Activation and detoxification metabolism of urban air pollutants 2-nitrobenzanthrone and carcinogenic 3-nitrobenzanthrone by rat and mouse hepatic microsomes

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Abstract

OBJECTIVES: 2-Nitrobenzanthrone (2-NBA) has recently been detected in ambient air particulate matter. Its isomer 3-nitrobenzanthrone (3-NBA) is a potent mutagen and suspected human carcinogen identified in diesel exhaust. Understanding which enzymes are involved in metabolism of these toxicants is important in the assessment of individual susceptibility. Here, metabolism of 2-NBA and 3-NBA by rat and mouse hepatic microsomes containing cytochromes P450 (CYPs), their reductase (NADPH:CYP reductase), and NADH:cytochrome b_5 reductase was investigated under anaerobic and aerobic conditions. In addition, using the same microsomal systems, 2-NBA and 3-NBA were evaluated to be enzymatically activated under anaerobic conditions to species generating 2-NBA- and 3-NBA-derived DNA adducts. **METHODS:** High performance liquid chromatography (HPLC) with ultraviolet (UV) detection was employed for the separation and characterization of 2-NBA and 3-NBA metabolites formed by hepatic microsomes of rats and mice under the anaerobic and aerobic conditions. Microsomal systems isolated from the liver of the control (untreated) rats and rats pretreated with Sudan I, β-naphthoflavone (β-NF), phenobarbital (PB), ethanol and pregnenolon 16α-carbonitrile (PCN), the inducers of cytochromes P450 (CYP) 1A1, 1A1/2, 2B, 2E1 and 3A, respectively, were used in this study. Microsomes of mouse models, a control mouse line (wild-type, WT) and Hepatic Cytochrome P450 Reductase Null (HRN) mice with deleted gene of NADPH:CYP reductase in the liver, thus absenting this enzyme in their livers, were also employed. To detect and quantify the 2-NBA- and 3-NBA-derived DNA adducts, the ^{32}P postlabeling technique was used.

RESULTS: Both reductive metabolite of 3-NBA, 3-aminobenzanthrone (3-ABA), found to be formed predominantly under the anaerobic conditions, and two 3-NBA oxidative metabolites, whose structures have not yet been investigated, were formed by several microsomal systems used in the study. Whereas a 3-NBA reductive metabolite, 3-ABA, was found only in the microsomal systems of control rats, the rats treated with β-NF and PB, and microsomes of WT and HRN mice, all hepatic microsomes tested in the study were capable of activating this carcinogen under the reductive conditions to form DNA adducts. A stability of a reactive intermediate of 3-NBA, *N*-hydroxy-3-aminobenzanthrone that is formed during 3-NBA reduction to 3-ABA, to form nitrenium (and/or carbenium) ions binding to DNA in individual microsomes as well as binding of these ions to proteins of these microsomes, might be the reasons explaining this phenomenon. In contrast to 3-NBA, its isomer 2-NBA was not metabolized by any of the used enzymatic systems both under the anaerobic and aerobic conditions. Likewise, no DNA adducts were detectable after reaction of 2-NBA in these systems with DNA.

CONCLUSIONS: The results found in this study, the first report on the metabolism of 2-NBA and 3-NBA by rat and mouse hepatic microsomes demonstrate that 3-NBA, in contrast to 2-NBA, is reductively activated to form 3-NBA-derived DNA adducts by these enzymatic systems. NADPH:CYP reductase can be responsible for formation of these DNA adducts in rat livers, while NADH:cytochrome b_5 reductase can contribute to this process in livers of HRN mice.

Abbreviations:

3-ABA	- 3-aminobenzanthrone
2-ABA	- 2-aminobenzanthrone
β-NF	- β-naphthoflavone
CYP	- cytochrome P450
dA-N^6-3-ABA	- 2-(2′-deoxyadenosin-N^6-yl)-3-aminobenzanthrone
dG-N^2-3-ABA	- *N*-(2′-deoxyguanosin-N^2-yl)-3-aminobenzanthrone
dG-C8-*N*-3-ABA	- *N*-(2′-deoxyguanosin-8-yl)-3-aminobenzanthrone
DMSO	- dimethyl sulfoxide
HPLC	- high performance liquid chromatography
HRN mice	- Hepatic cytochrome P450 reductase null mice
N-OH-2-ABA	- *N*-hydroxy-2-aminobenzanthrone
N-OH-3-ABA	- *N*-hydroxy-3-aminobenzanthrone
NADPH	- nicotinamidadeninedinucleotide phosphate (reduced)
2-NBA	- 2-nitrobenzanthrone
3-NBA	- 3-nitrobenzanthrone
NQO1	- NAD(P)H:quinone oxidoreductase 1
PB	- phenobarbital
PCN	- pregnenolon 16α-carbonitrile
r.t.	- retention time
RAL	- relative adduct labeling
TLC	- thin-layer chromatography
UV	- ultraviolet
WT	- wild-type

INTRODUCTION

The increased lung cancer risk after exposure to the environmental pollutants nitro-polycyclic aromatic hydrocarbons and their detection in the lungs of non-smokers with lung cancer has led to considerable interest in assessing their potential cancer risk (IARC 1989; Vineis & Husgafvel-Pursiainen 2005).

