

THE ENVIRONMENTAL POLLUTANT AND CARCINOGEN 3-NITROBENZANTHRONE AND ITS HUMAN METABOLITE 3-AMINOBENZANTHRONE ARE POTENT INDUCERS OF RAT HEPATIC CYTOCHROMES P450 1A1 AND -1A2 AND NAD(P)H:QUINONE OXIDOREDUCTASE

Marie Stiborová, Helena Dračínská, Jana Hájková, Pavla Kadeřábková, Eva Frei, Heinz H. Schmeiser, Pavel Souček, David H. Phillips, and Volker M. Arlt

Department of Biochemistry, Faculty of Science, Charles University, 128 40 Prague 2, Czech Republic (M.S., H.D., J.H., P.K.); Division of Molecular Toxicology, German Cancer Research Center, D-69120 Heidelberg, Germany (E.F., H.H.S.); Biotransformations Group, National Institute of Public Health, Center of Occupational Diseases, Prague 10, Czech Republic (P.S.); and Section of Molecular Carcinogenesis, Institute of Cancer Research, Sutton, Surrey SM2 5NG, UK (D.H.P., V.M.A)

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Address correspondence to: Marie Stiborová, Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, 128 40 Prague 2, Czech Republic. Phone: +420-2-21951285; Fax: +420-2-21951283; E-mail address: stiborov@natur.cuni.cz

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ABBREVIATIONS: 3-NBA, 3-nitrobenzanthrone; 3-ABA, 3-aminobenzanthrone; COX, cyclooxygenase; c_T , cycle threshold; 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; DMSO, dimethylsulfoxide; EROD, 7-ethoxyresorufin O -deethylation; HRP, horseradish peroxidase; LPO, lactoperoxidase; MPO, myeloperoxidase; N -Aco-ABA, N -acetoxy-3-aminobenzanthrone; N -OH-ABA, N -hydroxy-3-aminobenzanthrone; NQO1, NAD(P)H:quinone oxidoreductase; PVDF, polyvinylidene difluoride; NAT, N,O -acetyltransferase; SULT, sulfotransferase; CYP, cytochrome P450; dA, deoxyadenosine; dG, deoxyguanosine; acetyl-CoA, acetyl coenzyme A; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; RAL, relative adduct labelling; RT, real-time; PCR, polymerase chain reaction.

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ABSTRACT:

3-Nitrobenzanthrone (3-NBA), a suspected human carcinogen occurring in diesel exhaust and air pollution and its human metabolite 3-aminobenzanthrone (3-ABA) were investigated for their ability to induce biotransformation enzymes in rat liver and the influence of such induction on DNA adduct formation by the compounds. Rats were treated (i.p.) with 0.4, 4 or 40 mg/kg body weight of 3-NBA or 3-ABA. When hepatic cytosolic fractions from rats treated with 40 mg/kg body weight of 3-NBA or 3-ABA were incubated with 3-NBA, DNA adduct formation, measured by ³²P-postlabelling analysis, was 10-fold higher in incubations with cytosols from pretreated rats than with controls. The increase in 3-NBA-derived DNA adduct formation corresponded to a dose-dependent increase in protein levels and enzymatic activity of NAD(P)H:quinone oxidoreductase (NQO1). NQO1 is the major enzyme reducing 3-NBA in human and rat livers. Incubations of 3-ABA with hepatic microsomes of rats treated with 3-NBA or 3-ABA (40 mg/kg body weight) led to an up to 12-fold increase in 3-ABA-derived DNA adduct formation compared to controls. The observed stimulation of DNA adduct formation by both compounds was attributed to their potential to induce protein expression and enzymatic activity of cytochromes P450 1A1 and/or -1A2 (CYP1A1/2), the major enzymes responsible for 3-ABA activation in human and rat livers. Collectively, these results demonstrate for the first time that by inducing hepatic NQO1 and CYP1A1/2, both 3-NBA and 3-ABA increase the enzymatic activation of these two compounds to reactive DNA adduct forming species, thereby enhancing their own genotoxic potential.

