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In Vitro and *In Vivo* Studies on Oxygen Free Radical and DNA Adduct Formation in Rat Lung and Liver during Benzo[a]pyrene Metabolism

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Reactive oxygen species (ROS), possibly produced during the metabolic conversion of benzo(a)pyrene (B[a]P), could be involved in B[a]P-induced genotoxicity and, eventually, carcinogenicity. Therefore, ROS formation by rat lung and liver microsomes was studied *in vitro* by electron spin resonance (ESR/EPR) spectrometry. B[a]P-mediated generation of ROS was detected in incubations with rat lung, but not with liver microsomes. Inhibition of cytochrome P450 (CYP450) by the non isoform-specific inhibitor SKF-525A resulted in a complete inhibition of B[a]P-dependent ROS formation, whereas ROS formation was not affected by inhibition of prostaglandin H synthase by indomethacin. Subsequently, bulky DNA adduct formation and 8-oxo-dG levels after a single oral dose of B[a]P were examined *in vivo* in rat lung and liver, in combination with urinary excretion of 8-oxodG. B[a]P exposure resulted in increased urinary 8-oxo-dG levels. On the contrary, 8-oxo-dG levels decreased in liver and lung after B[a]P exposure. Bulky DNA adducts reached higher levels and were more persistent in rat lung than in liver. These results indicate that ROS are generated during the CYP450 dependent metabolism of B[a]P, particularly in the rat lung, but this does not necessarily result in increased levels of oxidative DNA damage *in vivo*, possibly by induction of DNA repair mechanisms.

Keywords: Benzo[a]pyrene; Reactive oxygen species; ESR; DNA damage; 8-Oxo-dG

Abbreviations: B[a]P, benzo(a)pyrene; CYP450, cytochrome P450; dG, 2′-deoxyguanosine; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DMSO, dimethylsulfoxide; ESR, electron spin resonance; HPLC-ECD, high performance liquid chromatography with electrochemical detection; ROS, reactive oxygen species; 8-oxodG, 7-hydro-8-oxo-2′-deoxyguanosine; PAH, polycyclic aromatic hydrocarbon; POBN, α-(1-oxy-4-pyridyl)-*N*-*tert*-butyl-nitrone

INTRODUCTION

Benzo[a]pyrene (B[a]P) is a well studied polycyclic aromatic hydrocarbon (PAH), which is known to be metabolized by cytochrome P450 (CYP450) to certain reactive diol-epoxides that can interact with DNA.[1] However, other metabolic routes have been described, which might be of biological relevance. For instance, B[a]P may be metabolized by the peroxidative activity of CYP450 or other enzymes (e.g. prostaglandin H synthase) via a one-electron oxidation pathway to a B[a]P-radical cation[2] that may covalently bind to DNA.[3] Additionally, B[a]P radical-cations are thought to be precursors for the labile phenol, 6-OH-B[a]P.[4] Auto-oxidation of this derivative may ultimately result in the formation of B[a]P quinones. B[a]P quinones are almost invariably found after metabolism of B[a]P, sometimes accounting for more than 50% of the total metabolic yield.[5] The B[a]P quinones formed are 6,12-, 1,6- and 3,6-B[a]P dione, which can undergo redox-cycling to their corresponding B[a]P diols and so may produce superoxide[6] (ROS) (Fig. 1) that can be converted into the more reactive hydroxyl radicals via the Haber–Weiss reaction. In particular, hydroxyl free

*Jan van Maanen died unexpectedly on November 5th, 2002. This article is to honor his memory.

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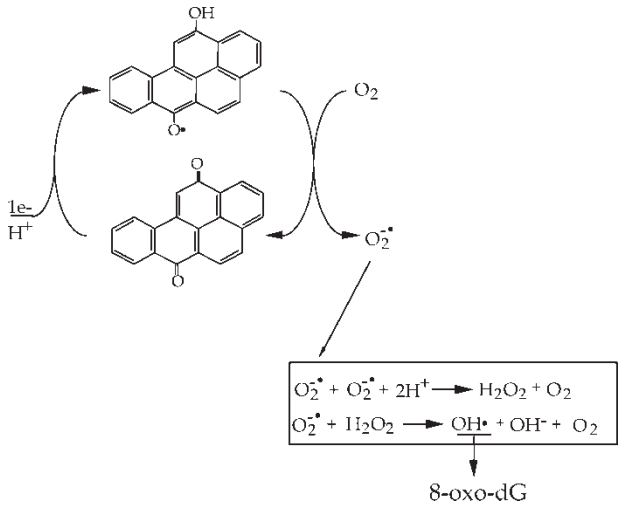

FIGURE 1 Mechanism for the formation of reactive oxygen species (ROS) and 8-oxo-dG by redox-cycling of B[a]P-diones.

radicals preferentially reacts with guanine, exhibiting the lowest ionization potential among nucleic acid components, and may subsequently give rise to oxidative DNA damage including its main product 7-hydro-8-oxo-2′-deoxyguanosine (8-oxo-dG).[7–9] Although it is generally accepted that exposure to B[a]P may lead to ROS and subsequently 8-oxo-dG formation, only few *in vivo* studies have actually addressed this issue. In fact, some recent studies from our group as well as from others showed that in lympho- or leucocytes of humans exposed to PAH's, a reduction of 8-oxo-dG levels were found.[10–12] It was therefore, the aim of this study to further investigate the impact of *in vitro* and *in vivo* B[a]P exposure on parameters of (oxidative) DNA damage. *In vitro*, utilizing rat liver and lung microsomal activation, B[a]P metabolism-dependent formation of oxygen free radical formation was detected by electron spin resonance (ESR or EPR) spectrometry. Also, after oral administration of a single dose of B[a]P to rats, *in vivo* formation of bulky DNA adducts and 8-oxo-dG formation in liver and lung were followed over time.