The nitroaromatic 3-nitrobenzanthrone (3-nitro-7*H*-benz[*de*]anthracen-7-one, 3-NBA, Figure 1) occurs in diesel exhaust and in airborne particulate matter (Enya *et al.* 1997; Seidel *et al.* 2002; Arlt 2005; Nagy *et al.* 2005). 3-NBA exhibits extremely high mutagenic activity (Enya *et al.* 1997; Arlt 2005) and is also a genotoxic carcinogen causing lung tumors in rats (Enya *et al.* 1997; Nagy *et al.* 2005). The predominant DNA adducts formed by 3-NBA after its metabolic activation by reduction of the nitro group (Arlt *et al.* 2003, 2005; Stiborova *et al.* 2006, 2008) are 2-(2'-deoxyguanosin-N^2-yl)-3-aminobenzanthrone and *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (Arlt *et al.* 2004a, 2004b, 2006; Bieler *et al.* 2005; Vom Brocke *et al.* 2009) and these are most probably responsible for the G to T transversion mutations induced by 3-NBA in Muta Mouse (Arlt *et al.* 2004c) and in *TP53* using human *TP53* knock-in (Hupki) murine embryonic fibroblasts (Vom Brocke, *et al.* 2009). The metabolic activation of 3-NBA is mediated primarily by cytosolic NAD(P)H:quinone oxidoreductase 1 (NQO1), while *N,O*-acetyltransferases (NATs) and sulfotransferases (SULTs) are the major activating phase II enzymes (Arlt *et al.* 2005, Stiborova *et al.* 2006, 2008) (Figure 1).

2-Nitrobenzanthrone (2-NBA), an isomer of 3-NBA, has been detected in ambient air particulate matter (Phousongphouang & Arey 2003). Using a semiempirical quantum mechanical approach recent studies indicated small differences between 2-NBA and 3-NBA in the reduction potential and the geometry of the nitro group attached to the benzanthrone skeleton (Takamura-Enya *et al.* 2006; Arlt *et al.* 2007; Reynisson *et al.* 2008; Nagy *et al.* 2007). Only differences in hydrophobicity were observed between these NBA isomers, indicating that penetration through cell membranes seems not to be identical for both compounds (Takamura-Enya *et al.* 2006; Arlt *et al.* 2007; Stiborova *et al.* 2010b). The mutagenic and genotoxic potential of 2-NBA is, however, much lower than that of 3-NBA (Takamura-Enya *et al.* 2006; Arlt *et al.* 2007). Although 2-NBA has been shown to be genotoxic *in vitro* (Arlt *et al.* 2007; Nagy *et al.* 2007), lack of genotoxicity *in vivo* in rats (*e.g.* DNA adduct formation) was reported (Arlt *et al.* 2007). Nevertheless, its higher abundance than 3-NBA in ambient air urges further investigation to assess its potential hazard to human health.

Recently, we have compared the efficiencies of human enzymatic systems such as hepatic cytosols and microsomes, NAD(P)H:quinone oxidoreductase 1 (NQO1), xanthine oxidase, NADPH:CYP reductase, *N,O*-acetyltransferases and sulfotransferases) and human primary hepatocytes to activate 2-NBA and its isomer 3-NBA to species forming DNA adducts (Stiborova *et al.* 2010b). We have found that in contrast to 3-NBA, 2-NBA was not metabolized at detectable levels

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by the tested human enzymatic systems and enzymes expressed in human hepatocytes and no DNA adducts were generated by 2-NBA. Therefore, we have also concluded that 2-NBA seems to possess a relatively lower risk to humans than 3-NBA (Stiborova *et al.* 2010b).

The aim of the present study was to investigate whether both studied environmental pollutants can be metabolized by other enzymatic systems, namely by hepatic microsomes of two animal models, rat and mice. Both reductive metabolism carried out under the anaerobic conditions and oxidative (aerobic) metabolism were examined. In addition, using the same microsomal systems, 2-NBA and 3-NBA were evaluated to be enzymatically activated under the anaerobic conditions to species generating 2-NBA- and 3-NBA-derived DNA adducts.