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Environmental factors and individual genetic susceptibility play an important role in many human cancers (Boffeta and Nyberg, 2003). Lung cancer is the most common malignant disease worldwide and is the major cause of death from cancer. Although tobacco smoking is the overwhelming cause of lung cancer, vehicular exhaust and ambient air pollution are also implicated as causative factors (IARC, 1989; Environmental Protection Agency, 2002; Vineis et al., 2004; Grashick et al., 2004). Nitro-aromatic compounds are widely distributed environmental pollutants found in vehicular exhaust from diesel and gasoline engines and on the surface of ambient air particulate matter. The increased lung cancer risk after exposure to these environmental sources and the detection of nitro-aromatics in the lungs of non-smokers with lung cancer has led to considerable interest in assessing their potential cancer risk to humans (IARC, 1989; Environmental Protection Agency, 2002; Vineis et al., 2004; Grashick et al., 2004).

3-Nitrobenzanthrone (3-nitro-7*H*-benz[*de*]anthracen-7-one, 3-NBA, Fig. 1), occurs in diesel exhaust and in airborne particulate matter (Enya et al., 1997; Seidel et al., 2002; Nagy et al., 2005; Arlt, 2005). 3-NBA might originate both from incomplete combustion of fossil fuels and from reaction of the parent aromatic hydrocarbon with nitrogen oxides in the atmosphere. The main metabolite of 3-NBA, 3-aminobenzanthrone (3-ABA, Fig. 1), has been found in urine samples of salt mine workers occupationally exposed to diesel emissions (Seidel et al., 2002), demonstrating that human exposure to 3-NBA in diesel emissions can be significant and is detectable. 3-NBA is carcinogenic in rats, causing lung tumours after intratracheal instillation (Nagy et al., 2005). It is also an exceptionally potent mutagen in the Ames *Salmonella typhimurium* assay, scoring more than 6 million revertants per nanomol in strain YG1024 expressing bacterial nitroreductase and *O*-acetyltransferase (Enya et al., 1997), and in the transgenic Muta Mouse assay (Arlt et al., 2004c). 3-NBA has also been shown to be genotoxic in several other short-term tests (Seidel et al., 2002). Its genotoxicity has been

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further documented by the detection of specific DNA adducts formed *in vitro* as well as *in vivo* in rodents (Arlt et al., 2001; 2002; 2003a; b; c; 2004c; 2005).

Most of the metabolic activation of 3-NBA *in vitro* is attributable to human and rat cytosolic NAD(P)H:quinone oxidoreductase (NQO1), while human *N,O*-acetyltransferase (NAT), NAT2, followed by NAT1, sulfotransferase (SULT), SULT1A1 and, to a lesser extent, SULT1A2 are the major phase II enzymes activating 3-NBA (Arlt et al., 2002; 2003a; 2005). Microsomal NADPH:cytochrome P450 (CYP) reductase is also effective in the activation of 3-NBA (Arlt et al., 2003c), but in a model organism, mice, 3-NBA is predominately activated by cytosolic nitroreductases such as NQO1 rather than microsomal NADPH:CYP reductase (Arlt et al., 2005) (Fig. 1). For 3-ABA, CYP1A1 and -1A2 (CYP1A1/2) enzymes are essential for the oxidative activation of 3-ABA in livers (Arlt et al., 2004b). However, cells in extrahepatic organs have the metabolic capacity to activate 3-ABA to form DNA adducts, independent of the CYP-mediated oxidation in the liver. Peroxidases such as prostaglandin H synthase (cyclooxygenase, COX), lactoperoxidase (LPO), myeloperoxidase (MPO), abundant in several extrahepatic tissues, generate DNA adducts, which are formed *in vivo* by 3-ABA or 3-NBA (Arlt et al., 2006a) (Fig. 1). The results suggest that both CYPs and peroxidases may play an important role in the oxidative metabolism of 3-ABA to reactive species that form DNA adducts, resulting in genotoxicity of 3-ABA and its parent compound.

It is known that several nitro-aromatics present in diesel exhaust and aromatic amines induce the expression of biotransformation enzymes participating in their metabolism (Chou et al., 1987; Djuric et al., 1988; Dagawa et al., 1998; Chen et al., 1998; Hatanaka et al., 2001; Iwanari et al., 2002; Hummerich et al., 2004). However, the potential of 3-NBA and 3-ABA to influence the expression and activities of enzymes metabolically activating these toxicants

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have not yet been investigated. The aim of the present study was to evaluate whether 3-NBA and 3-ABA induce the biotransformation enzymes involved in their metabolic activation.