MATERIALS AND METHODS

Materials

Glass capillaries (100 μl) were purchased from Brand AG (Wertheim, Germany). NADP and Glucose-6-phosphate were obtained from Boehringer Mannheim (Mannheim, Germany). Glucose-6-phosphate dehydrogenase was from Roche (Mannheim, Germany). All other chemicals, including spin traps, were from Sigma (St Louis, MO). Solutions of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) in nitrogen-flushed Milli-Q water were further purified by charcoal treatment. Stock concentrations of DMPO were determined spectrophotometrically by using the extinction coefficient $\epsilon = 7700\,M^{-1}\,cm^{-1}$ at 234 nm. S9 microsomal mixtures were prepared by differential centrifugation using standard procedures,[13] from lung and liver of Lewis rats after induction with phenobarbital/5,6-benzoflavone.

Electron Spin Resonance (ESR) Spectroscopy

ESR spectra were recorded at room temperature in glass capillaries on a Bruker EMX 1273 spectrometer equipped with an ER 4119HS high sensitivity cavity and 12 kW power supply operating at X band frequencies. The modulation frequency of the spectrometer was 100 kHz. Instrumental conditions for the recorded spectra were as follows: magnetic field, 3490 G; scan range, 60 G; modulation amplitude, 1 G; receiver gain, 1×10^5; microwave frequency, 9.85 GHz; power, 50 mW; time constant, 40.96 ms; scan time, 20.97 s; number of scans, 50. Radical formation was followed by performing incubations in duplicate with 10 μM B[a]P in Tris-buffer (40 mM, pH 8.0), 2 mM $MgCl_2$, 100 mM spin trap, either 4.0 or 0.8 mg/ml rat liver or rat lung microsomal fractions in the presence of a NADPH-generating system containing 0.5 mM NADP, 5 mM glucose-6-phosphate and 0.5 U glucose-6-phosphate dehydrogenase for 1 h at 37°C. In control experiments, B[a]P was replaced by equal amounts of the vehicle DMSO or methanol (1%). Where indicated, SKF-525A (1 mM), indomethacin (0.1 mM), mannitol (315 mM), superoxide dismutase (SOD, 1000 U) or catalase (1000 U) were added. Samples for ESR detection were taken from the incubates at 0, 30 and 60 min after start of the experiment. Quantitation of the spectra (in arbitrary units) was performed by peak height measurements using the WIN-EPR spectrum manipulation program. The rate of radical formation was calculated by performing linear regression of these data over the 60 min time interval.

In Vivo Experiments by Exposure of Rats to B[a]P

Fifteen male Lewis rats (250–350 g bodyweight (bw)) were acutely exposed to a single dose of B[a]P (10 mg B[a]P in tricapryline/kg bw) by oral administration. Rats were housed individually in metabolic cages to collect 24 h urine samples and they were provided with food (diet no. RSM-A, Hope Farms, Woerden, the Netherlands) and water *ad libitum*. Rats were killed at 1, 2, 4, 11 and 21 days (3 rats per time-point) after the treatment to study the time-course of formation of 8-oxo-dG and B[a]P-DNA adducts. Three control rats received vehicle only (tricapryline) and were killed after 1, 2 and 4 days, respectively after treatment. Liver, lung and heart tissue were removed and frozen at −20°C until DNA isolation.

Tissues were homogenized with a potter (1000 rpm) in 1% SDS/1 mM EDTA and solutions thus obtained were incubated overnight at 37°C with 0.5 mg Proteinase K. After the incubation period, DNA was isolated, in the presence of 8-hydroxyquinoline to prevent artificial 8-oxo-dG, by means of repetitive extraction with phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and chloroform/isoamyl alcohol (24 : 1). Subsequently, DNA was precipitated with two volumes of 100% cold ethanol and 1/30 volume of 3 M sodium acetate (pH 5.3). Precipitated DNA was rinsed with 70% ethanol, dissolved in 2 mM Tris (pH 7.4) and stored at −20°C until analysis. DNA samples were analyzed for the presence of 8-oxo-dG and B[a]P-DNA adducts by ^{32}P-postlabeling as described. Furthermore, 8-oxo-dG excretion in 24 h urine was studied using a competitive ELISA (obtained from the Japan Institute for the Control of Aging, Fukuroi City, Japan)[14,15]. A standard curve was used to calculate the level of 8-oxo-dG in urine samples. Analysis of each sample was performed in triplicate and the mean coefficient of variation was 4.5 ± 1.8%.

Determination of Oxidative DNA Damage (8-oxo-dG)

HPLC-ECD analysis of 8-oxo-dG was done as described earlier.[16] Briefly, after extraction, DNA was digested into deoxyribonucleosides by treatment with nuclease P1 [0.02 U/μl] and alkaline phosphatase [0.014 U/μl]. The digest was then injected into a Gynkotek 480 isocratic pump (Gynkotek, Bremen, Germany) coupled with a Midas injector (Spark Holland, Hendrik Ido Ambacht, the Netherlands) and connected to a Supelcosil TM LC-18S column (250 × 4.6 mm) (Supelco Park, Bellefonte, PA) and an electrochemical detector (Antec, Leiden, the Netherlands). The mobile phase consisted of 10% aqueous methanol containing 94 mM KH_2PO_4, 13 mM K_2HPO_4, 26 mM NaCl and 0.5 mM EDTA. Elution was performed at a flow rate of 1.0 ml/min with a lower detection limit of 40 fmol absolute for 8-oxo-dG, or 1.5 residues/10^6 2′-deoxyguanosine (dG). dG was simultaneously monitored at 260 nm.

