treated cells.

In accordance with these observations, the results of this thesis show that B[*a*]P, which is effectively metabolised by HepG2, failed to produce any significant levels of oxidative damage in HepG2 cells in sub-toxic concentration regardless the time of cell exposure. Similar results were obtained for HEL cells, where only significant increase of oxidative damage was found for levels of protein carbonyls after the 48 h incubation.

For DB[*a,l*]P, the results of the treatment of HepG2 cells at the sub-toxic concentration show significantly increased levels of 8-oxodG and 15-F_{2t}-isoprostanes after the 48 h incubation period. Solely the marker of protein oxidation remained at control levels. For HEL cells, the oxidative damage to macromolecules at sub-toxic concentrations of DB[*a,l*]P were not observed for any marker tested. To the best of my knowledge, this study is the only one analysing the effect of DB[*a,l*]P on oxidative stress induction. Thus, the results cannot be compared with observations of other authors.

The treatment of HepG2 cells with c-PAH mix resulted in increased levels of DNA and protein oxidation after 48 h of incubation. HEL cells failed to produce oxidative damage to DNA and lipids. The only increased marker was again the marker of protein oxidation but only for the higher concentration and 48 h incubation.

In general, the results for individual c-PAHs and c-PAH mix show differences between the two cell lines. The markers of oxidative stress often increased above control levels in HepG2 cells, but only after 48 h of incubation, while the overall ability of HEL cells to be oxidised by c-PAHs is very limited. These differences may be due to the different ability of the cell lines to metabolically activate c-PAHs. As already mentioned,

c-PAHs in HepG2 cells induce much higher levels of DNA adducts, which is probably caused by the higher activity of PAH-metabolising enzymes.

In both cell lines, a significant decrease in oxidative damage below the control level was observed for some doses of tested c-PAHs. This effect was particularly observed for lipid peroxidation even at sub-toxic concentrations. I have not found any similar results in scientific literature nor have I found any explanation for this phenomenon. But it shall be mentioned that the number of studies concerning the same issue is rather limited. Since this observation arises from independent experiments, I believe it is not a spurious finding and/or an experimental error.

The ability of the c-PAH mix to induce oxidative damage did not particularly differ from the results obtained for B[*a*]P, suggesting that the presence of other c-PAHs and/or their interactions do not substantially affect oxidative stress.

5.2 EOM

As already mentioned, many studies have investigated the effect of various types of particulate matter on induction of oxidative damage *in vitro*. The results of these studies were affected by the type of particles used and the method of their preparation. Most of them used aqueous suspensions of PM. Generally, the results show that the aqueous suspensions of PM are able to generate oxidative DNA damage: DANIELSEN *et al*. (2008) compared the effect of respirable authentic street particles (ASP) collected in Copenhagen, Denmark and that of standard reference material (SRM) 1650 and SRM 2975 on the induction of strand breaks at Fpg-sensitive sites in A549 cells (measured by a SCGE with Fpg) and 8-oxodG formation in CT DNA (measured by HPLC with electrochemical detection). ASP were obtained from filters by ultrasonication in ultrapure water. The results showed that while all types of particles induced DNA damage in A549 cells, in CT DNA only ASP were able to generate 8-oxodG. The transition metal content was substantially higher in ASP compared to SRM 1650 and 2975 (iron: 37.4 μg/mg, 0.0031 μg/mg and 0.0009 μg/mg; copper: 2.1 μg/mg, 0.020 μg/mg and not detectable, in ASP, SRM 1650 and 2975, respectively) while the PAH content was comparable (40 ng/mg, 47 ng/mg and 29 ng/mg, in ASP, SRM 1650 and 2975, respectively). Thus, the authors concluded that the

observed difference is due to higher levels of transition metals in ASP. SHI *et al.* (2003) also investigated the potential of urban air PM (fine and coarse fractions) to induce oxidative DNA damage to CT DNA and A549 cells. The PM samples were collected during various periods from July to December in Düsseldorf, Germany and subsequently resuspended in water. The induction of 8-oxodG was determined using an immunodotblot assay (CT DNA) and immunohistochemical staining (A549). The results showed that both PM size fractions were able to induce increased levels of 8-oxodG in both systems. PRAHALAD *et al*. (2001) tested the induction of 8-oxodG in free deoxyguanosine, CT DNA and human epithelial cells after the treatment with different types of PM. The levels of induced 8-oxodG (determined by HPLC with electrochemical detection) depended on the type and composition of the particles and the authors concluded that the level of oxidative DNA damage in all systems was dependent on the concentration of the water-soluble content of the PM. GARCON *et al*. (2006) investigated oxidative stress induction following the treatment of human lung epithelial cell line L132 with $PM_{2.5}$ collected in Dunquerque, a French city close to an industrial zone. The cells were incubated with two concentrations of PM suspension (18.84 and 75.36 μg PM/ml) for 24, 48 and 72 h and oxidative stress was determined. As a marker of lipid peroxidation, MDA production was evaluated using HPLC. Formation of 8-oxodG, measured by the 8-oxodG ELISA kit, served as a marker of oxidative damage to DNA. The authors observed concentration- and time-dependent changes in MDA and 8-oxodG levels. It was also noted that both inorganic (metals) as well as organic compounds (including PAHs) were detected in the PM. While the above-mentioned studies were mostly concerning the aqueous PM suspensions or water-soluble fractions of PM, only a couple of studies investigated the potential of organic extracts from PM to induce oxidative damage to macromolecules: GÁBELOVÁ *et al*. (2007) tested eight different EOM to determine their potential to induce oxidative damage to DNA (oxidised bases) in HepG2 cells. The levels of oxidative base lesions were analysed using a modified SCGE with Fpg. The EOM were extracted by dichloromethane from PM_{10} collected at four monitoring sites: Prague – Smíchov, Prague – Libuš, Košice and Sofia in the winter and summer seasons. The HepG2 cells were incubated with different concentrations of individual EOM ranging from 5 to 150 μg/ml for 2 h and then oxidative damage to DNA was determined. Based on preliminary experiments, the 2 h