Materials and Methods

Chemicals. NADPH, deoxyadenosine (dA) 3'-monophosphate, deoxyguanosine (dG) 3'-monophosphate, xanthine oxidase, acetyl coenzyme A (acetyl-CoA), 3'-phosphoadenosine-5'-phosphosulfate (PAPS), menadione (2-methyl-1,4-naphthoquinone), salmon testis and calf thymus DNA were obtained from Sigma Chemical Co (St Louis, MO, USA); Sudan I from BDH (Poole, UK); 7-ethoxyresorufin from Fluka Chemie AG (Buchs, Switzerland). All these and other chemicals were reagent grade or better.

Synthesis of 3-NBA and 3-ABA. 3-NBA and 3-ABA were synthesized as described recently (Arlt et al., 2003a) and their authenticity was confirmed by UV spectroscopy, electrospray mass spectra and high field proton NMR spectroscopy.

Animal Experiments. 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 is in compliance with Declaration of Helsinki. Male Wistar rats (100-125 g) were treated with a single dose of 0.4, 4 or 40 mg/kg body weight (n=3) of 3-NBA or 3-ABA by intraperitoneal injection. 3-NBA and 3-ABA was dissolved in sunflower oil at a concentration of 0.4 or 4 mg/ml. Three control animals received an equal volume of oil only. Rats were placed in cages in temperature and humidity controlled rooms. Standardized diet and water were provided *ad libitum*. The animals were killed 24 hours after treatment by cervical dislocation. Livers were removed immediately after death and used for isolation of mRNA and for preparation of microsomal and cytosolic fractions.

Preparation of Microsomal and Cytosolic Fractions. Microsomal and cytosolic fractions were isolated from the livers of rats, either uninduced or pretreated with 3-NBA or 3-ABA (see above) as described (Stiborová et al., 2003). Both subcellular preparations were analyzed for the presence of 3-NBA and/or 3-ABA using the HPLC on a CC 250/4 Nucleosil, 100-5 C18 HD column (Macherey-Nagel, 4 x 250 mm) preceded by a C-18 guard column.

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Eluent was 70% methanol, at a flow rate of 0.6 ml min⁻¹, and detection was at 254 nm. Standards of 3- ABA and 3-NBA were eluted with retention times of 8.2 and 24.5 min, respectively. Neither chemical was detectable in microsomal and cytosolic fractions from rat that had been pretreated with the compounds.

Isolation of CYPs and NADPH:CYP reductase. CYP2B4 and 2E1 enzymes were isolated from liver microsomes of rabbits induced with phenobarbital (CYP2B4) or ethanol (CYP2E1) by procedures described (Stiborová et al., 2002). Recombinant rat CYP1A1 protein was purified to homogeneity from membranes of *Escherichia coli* transfected with a modified *CYP1A1* cDNA (Stiborová et al., 2002), in the laboratory of H. W. Strobel (University of Texas, Medical School of Houston, Texas, USA) by P. Hodek (Charles University, Prague, Czech Republic). Human recombinant CYP3A4 was a gift of P. Anzenbacher (Palacky University, Olomouc, Czech Republic). Rabbit liver NADPH:CYP reductase was purified as described (Yasukochi et al., 1979).

Preparation of antibodies. Leghorn chickens were immunized subcutaneously three times a week with CYP antigens [rat recombinant CYP1A1, rabbit CYP2B4, rabbit CYP2E1, human recombinant CYP3A4, rabbit NADPH:CYP reductase, human recombinant NQO1 (Sigma, St Louis, MO, USA)] (0.1 mg/animal) emulsified in complete Freund's adjuvant for the first injection and in incomplete adjuvant for boosters. Immunoglobulin fraction was purified from pooled egg yolks using fractionation by polyethylene glycol 6000 (Stiborová et al., 2002).