MATERIAL AND METHODS

Chemicals and enzymes

Microsomes from rat livers were isolated and characterized for CYP activities as described (Stiborova *et al.* 2010a, 2011). NADPH, calf thymus DNA and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical Co (St Louis, MO, USA), Enzymes and chemicals for the ^{32}P-postlabeling assay were obtained from sources described (Arlt *et al.* 2004a, 2004b, 2004c, Phillips & Arlt 2007). All these and other chemicals were reagent grade or better.

Synthesis of 2-NBA, 3-NBA, 3-ABA and N-hydroxy-3-aminobenzanthrone (N-OH-3-ABA)

2-NBA was synthesized as reported (Suzuki *et al.* 1997). 3-NBA, 3-ABA and *N*-OH-3-ABA were synthesized as described (Arlt *et al.* 2003, 2004b, Osborne *et al.* 2005).

Their authenticity was confirmed by UV spectroscopy, electrospray mass spectrometry and high field ^{1}H NMR spectroscopy. Their purity was analyzed using HPLC, being >99.9% based on this method (Arlt *et al.* 2007).

Preparation of microsomes and assays

The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which complies with the Declaration of Helsinki. Microsomes from livers of ten male untreated Wistar rats and those of ten male rats pre-treated with Sudan I, β-naphthoflavone (β-NF), phenobarbital (PB), ethanol and pregnenolone-16α-carbonitrile (PCN) were prepared by the procedure described previously (Stiborova *et al.* 2003, 2011, Naiman *et al.* 2010). Protein concentrations in the microsomal fractions were assessed using the bicinchoninic acid protein assay with the bovine serum albumin as a standard (Weichelman *et al.* 1988). The concentration of CYP was estimated according to Omura and Sato (Omura & Sato 1964) based on absorption of the complex of reduced CYP with carbon monoxide. Hepatic microsomes of control (uninduced) rats and rats induced with Sudan I, β-NF, phenolbarbital (PB), ethanol and PCN contained 0.6, 1.8, 1.3, 2.7, 1.8 and 1.6 nmol CYP/mg protein, respectively. The activity of NADPH:CYP reductase in rat hepatic microsomes was measured according to Sottocasa *et al.* (1967) using cytochrome c as substrate (i.e., as NADPH:cytochrome c reductase). NADPH:CYP reductase activities in hepatic microsomes of control (uninduced) rats and rats induced with Sudan I, β-NF, PB, ethanol and PCN were 0.210, 0.202, 0.199, 0.325, 0.201 and 0.290 µmol/min/mg protein, respectively.
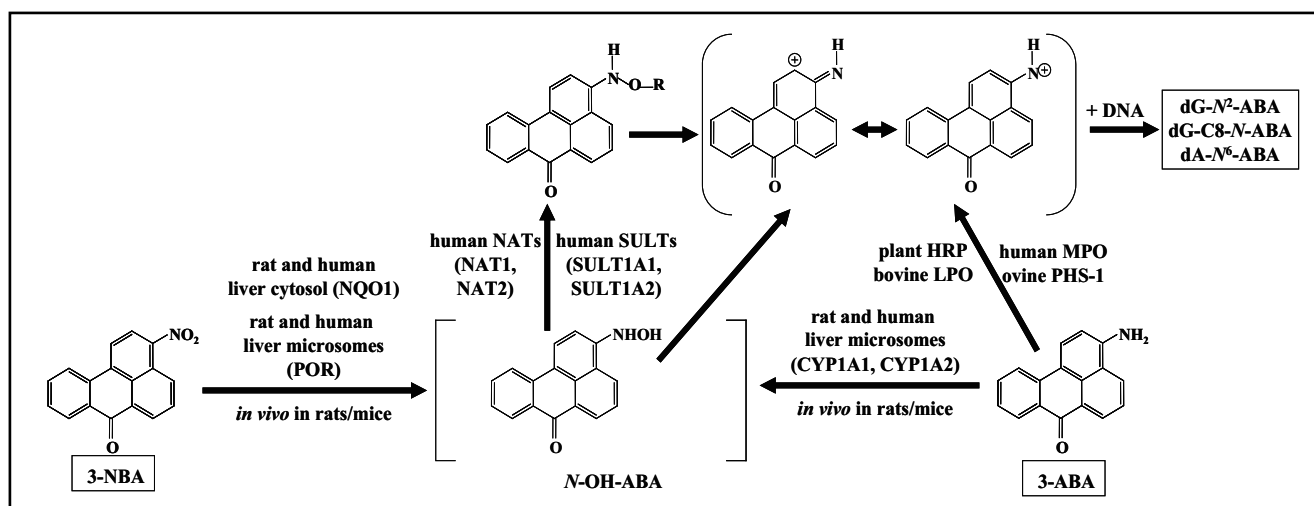