Analysis of B[a]P-DNA Adducts by ^{32}P-postlabeling Assay

The ^{32}P-postlabeling assay was performed as described earlier.[17] Briefly, 10 μg of DNA was digested to deoxyribonucleoside 3′-monophosphates using calf spleen phosphodiesterase (2 μg/μl) and micrococcal endonuclease (0.25 U/μl). Half of the digest was treated with NP1 (2.5 μg/μl) and subsequently, labeled with [γ-^{32}P]-ATP in the presence of T4-polynucleotide kinase. Radiolabeled adducted nucleotide biphosphates were separated by two-dimensional chromatography on polyethyleneimine (PEI)-cellulose sheets (Macherey Nagel, Düren, Germany) using the following solvent systems: D1, 1 M NaH_2PO_4, pH 6.5; D2, 8.5 M Urea, 5.3 M Lithiumformate, pH 3.5; D3, 1.2 M Lithiumchloride, 0.5 M Tris, 8.5 M Urea, pH 8.0, and D4, 1.7 M NaH_2PO_4, pH 6.0. To ensure the efficiency of NP1 treatment and ATP excess, an aliquot of the digest was one-dimensionally chromatographed on PEI-cellulose sheet (Merck, Darmstadt, Germany) using a solvent system of 0.12 M NaH_2PO_4, pH 6.8. For quantification purposes, two standards of [^{3}H]BPDE modified [1 adduct per 10^7 and 10^8 unmodified nucleotides] were run parallel in all experiments. Quantification was performed using a phosphor imager (Molecular Dynamics™, Sunnyvale, CA). The detection limit was estimated at 0.1 adduct per 10^8 nucleotides. Nucleotides quantification was done by labeling the remaining half of the digested DNA with [γ-^{32}P]-ATP in the presence of T4-polynucleotide kinase, followed by one-dimensional chromatography on PEI-cellulose sheet using a solvent system of 0.12 M NaH_2PO_4, pH 6.8. Results were expressed as numbers of adducts per 10^5 nucleotides.

Statistics

Statistical significance between the intensities of spin adducts, bulky DNA adduct and 8-oxo-dG levels were calculated by performing the one-sided Mann-Whitney *U* test. *P* values lesser than 0.05 were considered to be statistically relevant.

RESULTS

In Vitro Generation of Oxygen Free Radicals by B[a]P

To explore oxygen radicals formation after rat lung or liver microsomal activation of B[a]P, we made use of ESR spectrometry in combination with spin trapping techniques. At a protein concentration of 4 mg/ml, neither rat liver nor rat lung microsomal B[a]P activation resulted in increased α-(1-oxy-4-pyridyl)-*N*-*tert*-butylnitrone (POBN)-trapped radical formation (Fig. 2). However, when a lower microsomal protein concentration was used (i.e. 0.8 mg/ml), rat liver microsomes showed higher radical formation that was significantly decreased in the presence of B[a]P ($P = 0.043$). In contrast, in presence of rat lung microsomes (0.8 or 2.0 mg/ml), B[a]P significantly ($P = 0.025$ at 0.8 mg/ml) increased POBN-trapped free radical formation. In experiments where the spin trap POBN was replaced by DMPO, a six-line spectrum

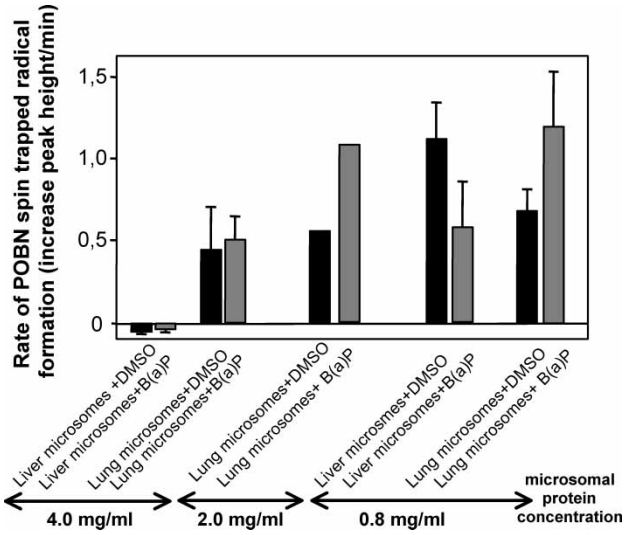

FIGURE 2 Levels of POBN spin-trapped radical formation by NADPH-supported rat liver or rat lung microsomes (protein concentration 4.0, 2.0 or 0.8 mg/ml) in the presence or absence of 10 μM B[a]P. All observations were from at least two experiments except for the rat lung microsomal protein concentration of 2.0 mg/ml. ESR settings were as described in "Materials and Methods".