Estimation of CYP, NADPH:CYP Reductase and NQO1 Protein Content in Microsomes and Cytosols of Rat Liver. Immunoquantitation of rat liver microsomal CYPs (CYP1A1, -1A2, -2B, -2E1 and -3A), NADPH:CYP reductase and of cytosolic NQO1 was done by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Samples containing 75 µg microsomal or cytosolic proteins were subjected to electrophoresis on

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SDS/10% polyacrylamide gels (Stiborová et al., 2002; 2005). After migration, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. Rat CYP, NADPH:CYP reductase and NQO1 proteins were probed with the chicken polyclonal antibodies as described above, as reported elsewhere (Stiborová et al., 2002; 2005). The antibody against rat recombinant CYP1A1 recognizes both CYP1A1 and -1A2 in rat liver microsomes (Stiborová et al., 2002). Rat recombinant CYP1A1 and -1A2 (in SupersomesTM, Gentest Corp., Woburn, MA, USA) and human recombinant NQO1 (Sigma Chemical Co, St Louis, MO, USA) were used as positive controls to identify the bands of CYP1A1 and -1A2 in microsomes and NQO1 in cytosols. The antigen-antibody complex was visualized with an alkaline phosphatase-conjugated rabbit anti-chicken IgG antibody and 5-bromo-4-chloro-3-indolylphosphate/nitrobluetetrazolium as dye (Stiborová et al., 2002; 2005).

CYP1A and NQO1 Enzyme Activity Assays. The microsomal samples were characterized for CYP1A activity using 7-ethoxyresorufin *O*-deethylation (EROD) activity and for the oxidation of Sudan I (a marker substrate for CYP1A1) (Stiborová et al., 2002; 2005). The cytosolic samples were characterized for NQO1 activity, using menadione (2-methyl-1,4-naphthoquinone) as a substrate (Stiborová et al., 2003). NQO1 activity was determined by following the oxidation of NADPH spectrophotometrically at 340 nm (Stiborová et al., 2003). The standard assay system contained 25 mM Tris-HCl (pH 7.4), 0.2% Tween 20, 0.07% bovine serum albumin, 400 mM NADPH and 100 mM menadione dissolved in methanol.

CYP1A and NQO1 mRNA Content in Rat Livers. Total RNA was isolated from frozen livers of three untreated rats and three rats pretreated with 40 mg/kg body weight of 3-NBA or 3-ABA using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the procedure supplied by the manufacturer. The quality of isolated RNA was verified by horizontal agarose gel electrophoresis, RNA quantity was assessed by UV-VIS spectrophotometry on a Carry

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300 spectrophotometer (Varian, Palo Alto, CA, USA). RNA samples (1 μg) were reversely transcribed using 200 U of reverse transcriptase per sample with random hexamer primers utilizing RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. The prepared cDNA was used for real-time (RT) polymerase chain reaction (PCR) performed in RotorGene 2000 (Corbett Research, Sydney, Australia) under the following cycling conditions: incubation at 50 °C for 2 min and initial denaturation at 95 °C for 10 min, then 50 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. Gain was set to 7 and fluorescence was acquired after elongation step. The PCR reaction mixtures (20 μl) contained 9 μl cDNA diluted 10-times in Milli-Q ultrapure water (Biocel A10, Millipore, Billerica, MA, USA), 10 μl TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 1 μl TaqMan Gene Expression Assay Mix (commercially available unlabeled PCR primers and FAMTM dye-labelled probe for rat *CYP1A1/2* or *NQO1* as target genes and β -actin as reference internal standard gene). Each sample was analysed in two parallel aliquots. Negative controls had the same compositions as samples but cDNA was omitted from the mixture. Data were analysed by the program RotorGene v6 (Corbett Research, Sydney, Australia) and evaluated by comparative cycle threshold (c_T) method for relative quantitation of gene expression. Cycle thresholds, at which a significant increase in fluorescence signal was detected, were measured for each sample. Then $\Delta\Delta c_T$ was evaluated according to following equations: $\Delta c_T = c_T$ (target) - c_T (internal standard), $\Delta\Delta c_T = \Delta c_{T\text{treated}} - \Delta c_{T\text{control}}$, where $\Delta c_{T\text{treated}}$ is Δc_T for treated rats and $\Delta c_{T\text{control}}$ is Δc_T for untreated rats. Δc_T is positive if the target is expressed at a lower level than the internal standard (β -actin), and negative if expressed at a higher level. The induction of mRNA expression of studied target genes in pretreated animals was evaluated as $2^{-(\Delta\Delta c_T)}$.