Fig. 1. Pathways of metabolic activation and DNA adduct formation of 3-nitrobenzanthrone and 3-aminobenzanthrone. See text for details. 3-NBA, 3-nitrobenzanthrone; 3-ABA, 3-aminobenzanthrone; NQO1, NAD(P)H:quinone oxidoreductase; NAT, *N*,*O*-acetyltransferases; SULT, sulfotransferase; CYP, cytochrome P450; HRP, horseradish peroxidase; LPO, lactoperoxidase; MPO, myeloperoxidase; PHS-1, prostaglandin H synthase 1 (cyclooxygenase 1); POR, NADPH:CYP oxidoreductase; R = -COCH$_3$ or -SO$_3$H; dA-*N*6-ABA, 2-(2′-deoxyadenosin-*N*6-yl)-3-aminobenzanthrone; dG-*N*2-ABA, *N*-(2′-deoxyguanosin-*N*2-yl)-3-aminobenzanthrone; dG-C8-*N*-ABA, *N*-(2′-deoxyguanosin-8-yl)-3-aminobenzanthrone.

Mouse hepatic microsomes from livers of four HRN (Hepatic Cytochrome P450 Reductase Null) (*Por*[lox/lox] + *Cre*[ALB]) mice (Henderson *et al.* 2003) and four wild-type (WT) mice were isolated using the same procedures as described above. Hepatic microsomes of WT and HRN mice contained 0.3 and 0.4 nmol CYP/mg protein, respectively. NADPH:CYP reductase activity in hepatic microsomes of WT mice was 0.120 μmol/min/mg protein, but it was not detectable in microsomes of HRN mice.

Incubations

Incubation mixtures used for activation of 2-NBA and 3-NBA metabolism by rat and mouse hepatic microsomes to species forming DNA adducts were carried out as described previously (Arlt *et al.* 2003; Stiborova *et al.* 2010b). Briefly, the deaerated and argon-purged incubation mixtures, in a final volume of 750 μl, consisted of 50 mM Tris/HCl buffer (pH 7.4), 1 mM NADPH, rat or mouse hepatic microsomes (1 mg of protein), 0.5 mg of calf thymus DNA and 100 μM 2-NBA or 3-NBA (dissolved in 12.5 μl dimethyl sulfoxide – DMSO). This relatively high concentration of DMSO in reaction mixtures, which might inhibit activities of some CYPs, was necessary to be used, because of the very low solubility of 2-NBA or 3-NBA. The same volume of DMSO was added into the control sample (without 2-NBA or 3-NBA). In the case of mouse hepatic microsomes, incubation mixtures of the same composition, but containing of 1 mM NADH instead of 1 mM NADPH, were also used. The reaction was initiated by adding 2-NBA or 3-NBA. Incubations were carried out at 37 °C for 2 hr; 3-NBA-derived DNA adduct formation was found to be linear up to 3 hr (Arlt *et al.* 2003). Control incubations were carried out (*i*) without activating system (microsomes), (*ii*) without NADPH, (iii) without DNA or (*iv*) without 3-NBA or 2-NBA. After the incubation and extraction with ethyl acetate, DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described (Arlt *et al.* 2003).

Incubations mixtures used to study 2-NBA and 3-NBA metabolism by rat and mouse hepatic microsomes, containing final volume of 500 μl, consisted of 50 mM Tris/HCl buffer (pH 7.4), 1 mM NADPH, 0.5 mg of microsomal protein and 50 μM 2-NBA or 3-NBA (dissolved in DMSO). The reaction was initiated by adding 2-NBA or 3-NBA. Reaction mixtures were incubated at 37 °C for 60 min both under the aerobic conditions (in open tubes) and under the anaerobic conditions that are described above (see incubation conditions used to study activation of 2-NBA and 3-NBA to form DNA adducts). In control incubations NADPH was omitted from the mixtures. After incubation, the mixtures were extracted twice with ethyl acetate (2×1 ml) and 5 μl of 1 mM phenacetine in methanol was added as an internal standard. The extracts were evaporated to dryness; residues were

dissolved in 30 μl of methanol, and subjected to reverse phase-HPLC to evaluate the amounts of 2-aminobenzanthrone (2-ABA) or 3-ABA formed.

HPLC

HPLC was performed with a Dionex HPLC pump P580 with UV/VIS UVD 170S/340S spectrophotometric detector set at 254 nm, and peaks were integrated with a Chromeleon™ 6.01 integrator (Stiborova *et al.* 2010b; Klejdus *et al.* 2005). The column used was a Nucleosil 100-5 C$_{18}$ (Macherey-Nagel, Düren, Germany, 25 cm × 4.6 mm, 5 μm), preceded by a C-18 guard column. Chromatography was under isocratic conditions of 70% methanol in water, with a flow rate of 0.6 ml/min. Recoveries of 2-NBA, 3-NBA and 3-ABA were around 95%. 3-ABA, 3-NBA and 2-NBA were eluted with retention times (r.t.) of 7.8, 24.6 and 25.4 minutes, respectively (Figure 2)