was detected (Fig. 3). The hyperfine splitting constants in the DMPO ($A_N = 16.2$ G, $A_H = 23.1$ G) and POBN ($A_N = 15.9$ G, $A_H = 2.8$ G) spin-trapped free radical signal revealed that carbon-centered radicals were formed, that most likely will be derived from the reaction of DMSO with oxygen radicals resulting in the formation of methyl radicals. To prove that indeed oxygen radicals were generated during activation of B[a]P by rat lung microsomes, experiments were performed in the presence of scavengers of ROS like mannitol, superoxide dismutase and superoxide dismutase in combination with catalase. For these experiments, B[a]P was dissolved in methanol in order to prevent a rapid reaction (scavenging) of oxygen radicals with DMSO that would compete with

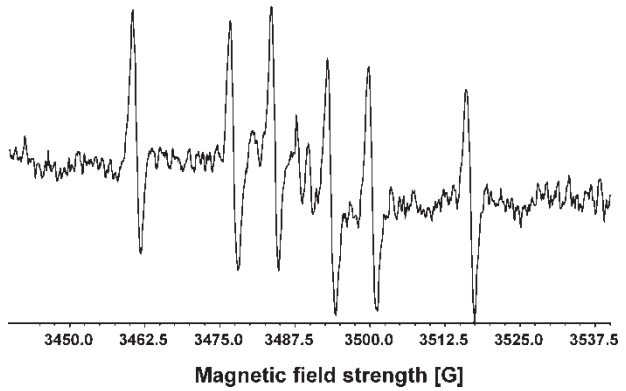

FIGURE 3 A typical example of an ESR spectrum detected after a 60 min incubation of NADPH-supported rat lung microsomes (protein concentration 0.8 mg/ml) in the presence of 10 μM B[a]P and the spin trap DMPO (100 mM). ESR settings were as described in "Materials and Methods" section.

the possible inhibiting effect of scavengers for ROS. All these scavengers inhibited POBN-trapped radical formation (Table I). Furthermore, B[a]P activation mediated ROS formation was almost completely inhibited by the non isoform-specific inhibitor of CYP450, SKF-525A[18] (Table II). No influence on ROS formation was found in the case where SKF-525A was replaced by indomethacin, an inhibitor of prostaglandin H synthase activity.

Oxidative DNA Damage in Rats Exposed to B[a]P

In the control rats killed at day 1, 2 or 4, average 8-oxo-dG levels were respectively 1.5 ± 0.3 per 10^5 dG in liver and 1.2 ± 0.3 in lung DNA. One day after a single oral dose of B[a]P, 8-oxo-dG levels were 0.8 ± 0.3 per 10^5 dG in liver ($P = 0.04$, a significant decrease of 45%) and 1.0 ± 0.1 in lung ($P = 0.12$). 8-Oxo-dG levels further decreased in liver (0.6 ± 0.1 at $t = 11$ days, $P = 0.004$) and lung (0.5 ± 0.2 at $t =$ 11 days, $P = 0.05$, Fig. 4A,B). In contrast, 8-oxo-dG levels in heart tissue was 0.9 ± 0.1 after one day and did not change (i.e. 1.0 ± 0.4 and 1.0 ± 0.2 per 10^5 dG, respectively at $t = 4$ or 11 days after B[a]P exposure). Simultaneously, excretion of 8-oxo-dG in 24 h urine of exposed rats increased from day 1 (1.1 ± 0.4 μg 8-oxo-dG per 24 h) to day 21 (2.0 ± 0.5 μg 8-oxo-dG per 24 h, $P = 0.002$). Average 8-oxo-dG in 24 h urine of control rats that received vehiculum only, was 0.7 ± 0.2 μg 8-oxo-dG per 24 h (Fig. 5).

Bulky DNA Adducts in Rats Exposed to B[a]P

In control rats, no bulky DNA adducts could be detected. In rats exposed to a single dose of B[a]P, bulky DNA adducts predominantly consisting of BPDE-adducts, were detected in liver and lung (Fig. 4C,D). Rat lung total bulky DNA adduct levels as well as BPDE adduct levels were significantly higher at all time-points as compared to this levels in rat liver ($P < 0.01$ for all time-points). The levels in both organs showed a comparable time-course in which the highest levels were detected 2 days after acute oral exposure ($2.7 \pm 0.4 \times 10^{-3}$ per 10^5 dG in rat liver and $6.9 \pm 2.3 \times 10^{-3}$ in lung DNA). However, the decline of DNA adducts after day 2 was more pronounced in rat liver than in rat lung.

DISCUSSION

Metabolic activation of B[a]P by CYP450 results in the formation of reactive metabolites capable of reacting with the encoding bases of DNA, finally resulting in genetic alterations involved in the development of pathologies e.g. carcinogenesis. One metabolic

TABLE I Inhibition of free radical formation by different ROS scavengers in NADPH-supported rat lung microsomes in the presence of B[a]P

Incubation conditions with rat lung microsomes	Rate of radical formation (increase in peak height/min)	Inhibition (%)
Methanol	0.3 ± 0.1	
B[a]P (10 μM)	0.8 ± 0.1	
+ Mannitol (315 mM)		42 ± 1
+ Catalase (1000 U)		49 ± 3
+ Catalase (1000 U) + superoxide dismutase (1000 U)		85 ± 5

Free radical formation was followed by ESR in incubations containing 0 or 10 μM B[a]P in Tris-buffer (40 mM, pH 8.0), $MgCl_2$, (2 mM), POBN (100 mM), rat lung microsomes (protein concentration 0.8 mg/ml), NADP (0.5 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (0.5 U) and methanol (1%) for 1 h at 37°C. ESR measurements were performed on samples taken at 0, 30 and 60 min. ESR settings and spectra calculations were performed as described in "Materials and Methods" section. Inhibition of oxygen free radical formation by different ROS scavengers was expressed as percentage of total oxygen free radical formation.