Cytosolic Incubations. The deaerated and argon-purged incubation mixtures, in a final volume of 750 μl , consisted of 50 mM Tris-HCl buffer (pH 7.4), containing 0.2% Tween 20,

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cofactors for cytosolic enzymes (1 mM NADPH; 2 mM acetyl-CoA; 100 μ M PAPS), pooled hepatic cytosolic sample from 3 rats, either untreated (control) or treated with 40 mg/kg body weight of 3-NBA or 3-ABA (1 mg of cytosolic protein), 30 μ M 3-NBA [dissolved in 12.5 μ l dimethylsulfoxide (DMSO)] and 0.5 mg of calf thymus DNA. The reaction was initiated by adding 3-NBA. Incubations with rat cytosols were carried out at 37°C for 3 hours; the cytosol-mediated 3-NBA-derived DNA adduct formation was found to be linear up to 4 hr of incubation (Arlt et al. 2005). Control incubations were carried out either (i) without activating system (cytosol) or (ii) without DNA or (iii) without 3-NBA. After the incubation, DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described (Arlt et al. 2005).

Incubations used to evaluate the activation of 3-ABA by peroxidases in rat hepatic cytosolic samples contained, in a final volume of 750 μ l, 50 mM Tris-HCl buffer (pH 7.4), containing 0.2% Tween 20, 100 μ M 3-ABA (dissolved in 7.5 μ l DMSO) and 200 μ M H₂O₂.

Microsomal Incubations. Incubation mixtures, in a final volume of 750 μ l, consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, pooled hepatic microsomal sample from 3 rats, either untreated (control) or treated with 40 mg/kg body weight of 3-NBA or 3-ABA (1 mg of microsomal protein), 100 μ M 3-ABA (dissolved in 7.5 μ l DMSO) and 0.5 mg of calf thymus DNA. The reaction was initiated by adding 3-ABA. Incubations with rat microsomes were carried out at 37°C for 2 h; the microsomal-mediated 3-NBA-derived DNA adduct formation was found to be linear up to 3 hr of incubation. Control incubations were carried out either (i) without activating system (microsomes) or (ii) with activating system and 3-ABA, but without DNA or (iii) with activating system and DNA but without 3-ABA. After the incubation, DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described (Arlt et al., 2004b).

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³²P-Postlabelling analysis and HPLC analysis of ³²P-labelled 3',5'-deoxyribonucleoside bisphosphate adducts. ³²P-Postlabelling analysis using butanol extraction, and thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) were performed as described (Arlt et al., 2002; 2003b). Enrichment by butanol extraction has been shown to yield more adduct spots and a better recovery of 3-ABA-derived DNA adducts than using enrichment by nuclease P1 digestion (Arlt et al., 2004b). DNA adduct spots were numbered as reported (Arlt et al., 2003b; 2004a; b). As reference compounds deoxyadenosine (dAp) and deoxyguanosine (dGp) 3'-monophosphates (4 μmol/ml) (Sigma) were incubated with 3-NBA (300 μM) activated by xanthine oxidase (1 U/ml) (Sigma) in the presence of hypoxanthine and analysed as described previously (Arlt et al., 2001). DNA adduct standard samples of 3-NBA, 2-(2'-deoxyadenosin-*N*⁶-yl)-3-aminobenzanthrone-3'-phosphate (dA3'p-*N*⁶-ABA), *N*-(2'-deoxyguanosin-*N*²-yl)-3-aminobenzanthrone-3'-phosphate (dG3'p-*N*²-ABA) and *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone-3'-phosphate (dG3'p-C8-*N*-ABA), were prepared by reacting *N*-acetoxy-3-aminobenzanthrone (*N*-Aco-ABA) with dAp or dGp and analysed as described recently (Arlt et al., 2006b).

Results

The Effect of 3-NBA and 3-ABA on Expression of Biotransformation Enzymes.