^{32}P-Postlabeling analysis of DNA adducts

^{32}P-Postlabeling analysis using n-butanol extraction, and thin layer chromatography (TLC) and HPLC were performed as described (Arlt *et al.* 2006; Stiborova *et al.* 2010b). Enrichment by *n*-butanol extraction has been shown to yield more adduct spots and a better recovery of 3-NBA-derived DNA adducts than enrichment by nuclease P1 digestion (Arlt *et al.* 2004a, 2004b, 2004c, 2006; Stiborova *et al.* 2006). The detection limit certain individual adducts was around 1 adduct per 10^{10} nucleotides. 3-NBA-derived DNA adducts were identified using standards of 2-(2'-deoxyadenosin-N^6-yl)-3-aminobenzanthrone-3'-phosphate, *N*-(2'-deoxyguanosin-N^2-yl)-3-aminobenzanthrone-3'-phosphate and *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone-3'-phosphate as described (Arlt *et al.* 2006; Stiborova *et al.* 2010b).

RESULTS AND DISCUSSION

Metabolism of 2-NBA and 3-NBA by rat and mouse hepatic microsomes

In initial experiments, 3-NBA was incubated with rat hepatic microsomes in the presence of NADPH, a cofactor of CYP- and NADPH:CYP reductase-mediated reactions, under the anaerobic conditions. Using HPLC with UV detection, one major 3-NBA reduction product (see Figure 2 for hepatic microsomes of rats treated with PB) was found. Using co-chromatography with synthetic standards, this peak was identified to be the 3-NBA reduction product, namely 3-ABA (Figure 2). No 3-ABA was detectable when NADPH was omitted from the incubation mixtures. This finding suggests that either NADPH:CYP reductase or CYP enzymes might be responsible for reduction of 3-NBA to 3-ABA. A time-dependent decrease in 3-NBA in incubation mixture corresponded to an increase in 3-ABA formation (data not shown). No *N*-OH-3-ABA was detectable by HPLC, although it should be formed during 3-NBA
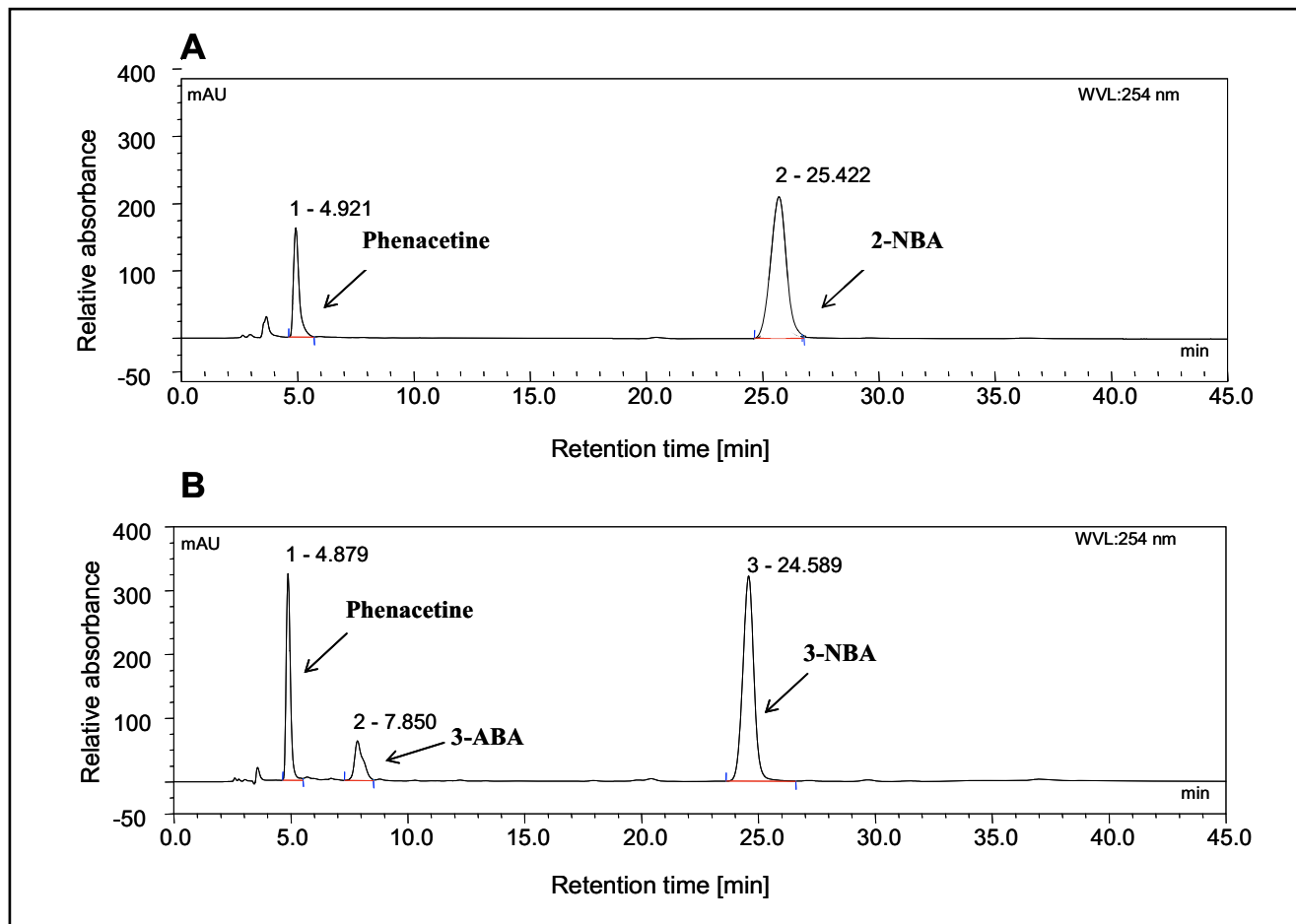
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Fig. 2. HPLC chromatographs of 2-NBA (A) and 3-NBA (B) metabolites produced by human NQO1 under the anaerobic conditions. Experimental conditions are described in Material and methods.