treatment period was selected, because 24 h and 48 h treatment did not result in increased levels of oxidative DNA damage but lead to cytotoxicity. From all EOM tested, only the one from Košice summer sampling period induced significant increase in oxidative DNA lesions in three (20, 50 and 100 μg/ml) from total six concentrations applied. As authors did not find any consistent effect, they concluded that EOM probably plays only a marginal role in oxidative stress generation. On the contrary, LAZAROVÁ and SLAMEŇOVÁ (2004) found a significant oxidative DNA damage (oxidised bases, measured also by the modified SCGE with Fpg) in HepG2 cells following the 24 h treatment with different concentrations of EOM extracted from PM_{10} collected in Teplice during summer and winter periods. The pre-treatment of the cells with vitamins C and E significantly reduced the oxidative DNA damage caused by EOM exposure. As the c-PAH content in EOM from Teplice from winter and summer seasons was comparable with the c-PAH content in EOM tested by GÁBELOVÁ, the positive effect found by LAZAROVÁ was not probably caused by c-PAHs.

The summary of above-mentioned studies may indicate that the composition of PM, particularly the presence of water-soluble compounds, is a critical parameter affecting the ability of PM to induce oxidative stress. However, the results of this thesis in accordance with the results of LAZAROVÁ and SLAMEŇOVÁ (2004) suggest that some components present in the organic fraction are also capable of causing oxidative damage to DNA, lipids and proteins, specifically in HepG2 cells.

It should be mentioned that the concentration of B[*a*]P (and the other c-PAHs as well) in the EOM used for the treatment, was much lower than the concentration of B[*a*]P alone used for the experiments. The concentrations of B[*a*]P in the 10 μg/ml EOM used for treatment were as follows: summer 2000 – 2 nM B[*a*]P; winter 2001 – 9.3 nM and winter 2005 – 19.5 nM B[*a*]P.

Similar to the results obtained for individual c-PAHs and the c-PAH mix, the induction of oxidative damage to macromolecules in HEL cells was minimal. Unexpectedly, in HEL cells a significant decrease in 15-F_{2t}-IsoP levels for all concentrations and EOM tested was observed. This pattern was similar to that observed for individual c-PAHs and the c-PAH mix, but was even more pronounced. Again, I have not found any explanation for these results supported by the experimental work. I can only

speculate that changes in the redox status of the cell caused by compounds present in PM could induce the reducing environment within the cells, which in turn resulted in the decreased levels of lipid peroxidation. This speculation will be further investigated in future studies.

In HepG2 cells, a significant increase of oxidative damage to DNA and lipids was observed for almost all concentrations and types of EOM and for both treatment periods. Protein oxidation was induced only after 48 h treatment and generally with the higher tested concentrations. This observation might be explained by the fact that carbonyl groups are relatively difficult to induce compared to other products of oxidative stress and thus they may be reflective of more severe oxidative stress (DALLE-DONNE *et al*. 2003).

The c-PAHs content in the three tested EOM was substantially different: the EOM from summer 2000 contained the lowest concentration of c-PAHs while the highest concentration per mg EOM was present in the winter 2005 EOM (Table 1). One could say that in accordance with this data, there are some results with corresponding differences in the levels of oxidative stress. For example, 8-oxodG levels induced by 10 μg/ml EOM after 24 h treatment in HepG2 cells were lowest for the summer 2000 EOM, followed by the winter 2001 and with highest levels found for winter 2005. For 15-F_{2t}-IsoP, this trend was also observed with the higher concentration of EOM (25 μg/ml). However, this effect was restricted only for some markers and treatment periods. It was not found for protein oxidation, or for 48 h treatment periods for either marker. Since there was not a similar response for B[*a*]P alone, or for the c-PAH mix, it is probable that other, unidentified components of EOM were responsible for the induction of oxidative stress in HepG2 cells.

6 SUMMARY

In this master thesis the effect of c-PAHs, their artificial mixture and EOM on oxidative damage to macromolecules in two cell lines was investigated. The most important conclusions are listed below:

- The results demonstrate that EOM from urban air particulate matter posses the ability to induce oxidative damage to DNA, lipids and proteins in HepG2 cells.

- Since B[*a*]P, DB[*a,l*]P and the artificial mixture of c-PAHs had lower ability to cause oxidative damage that was limited only to longer incubation periods, there might be some components of EOM other than c-PAHs responsible for increased levels of 8-oxodG, 15-F_{2t}-IsoP, and protein carbonyls in EOM-treated HepG2 cells.

- The response of the two cell lines to the treatment with EOM is substantially distinct. In HepG2 cells the increase in oxidative damage to macromolecules following the treatment with EOM was observed, in HEL cells this phenomenon was not seen.

- In HepG2 cells, oxidative damage to DNA and lipids was induced already after 24 h of incubation with EOM, while protein oxidation was increased after 48 h treatment. Because the induction of oxidative damage to individual macromolecules may be different, simultaneous analyses of more oxidative stress markers should be performed.

These conclusions demonstrate that the aims of the master thesis have been accomplished.

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