pathway results in the formation of B[a]P-7,8-dihydrodiol-9,10-epoxide (BPDE), a major carcinogen that reacts with DNA into bulky BPDE-DNA adducts. In addition, B[a]P can also undergo one-electron oxidation, and via subsequent reactions, the B[a]P quinones 6,12-, 1,6- and 3,6-B[a]P dione are formed, which can undergo redox-cycling to their corresponding B[a]P diols and so may produce ROS.[6] This includes formation of different types of oxygen radicals that are capable of oxidatively damaging DNA by reacting with its bases. In particular, the most reactive form of this type of radicals, the hydroxyl radical (˙OH), is known to cause chemical modifications to DNA, resulting in the formation of the major oxidative DNA product, 8-oxodG.[7] In previous *in vitro* studies, increased levels of oxidatively damaged bases were observed in cells treated with B[a]P with[19] or without[20] additional exposure to light. However, these observations in cells did not distinguish between direct B[a]P-induced, and thus metabolism independent, and B[a]P metabolism dependent effects.

In order to elucidate the question whether B[a]P metabolism may result in increased ROS formation, we used ESR in combination with spin trapping techniques to detect free radical formation. Activation of B[a]P by rat lung microsomes at 2.0 and 0.8 mg/ml protein concentration resulted in significantly increased spin-trapped free radical formation that was partly inhibited by scavengers of ROS including mannitol (˙OH radical scavenger), catalase (hydrogen peroxide scavenger) and superoxide dismutase (superoxide radical scavenger) in combination with catalase. Thus, in the presence of rat lung microsomal fraction and with ESR spectrometry, we were able to demonstrate that B[a]P activation results in increased formation of different types of ROS, namely hydrogen peroxide, hydroxyl- and superoxide radicals. Additionally, at a higher rat lung microsomal protein concentration (4.0 mg/ml), the increased oxygen free radical formation by B[a]P disappeared. As can be also seen in Fig. 2, spin-trapped radical formation in both rat lung and liver microsomes decreased at this higher microsomal protein concentration, indicating that this disappearance could most probably be attributed to rapid radical scavenging by nucleophillic sites in proteins.

Increased ROS formation via rat lung microsomal activation of B[a]P was completely inhibited by SKF-525A, an inhibitor of different isoforms of CYP450.[18] Other enzymes such as peroxidases and prostaglandin H synthase have been reported to be involved in activation of B[a]P via the one-electron pathway[2] that ultimately results in ROS formation via redox-cycling of B[a]P quinones. This enzymatic activation could be important in organs expressing relatively low levels of CYP450, like lung. However, in our *in vitro* study, inhibition of prostaglandin H synthase by indomethacin did not result in lower ROS formation by B[a]P, so we only found evidence for the involvement of CYP450 in ROS formation by B[a]P after rat lung microsomal activation. Furthermore, B[a]P activation by rat liver microsomes resulted in decreased spin-trapped

TABLE II Effect of enzyme inhibitors on B[a]P-dependent oxygen free radical formation in NADPH-supported rat lung microsomes

Incubation conditions with rat lung microsomes	Rate of radical formation (increase in peak height/min)	Inhibition (%)
DMSO	0.7 ± 0.1	
B[a]P (10 μM)	1.3 ± 0.3	
+ SKF-525A (1 mM)		85 ± 14
+ Indomethacin (0.1 mM)		0 ± 20

Free radical formation was followed by ESR in incubations containing 0 or 10 μM B[a]P in Tris-buffer (40 mM, pH 8.0), $MgCl_2$, (2 mM), POBN (100 mM), rat lung microsomes (protein concentration 0.8 mg/ml), NADP (0.5 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (0.5 U) and DMSO (1%) for 1 h at 37°C. ESR measurements were performed on samples taken at 0, 30 and 60 min. ESR settings and spectra calculations were performed as described in "Materials and Methods" section. Inhibition of oxygen free radical formation was expressed as percentage of B[a]P-dependent free radical formation.

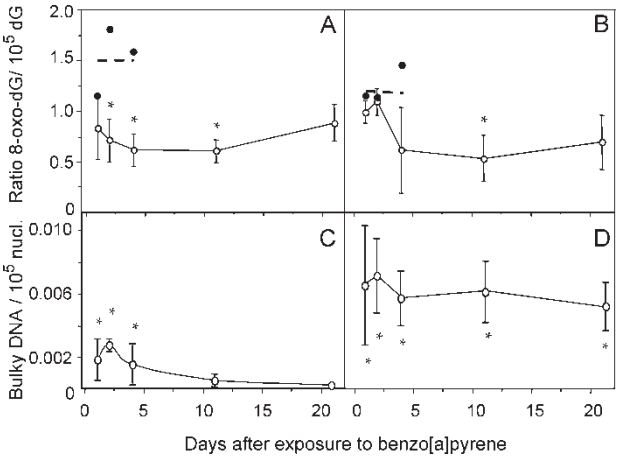

FIGURE 4 Levels of 8-oxo-dG (per 10^5 dG) in rat liver (A) and lung (B), and bulky DNA adducts (per 10^5 nucleotides) in rat liver (C) or lung (D) at several time points after a single oral dose of B[a]P (10 mg/ kg body weight). Black dots indicate 8-oxodG levels in control rats treated with tricapryline (vehiculum for B[a]P) only. Dotted line is the average 8-oxo-dG level in control rats. No bulky DNA adducts were measured in liver and lung of control rats. * $P < 0.05$ as compared with controls.