reduction to 3-ABA and as a reactive intermediate binding to DNA (Arlt *et al.* 2003, 2005). Because of the reactivity of this intermediate, it is easily converted to the nitrenium and/or carbenium ion (Figure 1), which may be scavenged by the microsomal proteins present in the incubation mixtures. In the case of a rat animal model, besides hepatic microsomes of control (untreated) rats, we have also used hepatic microsomes of rats treated with several CYP inducers such as Sudan I and β-NF, which are inducers of CYP1A1/2, PB, which is an inducer of CYP2B, ethanol, which increases the levels of CYP2E1 and PCN, which induces CYP3A. Under the anaerobic conditions, hepatic microsomes of control (untreated) rats and microsomes of rats treated with β-NF and PB were capable of reducing 3-NBA to 3-ABA, having almost the same efficiencies to form this 3-NBA metabolite (Table 1). Surprisingly, hepatic microsomes of rats treated with β-NF reduced 3-NBA even under the aerobic conditions (Table 1). This unexpected result remains to be explained. In contrast to these results, the final reduction metabolite of 3-NBA, 3-ABA, was not detectable in other microsomes tested in this study (Table 1). Amounts of 3-ABA formed by individual rat hepatic microsomes did not correspond

either to specific contents of CYP or to activities of NADPH:CYP reductase in these microsomes. This finding suggests that amounts of 3-ABA detectable in microsomal systems does not depend only on the activities of both types of enzymes, but also on other, still unknown, factors. A stability of a reactive intermediate of 3-NBA, *N*-OH-3-ABA that is formed during 3-NBA reduction to 3-ABA to form nitrenium (and/or carbenium) ions binding to DNA in individual microsomes, as well as binding of these ions to microsomal proteins, might be the reasons explaining this phenomenon. These results also indicate that the determination of amounts of the final reductive metabolite of 3-NBA, 3-ABA, seems not to be a suitable marker which should be used to evaluate efficiencies of enzymatic systems to reduce 3-NBA, the crucial reaction leading to 3-NBA-derived DNA adducts (Figure 1).

Hepatic microsomes of WT and HRN mice were also able to reduce 3-NBA to 3-ABA, both under the anaerobic and aerobic conditions. Lower amounts of 3-ABA was formed by hepatic microsomes of HRN mice, but these microsomes were more effective in this reaction under the aerobic than the anaerobic conditions (Table 1).

Under the aerobic conditions, hepatic microsomes of control rats and rats pretreated with PB, ethanol and PCN were also capable of oxidizing 3-NBA. Using HPLC with UV detection, up to two 3-NBA oxidation products, eluted with the retention times (r.t.) of 13 and 18 min, were found (data not shown). Hepatic microsomes of control rats generated both these metabolites, whereas microsomes of rats treated with PB formed only the metabolite eluting at 13 min and microsomes of rats pretreated with ethanol and PCN formed the metabolite eluting at 18 min. Structures of these metabolites remain to be characterized. No such metabolites were generated by hepatic microsomes of the rats treated with other CYP inducers and microsomes of both mouse models.

In contrast to 3-NBA that is reduced by several microsomal systems to 3-ABA, no 2-aminobenzanthrone (2-ABA) was found to be generated from 2-NBA by all hepatic microsomes used in the experiments (see Figure 2). Likewise, no oxidation product peaks were detectable by HPLC employed to separate the 2-NBA metabolites.