Western blots with chicken polyclonal antibodies raised against various CYPs (CYP1A1, -2B4, -2E1 and -3A4), NADPH:CYP reductase and NQO1 showed that the expression of hepatic CYP1A1/2 and NQO1 were induced in rats by both compounds (Figs. 2 and 3). The expression of CYP2B, -2E1, -3A and NADPH:CYP reductase, was essentially not altered by treating rats with 3-NBA or 3-ABA (data not shown). The efficiency to induce hepatic CYP1A1 and -1A2 enzymes was higher for 3-NBA than for 3-ABA (16-fold *versus* 3-fold for CYP1A1 and 3-fold *versus* 2-fold for CYP1A2 at 40 mg/kg body weight) (Fig. 2A,B,E,F). EROD activity, a marker for CYP1A1 and -1A2, as well as oxidation of Sudan I, a marker substrate of CYP1A1 (Stiborová et al., 2002; 2005), were increased in rats treated with both compounds; a statistically significant increase in these marker activities was found in hepatic microsomes of rats treated with the highest dose of the toxicants (40 mg/kg body weight) (Fig. 2C,D,G,H). The level of hepatic NQO1, the major enzyme reductively activating 3-NBA in rat and human livers (Arlt et al., 2005), was significantly enhanced by pretreatment rats with 3-NBA and 3-ABA. The increase in the expression of NQO1 was concentration-dependent; a more than 10-fold increase in NQO1 expression was caused by treating rats with 4 and 40 mg/kg body weight of 3-NBA or 3-ABA (Fig. 3). The increase in NQO1 activities in hepatic cytosols depended on administered dose and was parallel to the protein content (Fig. 3). A 1.9-, 2.5- and 3.4-fold increase in NQO1 activity measured with menadione was found in livers of rats treated with 0.4, 4 and 40 mg/kg of 3-NBA, respectively (Fig. 3B). For 3-ABA only the doses 4 and 40 mg/kg body weight resulted in a significant increase in menadione reduction (Fig. 3D).

Besides evaluating the effects of 3-NBA and 3-ABA on protein levels and enzyme activities of CYP1A1, -1A2 and NQO1, modulation of their mRNA expression by these

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compounds was also investigated. The relative amounts of CYP1A subfamily and NQO1 mRNA were measured by RT-PCR analysis. As shown in Table 1, treatment of rats with 40 mg/kg body weight of 3-NBA induced a 4.8- and 4.4-fold increase in mRNA expression levels of hepatic CYP1A1 and NQO1, respectively, while no increase in mRNA expression was observed for CYP1A2. However, in contrast to enhancement of protein levels and enzyme activities of CYP1A and NQO1 by 3-ABA, no increase in expression of their mRNAs were detectable under the conditions used in the experiments.

Activation of 3-NBA by Rat Hepatic Cytosols. We compared the formation of DNA adducts in calf thymus DNA by 3-NBA incubated with cytosols from the livers of rats, either control (uninduced) or pretreated either with 3-NBA itself or with its reductive metabolite, 3-ABA. Cytosolic samples from uninduced rats were capable of activating reductively 3-NBA to form DNA adducts (Arlt et al., 2005). The DNA adduct pattern generated by 3-NBA consisted of a cluster of up to five adducts (spots 1–5 in Figure 4) essentially identical to that observed *in vivo* in rats and mice treated with 3-NBA (Arlt et al., 2001; 2003b; 2005; Bieler et al., 2005), and in *in vitro* incubations using human hepatic cytosols (Arlt et al., 2005) or rat and human hepatic microsomes (Arlt et al., 2003c). Cochromatographic analysis of individual spots on HPLC confirmed that adduct spots 1–5 that are formed with rat hepatic cytosols 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 -ABA; spot 1), *N*-(2'-deoxyguanosin- N^2 -yl)-3-aminobenzanthrone (dG- N^2 -ABA; spot 3) and *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG-C8-*N*-ABA; spots 4/5). Thin-layer chromatograms of ^{32}P -labelled DNA from control incubations carried out in parallel without cytosol, without DNA, or without 3-NBA were devoid of adduct spots in the region of interest (data not shown).

As with human hepatic cytosols (Arlt et al., 2005), the addition of cofactors of phase II enzymes to the incubations containing 3-NBA, rat hepatic cytosol and NADPH stimulated the

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formation of the 3-NBA-DNA adducts (Fig. 5 and Supplementary Table S1). The addition of cofactors of NATs and SULTs, acetyl-CoA (King et al., 1998) and PAPS (Glatt et al., 2001), increased the levels of 3-NBA adducts by 3-fold and 38-fold, respectively, over the activity with NADPH alone (Fig. 5 and Supplementary Table S1).