radical formation. The only reasonable explanation at this moment, is contribution of the previously reported rat liver microsomal metabolic conversion of the methyl radical adduct into ESR silent forms.[21] In conclusion, combining our *in vitro* results, activation of B[a]P by lung microsomes resulted in the ESR detectable formation of ROS, whereas rat liver microsomes did not. The question, to what extent ROS formation is able to damage DNA or other biological macromolecules, like proteins or lipids, remains unanswered. Interestingly, in a recent paper, it is shown that B[a]P treatment increased lipid peroxidation and lowered antioxidant capacity in the lung of a mouse model of lung cancer.[22]

To further address the issue of B[a]P-induced DNA damage *in vivo*, we performed a study in which rats were acutely exposed to an oral dose of B[a]P. The formation of bulky DNA adduct and 8-oxodG levels in rat liver and lung were followed over time together with the urinary 8-oxo-dG excretion. Kim *et al.*[23] reported in an *in vivo* study that 8-oxo-dG levels were significantly increased in liver DNA of female Sprague–Dawley rats at 6–12 h after oral exposure to B[a]P. Subsequently, 8-oxo-dG levels were reduced to baseline levels within 24–96 h, indicating that 8-oxo-dG is rapidly removed from liver DNA. However, in their experiment, 8-oxo-dG levels did not fall below background levels as observed in the present study in Lewis rats. Some major differences in experimental conditions may explain this discrepancy. The most important one being the dose of B[a]P, which was 7.5 times higher in their experiment than in the present study. This may result in other metabolic routes and/or higher levels of ROS generation. Furthermore, in the present work, male rats were used which may differ in DNA-repair capacity as compared to female rats.[24]

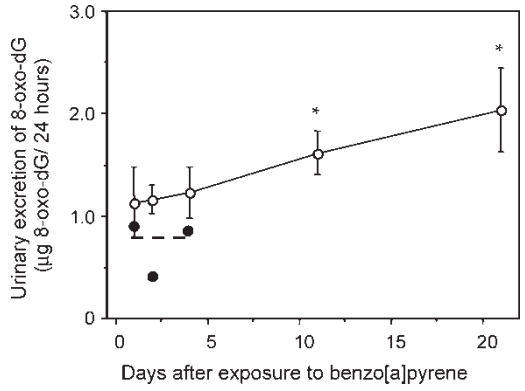

FIGURE 5 Total urinary excretion of 8-oxo-dG per 24 h (µg 8-oxo-dG/24 h) after acute oral exposure to B[a]P. Black dots indicate the total urinary excretion of 8-oxo-dG per 24 h levels in control rats treated with tricapryline (vehiculum for B[a]P) only. Dotted line is the average total urinary excretion of 8-oxo-dG per 24 h level in control rats. * $P < 0.05$ as compared with controls.

8-Oxo-dG levels in tissues are the result of the balance between the rates of damage and repair.[25] DNA repair enzymes remove 8-oxo-dG from DNA, which is subsequently excreted in urine. In the present study, higher urinary levels of 8-oxo-dG were observed in B[a]P-exposed rats as compared to controls, whereas in the organs a decrease was observed. Changes in tissue levels of 8-oxo-dG were in agreement with the reported ranking order of DNA repair activities in different organs,[26] namely liver, greater than lung. This organ-specific difference in DNA repair is also reflected in the higher and sustained level of B[a]P-DNA adduct level in the rat lung compared to liver. Although our *in vitro* experiments with rat lung microsomes in combination with ESR spectroscopy clearly showed that in this organ, ROS will be formed during the metabolic conversion of B[a]P, it seems that *in vivo* the balance was in favor of removal of 8-oxo-dG rather than its formation. The maximal B[a]P-DNA adduct levels in rat lung and liver is found in both organs at 2 days after exposure, but this level is higher in rat lung. This, as well as the much faster disappearance of these adducts in liver compared to lung initiated after day 2, were also in line with the reported higher tissue specific DNA repair rates in the liver.[26] In rat liver, lowering in B[a]P-DNA adduct and 8-oxo-dG levels followed a similar pattern, while in rat lung B[a]P-DNA adduct levels remains increased over 21 days, but 8-oxo-dG levels decreased much faster. As indicated by our *in vitro* ESR experiments, ROS formation by B[a]P metabolism in the rat lung is much higher than that in rat liver, and this probably also induces much faster 8-oxo-dG repair. Also, we cannot exclude that *in vivo* B[a]P induced additional processes including up regulation of different

enzymes activities (e.g. CYP's, peroxidases) resulting in additive oxidative stress.