Activation of 2-NBA and 3-NBA to species forming DNA adducts

In initial experiments, we compared the DNA adduct formation by 2-NBA and 3-NBA using hepatic microsomal systems under standardized experimental conditions, in the presence of NADPH, a cofactor of microsomal enzymes (NADPH:CYP reductase and CYPs). The same rat and mouse hepatic microsomes that were used to study metabolism of 2-NBA and 3-NBA were utilized. The formation of DNA adducts was analyzed using the butanol extraction version of the ^{32}P-postlabeling method.

In accordance with our previous *in-vitro* studies (Bieler *et al.* 1999; Arlt *et al.* 2003, 2005; Stiborova *et al.* 2006, 2008), the DNA adduct pattern generated from 3-NBA by these enzymatic systems, consisted of a cluster of at least five adducts (spots indicated with arrows in Figure 3) essentially identical to those found previously *in vivo* in rats and mice treated with this carcinogen (Arlt *et al.* 2003, 2005; Bieler *et al.* 2005, 2007). Co-chromatographic analysis of individual spots on HPLC confirmed that adduct spots are derived from 3-NBA by nitroreduction (data not shown). Three of these adducts were identified as 2-(2'-deoxyadenosin-N^6-yl)-3-aminobenzanthrone (dA-N^6-3-ABA, spot 1), 2-(2'-deoxyguanosin-N^2-yl)-3-aminobenzanthrone (dG-N^2-3-ABA, spot 3) and N-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG-C8-N-3-ABA, spots 4,5) (see Figure 3). TLC autoradiograms of ^{32}P-labeled DNA from control incubations carried out in parallel without activation systems, without DNA, or without 3-NBA or without NADPH, a cofactor of microsomal enzymes (CYPs and NADPH:CYP reductase), were devoid of adduct spots in the region of interest (data not shown). These results indicate that both CYPs and

Tab. 1. Amounts of 3-ABA formed from 3-NBA during its incubation with different rat or mouse hepatic microsomes.

Hepatic microsomal system of	Experimental conditions	3-ABA (mmol/mg protein)
control rats	Anaerobic	5.2±0.1
	Aerobic	0
rats treated with Sudan I	Anaerobic	0
	Aerobic	0
rats treated with β-NF	Anaerobic	4.6±0.5
	Aerobic	4.6±0.1
rats treated with PB	Anaerobic	5.8±0.9
	Aerobic	0
rats treated with ethanol	Anaerobic	0
	Aerobic	0
rats treated with PCN	Anaerobic	0
	Aerobic	0
control mice (WT mice)	Anaerobic	3.1±0.8
	Aerobic	3.6±0.3
HRN mice	Anaerobic	1.5±0.2
	Aerobic	2.8±0.3

Experimental conditions are described in Material and methods. NADPH was used in all incubations mixtures. Values in the table are averages and standard deviations of three determinations.
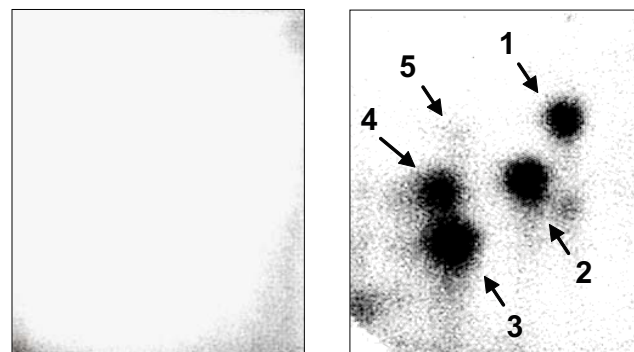

Fig. 3. Autoradiographic profiles of DNA adducts generated by 100 μM 2-NBA (A) and 100 μM 3-NBA (B) after activation with hepatic microsomes of rats pretreated with PCN. Experimental conditions are described in Material and methods. Spot 1, dA-N^6-3-ABA, spot 3, dG-N^2-3-ABA, and spots 4,5, dG-C8-N-3-ABA.

NADPH:CYP reductase might be responsible for the reductive activation of 3-NBA leading to the formation of these DNA adducts.

In the species comparison, microsomes from rats were more efficient to activate 3-NBA than those of mice, mainly the microsomes of HRN mice. The efficiency of rat and mouse hepatic microsomes to activate 3-NBA corresponded to the NADPH:CYP reductase activity (Table 2). Incubations of 3-NBA and DNA with

Tab. 2. Total levels of DNA adducts formed after activation of 3-NBA by different rat or mouse hepatic microsomes in the presence of NADPH.