Hepatic cytosolic samples from rats pretreated with 40 mg/kg body weight of 3-NBA or 3-ABA were more effective in formation of 3-NBA-DNA adducts in the presence of NQO1 cofactor, NADPH, than the hepatic cytosol of untreated (control) rats. Formation of 3-NBA-DNA adducts was 10-fold higher with each inducer than in cytosols of control rats. Addition of acetyl-CoA and PAPS to the incubations with induced cytosols had essentially the same stimulatory effects on 3-NBA adduct formation as in control cytosols; a 3- and 40-fold increase was observed in comparison to the activity using NADPH (Fig. 5 and Supporting Table S1).

Activation of 3-ABA by Rat Hepatic Microsomes. Comparison of the efficiencies of microsomes isolated from livers of rats either uninduced or pretreated with 3-NBA or 3-ABA to activate 3-ABA to species binding to DNA was also performed. In the presence of NADPH all these hepatic microsomes were capable of oxidizing 3-ABA to form DNA adducts. Microsomal activation of 3-ABA generated a pattern of DNA adducts consisting of a cluster of five adducts (spots 1-5 in Figure 4), similar to those formed by 3-ABA and 3-NBA *in vitro* and *in vivo* (Arlt et al., 2001; 2002; 2003a; b; c; 2004b; c; 2005; 2006a; b; Bieler et al., 2005). In the presence of NADPH, hepatic microsomes isolated from rats treated with 3-NBA or 3-ABA (40 mg/kg body weight) exhibited 11.8- and 10-fold higher efficiencies, respectively, to activate 3-ABA, than hepatic microsomes from untreated rats (Fig. 6A and Supplementary Table S2). Chromatograms of DNA digests from control incubations carried out in parallel without microsomes, without NADPH, without DNA, or without 3-ABA were devoid of adduct spots in the region of interest (data not shown).

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Activation of 3-ABA by Rat Hepatic Cytosols. In the presence of hydrogen peroxide as a cofactor for peroxidases, the hepatic cytosols from rats treated with 3-NBA and 3-ABA were capable of activating 3-ABA to form DNA adducts. In this activation system, four 3-ABA-DNA adducts were detected (spots 1-4 in Figure 4), chromatographically (TLC, HPLC) indistinguishable from those formed by 3-NBA and 3-ABA *in vitro* and *in vivo* (Arlt et al., 2001; 2002; 2003a; b; c; 2004b; c; 2005; 2006a; b; Bieler et al., 2005); adduct spot 5 was not detectable. The levels of DNA adducts formed by 3-ABA incubated with hepatic cytosols of rats treated with 3-NBA and 3-ABA were 4.4 and 6.5 adducts in 10^8 normal nucleotides, respectively (Fig. 6B and Supplementary Table S3). No adducts were detectable when 3-ABA was incubated with hepatic cytosols of control rats or with cytosols of rats treated with 3-NBA or 3-ABA, but without hydrogen peroxide (data not shown).

Discussion

Epidemiological studies have shown that exposure to diesel exhaust and urban air pollution is associated with an increased risk of lung cancer. The risk to human health associated with exposure to diesel exhaust has generally been considered a particle problem and the role of chemicals bound to the particles may have been underestimated (Hatanaka et al. 2001). 3-NBA, an extremely potent mutagen and suspected human carcinogen, is one of several nitro-aromatics identified in diesel exhaust and air pollution (Enya et al., 1997; Seidel et al., 2002; Nagy et al., 2005; Arlt 2005). While the enzymes responsible for the activation of this carcinogen and its reductive metabolite, 3-ABA, to the DNA binding species have already been identified (Arlt et al., 2002; 2003a; c; 2004b; 2005; 2006a), there has, until now, been no information on the effects of 3-NBA and 3-ABA exposure on the expression and activities of these enzymes.