The removal of 8-oxo-dG from lung and liver might be due to DNA repair mechanisms, which were primarily induced to remove B[a]P-DNA adducts, but may simultaneously repair endogenously formed 8-oxo-dG. Bulky DNA adducts are repaired by nucleotide excision repair (NER), which has also been implicated in the removal of oxidative DNA damage. NER removes bulky DNA adducts with a half life time of about 24 h.[27] Base excision repair (BER) is much faster with a half life time of about 1 h[28] and is considered to be the main pathway for the removal of 8-oxo-dG, but NER can be considered as a back-up system.[29] By taking our first time point at 24 h after B[a]P exposure, we mainly focused on changes in DNA adduct levels induced by NER including the simultaneous removal of 8-oxodG during bulky adduct repair in rat liver and lung. Also, increased levels of 8-oxo-dG were detected in urine suggesting repair by NER, because BER (i.e. specific DNA repair glycosylases) will release 8-oxo-guanine rather than 8-oxo-dG. However, the antibodies which were used to assess urinary 8-oxo-dG may cross-react with 8-oxo-like structures and therefore, this assay is presently too unspecific to make firm conclusions on repair pathways.[30]

Although our ESR data may indicate otherwise, levels of oxidative DNA damage decreased in lung and liver after acute exposure to B[a]P, whereas the urinary excretion of 8-oxo-dG was found to be increased. Our *in vitro* results indicate that especially in rat lung, exposure to B[a]P favors ROS formation. However, this will not automatically result in increased levels of 8-oxo-dG, because the removal of DNA oxidation products also seems to be induced. Increased removal could probably not only account for our results in rats after B[a]P exposure, but also for the reduced 8-oxodG levels found in humans after PAH exposure.[10–12] But the question, to what extent the B[a]P metabolism-induced increased ROS formation in the lung will participate in pathologies as carcinogenesis e.g. lung cancer development, remains unanswered.

References

[1] Hall, M. and Grover, P.L. (1990) "Polycyclic aromatic hydrocarbons: metabolism activation and tumour initiation", In: Cooper, C.S. and Grover, P.L., eds, *Chemical Carcinogenesis and Mutagenesis* (Raven Press, New York) Vol. I, pp 327–372.

[2] Cavalieri, E.L., Devanesan, P.D. and Rogan, E.G. (1988) "Radical cations in the horseradish peroxidase and prostaglandin H synthase mediated metabolism and binding of benzo[a]pyrene to deoxyribonucleic acid", *Biochem. Pharmacol.* **37**, 2183–2187.

[3] Rogan, E.H., Cavalieri, E.L., Tibbels, S.R., Cremonesi, P., Warner, C.D., Nagel, D.J., Tomer, K.B., Cerny, R.L. and Gross, M.L. (1988) "Synthesis and identification of benzo(a)pyrene-guanine nucleoside adducts formed by electrochemical oxidation and by horseradish peroxidase catalyzed reaction of benzo(a)pyrene with DNA", *J. Am. Chem. Soc.* **110**, 4023–4029.

[4] Cavalieri, E.L., Rogan, E.G., Cremonesi, P. and Devanesan, P.D. (1988) "Radical cations as precursors in the metabolic formation of quinones from benzo[a]pyrene and 6-fluorobenzo[a]pyrene. Fluoro substitution as a probe for one-electron oxidation in aromatic substrates", *Biochem. Pharmacol.* **37**, 2173–2182.

[5] Wang, I., Rasmussen, R. and Crocker, T. (1974) "Metabolism of benzo(a)pyrene by microsomes from tissue of pregnant and fetal hamsters", *Life Sci.* **15**, 1291–1300.

[6] Lourentzen, R.J. and Tso, P.O.P. (1977) "Benzo(a)pyrene-dione/benzo(a)pyrenediol oxidation reduction couples and the generation of reactive reduced molecular oxygen", *Biochemistry* **16**, 1467–1473.

[7] Chatgilialoglu, C. and O'Neill, P. (2001) "Free radicals associated with DNA damage", *Exp. Geront.* **36**, 1459–1471.

[8] Cadet, J., Douki, T., Gasparutto, D. and Ravanat, J-L. (2003) "Oxidative damage to DNA: formation, measurement and biochemical futures", *Mutat. Res.* **531**, 5–23.

[9] Martinez, G.R., Loureiro, A.P.M., Marques, S.A., Miyamoto, S., Yamaguchi, L.F., Onuki, J., Almeida, E.A., Garcia, C.C.M., Barbosa, L.F., Medeiros, M.H.G. and Di Mascio, P. (2003) "Oxidative and alkylating damage in DNA", *Mutat. Res.* **544**, 115–127.

[10] Toraaso, M., Hayden, C., Marlow, D., Rinehart, R., Mathias, P., Werren, D., DeBord, D.G. and Reid, T.M. (2001) "DNA strand breaks, oxidative damage, and 1-OH pyrene in roofers with coal-tar pitch dust and/or asphalt fume exposure", *Int. Arch. Occup. Environ. Health* **74**, 396–404.

[11] Van Zeeland, A.A., de Groot, A.J., Hall, J. and Donato, F. (1999) "8-Hydroxydeoxyguanosine in DNA from leukocytes of healthy adults: relationship with cigarette smoking, environmental tobacco smoke, alcohol and coffee consumption", *Mutat. Res.* **439**, 249–257.

[12] Nia, A.B., van Schooten, F.J., Schilderman, P.A.E.L., de Kok, T.M.C.M., Haenen, G.R., van Herwijnen, M.H., van Agen, E., Pachen, D. and Kleinjans, J.C.S. (2001) "A multi-biomarker approach to study the effects of smoking on oxidative DNA damage and repair and antioxidative defense mechanisms", *Carcinogenesis* **22**, 395–401.

[13] Schramm, H., Robertson, L.W. and Oesch, F. (1985) "Differential regulation of hepatic glutathione transferase and glutathione peroxidase activities in the rat", *Biochem. Pharmacol.* **34**, 3735–3739.