Hepatic microsomal system of	Experimental conditions	Total levels of 3-NBA-DNA adducts (RAL/10^8 nucleotides)
control rats	Anaerobic	4.2±0.4
rats treated with β-NF	Anaerobic	3.4±0.3
rats treated with PB	Anaerobic	12.1±1.0
rats treated with ethanol	Anaerobic	3.9±0.4
rats treated with PCN	Anaerobic	15.9±1.3
control mice (WT mice)	Anaerobic	0.66±0.05
HRN mice	Anaerobic	0.54±0.05

Experimental conditions are described in Material and methods. 1 mM NADPH was used in all incubations mixtures. Values in the table are averages and standard deviations of three determinations. RAL – relative adduct labeling.

Tab. 3. Total levels of DNA adducts formed after activation of 3-NBA by mouse hepatic microsomes in the presence of NADH.

Hepatic microsomal system of	Experimental conditions	Total levels of 3-NBA-DNA adducts (RAL/10^8 nucleotides)
control mice (WT mice)	Anaerobic	0.25±0.03
HRN mice	Anaerobic	0.91±0.08

Experimental conditions are described in Material and methods. 1 mM NADH was used in incubations mixtures. Values in the table are averages and standard deviations of three determinations. RAL – relative adduct labeling.

hepatic microsomes of rats pretreated with inducers of NADPH:CYP reductase, PCN and PB, led to a 4- and 3-fold increase, respectively, in the formation of 3-NBA-DNA adducts, while lower activities of this enzyme in mouse hepatic microsomes resulted in a decrease in levels of 3-NBA-DNA adducts (Table 2). These results suggest that NADPH:CYP reductase might be the major enzyme responsible for reductive activation of 3-NBA to form DNA adducts in hepatic microsomes (Arlt *et al.* 2003). Interestingly, 3-NBA-derived DNA adducts were also formed in hepatic microsomes of HRN mice, where no activity of NADPH:CYP reductase was detectable. Therefore, another enzyme should be responsible for 3-NBA activation in these microsomes. Using NADH, a cofactor of NADH:cytochrome b$_5$ reductase in incubation mixtures containing hepatic microsomes of HRN mice, higher levels of 3-NBA-derived DNA adducts were found (Table 3). Hence, NADH:cytochrome b$_5$ reductase can substitute the NADPH:CYP reductase in reductive activation of 3-NBA in hepatic microsomes of HRN mice.

In contrast to the results found with 3-NBA, no 2-NBA-derived DNA adducts were detected under the same experimental conditions with any of the hepatic microsomes used in the experiments. This finding corresponds fully with the lack of 2-NBA reduction in these hepatic microsomal enzymatic systems.

CONCLUSIONS

The results of this study show that in contrast to 3-NBA, 2-NBA is not a substrate of the enzymes present in the rat and mouse hepatic microsomal systems tested in our experiments. Using the ^{32}P-postlabeling assay, which is in principle sensitive enough to detect DNA adduct levels as low as 1 adduct per 10^{10} normal nucleotides (Phillips & Arlt 2007), we were unable to detect 2-NBA-derived DNA adducts catalyzed by any of the microsomal enzymatic systems utilized. Our results correspond to data published previously showing that 2-NBA-derived DNA adducts were not detected both *in vitro*, in several human enzymatic systems (Stiborova *et al.* 2010b) and in Wistar rats treated with 2-NBA *in vivo* (Arlt *et al.* 2007). Only after the administration of the reactive *N*-hydroxylated intermediate *N*-hydroxy-2-aminobenzanthrone (*N*-OH-2-ABA), 2-NBA-derived DNA adducts were detectable in several rat organs such as lung, liver, kidney, colon and pancreas (Arlt *et al.* 2007).

In the case of 3-NBA, this carcinogen is reduced by rat and mouse hepatic microsomes to species forming 3-NBA-derived DNA adducts. NADPH:CYP reductase seems to be the major enzyme reductively activating 3-NBA in rat and/or mouse liver microsomes. However, when this enzyme is absenting in the microsomes, like it is in hepatic microsomes of HRN mice, NADH:cytochrome b$_5$ can substitute NADPH:CYP reductase in this activation reaction. The results found in the experiments determining the amounts of the final reductive metabolite of 3-NBA, 3-ABA, and levels of 3-NBA-derived DNA adducts strongly suggest that determination of the levels of these DNA adducts are a more suitable method to evaluate the enzymes responsible for reductive activation of this carcinogen than that of amounts of formed 3-ABA.

Finally, the present study has increased our knowledge on the potential of rat and mouse hepatic microsomal enzymatic systems to reductively activate the two NBA urban air pollutants, 2-NBA and 3-NBA. In accordance with previous data our results indicate that 3-NBA is a potent genotoxin and carcinogen (Arlt *et al.* 2003, 2005; Bieler *et al.* 2007; Stiborova *et al.* 2006, 2008, 2010b), supporting our efforts in investigating the mechanisms of 3-NBA carcinogenicity.

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