[14] Erhola, M., Toyokuni, S., Okada, K., Tanaka, T., Hiai, H., Ochi, H., Uchida, K. and Osawa, T. (1997) "Biomarker evidence of DNA oxidation in lung cancer patients: association of urinary 8-hydroxy-2′-deoxyguanosine excretion with radiotherapy, chemotherapy, and response to treatment", *FEBS Lett.* **409**, 287–291.

[15] Tsuboi, H., Kouda, K., Takeuchi, H., Takigawa, M., Masamoto, Y., Tackeuchi, M. and Ochi, H. (1998) "8-Hydroxydeoxyguanosine in urine as an index of oxidative damage to DNA in the evaluation of atopic dermatitis", *Br. J. Dermatol.* **138**, 1033–1035.

[16] De Kok, T.M.C.M., Ten Vaarwerk, F.J., Zwingman, I., van Maanen, J.M.S. and Kleinjans, J.C.S. (1994) "Peroxidation of linoleic, arachidonic and oleic acid in relation to the induction oxidative DNA damage and cytogenetic effects", *Carcinogenesis* **15**, 1399–1404.

[17] Nia, A.B., Maas, L.M., van Breda, S.G., Curfs, D.M., Kleinjans, J.C.S., Wouters, E.F.M. and van Schooten, F.J. (2000) "Applicability of induced sputum for molecular dosimetry of exposure to inhalatory carcinogens: ^{32}P-postlabeling of lipophilic DNA adducts in smokers and nonsmokers", *Cancer Epidem. Biomar. Prev.* **9**, 367–372.

[18] Ono, S., Hatanaka, T., Hotta, H., Satoh, T., Gonzales, F.J. and Tsutsui, M. (1996) "Specificity of substrate and inhibitor probes for cytochrome P450s: evaluation of *in vitro* metabolism using cDNA-expressed human P450s and human liver microsomes", *Xenobiotica* **26**, 681–693.

[19] Mauthe, R.J., Cook, V.M., Coffing, S.L. and Baird, W.M. (1995) "Exposure of mammalian cell cultures to benzo[a]pyrene and light results in oxidative DNA damage as measured by

8-hydroxydeoxyguanosine formation", *Carcinogenesis* **16**, 133–137.
[20] Leadon, S., Stampfer, M.R. and Bartley, J. (1988) "Production of oxidative DNA damage during the metabolic activation of benzo[a]pyrene in human mammary epithelial cells correlates with cell killing", *Proc. Natl Acad. Sci. USA* **85**, 4365–4368.
[21] Novakov, C.P. and Stoyanovsky, D.A. (2002) "Comparative metabolism of *N-tert*-butyl-N-[1-(1-oxy-pyridin-4-yl)-ethyl]- and *N-tert*-butyl-N-(1-phenyl-ethyl)-nitroxide by the cytochrome P450 monooxygenase system", *Chem. Res. Toxicol.* **15**, 749–753.
[22] Selvendiran, K., Prince Vijeya Singh, J., Baba Krishnan, K. and Sakthisekaran, D. (2003) "Cytoprotective effect of piperine against benzo[a]pyrene induced lung cancer with reference to lipid peroxidation and antioxidant system in Swiss albino mice", *Fitoterapia* **74**, 109–115.
[23] Kim, K.B. and Lee, B.M. (1997) "Oxidative stress to DNA, protein, and antioxidant enzymes (superoxide dismutase and catalase) in rats treated with benzo(a)pyrene", *Cancer Lett.* **113**, 205–212.
[24] Shugart, L. and Kao, J. (1985) "Examination of adduct formation *in vivo* in the mouse between benzo(a)pyrene and DNA of skin and hemoglobin of red blood cells", *Environ. Health Perspect.* **62**, 223–226.
[25] Loft, S., Vistisen, K., Ewerzt, M., Tjonneland, A., Overvad, K. and Poulsen, H.E. (1992) "Oxidative DNA damage estimated by 8-hydroxydeoxyguanosine excretion in humans: influence of smoking, gender and body mass index", *Carcinogenesis* **13**, 2241–2247.
[26] De Flora, S., Izzotti, A., Randerath, K., Randerath, E., Bartsch, H., Nair, J., Balansky, R., van Schooten, F.J., Degan, P., Fronza, G., Walsh, D. and Lewtas, J. (1996) "DNA adducts and chronic degenerative disease. Pathogenetic relevance and implications in preventive medicine", *Mutat. Res.* **366**, 197–238.
[27] Stierum, R.H., van Herwijnen, M.H.M., Maas, L.M., Hageman, G.J. and Kleinjans, J.C.S. (1994) "Measurement by ^{32}P-postlabeling of (±)anti-benzo[*a*]pyrene-diolepoxide-N^2-deoxyguonosine adduct persistence in unstimulated human peripheral blood lymphocytes", *Mutat. Res.* **325**, 31–37.
[28] Jaruga, P. and Dizdaroglu, M. (1996) "Repair of products of oxidative DNA base damage in human cells", *Nucl. Acids Res.* **24**, 1389–1394.
[29] De Boer, J. and Hoeijmakers, J.H. (2000) "Nucleotide excision repair and human syndromes", *Carcinogenesis* **21**, 453–460.
[30] Priemé, H., Loft, S., Cutler, R.G. and Poulsen, H.E. (1996) "Measurement of oxidative DNA injury in humans: evaluation of a commercially available ELISA assay", In: Kumpulainen, J.T. and Salonen, J.T., eds, *Natural Antioxidants and Food Quality in Atherosclerosis and Cancer Prevention* (The Royal Society of Chemistry, London), pp 78–82.