In the present study, we have shown that 3-NBA and 3-ABA are efficient inducers of the enzymes involved in their own metabolic activation to species that form DNA adducts. Expression of NQO1 protein, the major enzyme reductively activating 3-NBA in mouse liver *in vivo* and in *in-vitro* incubations with rat and human hepatic cytosols (Arlt 2005; Arlt et al., 2005), was significantly induced by 3-NBA and 3-ABA in livers of rats treated (i.p.) with a single dose of 0.4, 4 or 40 mg/kg body weight of either compounds. The reductive activation of 3-NBA by hepatic cytosolic samples from rats pretreated with 3-NBA or 3-ABA lead to 10-fold higher 3-NBA-DNA adduct levels than in hepatic cytosol from control rats. This increase was more than 4-times higher than the increase in NQO1 enzyme activity measured with menadione as a substrate. Pretreatment of rats with 3-NBA or 3-ABA had no effect on conjugating enzymes participating in 3-NBA activation (SULTs and NATs) (Arlt et al., 2005). The addition of cofactors of these enzymes, acetyl-CoA for NATs and PAPS for SULTs, to the incubations with the 3-NBA- and 3-ABA-rat hepatic cytosols had practically

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the same effects on 3-NBA adduct formation as in the control cytosols. The degrees of stimulation on 3-NBA-derived DNA adduct formation in rat hepatic cytosols by acetyl-CoA and PAPS were similar to those in human hepatic cytosols (Arlt et al., 2005). However, because of the induction of NQO1 in rat livers, the overall levels of 3-NBA adducts formed in these induced cytosols in the presence of acetyl-CoA and PAPS were one order of magnitude higher than in human hepatic cytosols [1.6-1.8 adducts in 10^4 normal (unmodified) nucleotides].

Because CYP1A1 and -1A2 are the predominant enzymes activating the metabolite of 3-NBA, 3-ABA, to species forming DNA adducts (Arlt et al., 2004b; 2006a) (Fig. 1), we have investigated whether their expression might also be influenced by the treatment of rats with 3-NBA or 3-ABA. Here, we have found that the expressions of CYP1A1 and -1A2 proteins as well as their enzymatic activities (EROD, a marker for CYP1A1/2, and Sudan I oxidation, a marker for CYP1A1) are induced in rats pretreated with 3-NBA or 3-ABA. The induction by 3-NBA resulted in one order of magnitude-fold higher 3-ABA-derived DNA adduct levels in incubations of 3-ABA with hepatic microsomes of rats treated with 3-NBA than in incubations with control microsomes. This increase was essentially analogous to the increase in EROD activity, but Sudan I oxidation catalyzed by rat 3-NBA-induced microsomes was only 2-times higher than oxidation of this CYP1A1 substrate by microsomes of control rats. This finding is difficult to explain at the present time. The investigation whether the CYP1A1 activity to Sudan I as a substrate is more sensitive to handling of rat microsomes might shed light on this observation. Even though 3-ABA exhibited a lower potency to induce CYP1A1/2 enzyme activity (up to 3-fold), hepatic microsomes of rats pretreated with this compound produced a 10-fold increase in 3-ABA-derived DNA adduct formation. This indicates that not only the activities of the CYP1A enzymes, but also other microsomal enzymes such as NADPH-dependent flavine-containing monooxygenases, known to be active in *N*-

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hydroxylation of several arylamines (Kim and Guengerich 2005), might be induced by 3-ABA and participate in its own activation in rat hepatic microsomes. However, this suggestion needs to be confirmed by further studies.

The increases both in expressions of NQO1 and CYP1A1 proteins and enzymatic activities induced by 3-NBA corresponded to elevated mRNA levels of these enzymes. However, levels of CYP1A2 mRNA were unaffected. Likewise, induction of NQO1 and CYP1A1/2 proteins and activities by 3-ABA did not correlate with mRNA levels. Similar discrepancies between induction of CYP1A mRNAs and protein levels were observed also by others (Dagawa et al., 1998; Chen et al. 1998; Dickins, 2004). It has been reported that some inducers might prolong half-lives of mRNAs, while others increase transcription. Moreover, half-lives of mRNAs are usually much shorter than those of proteins (Pan et al, 1993; Dagawa et al., 1998; Dickins, 2004). Detailed analyses of the time-dependence of the expression levels of mRNAs and proteins of the tested enzymes were not performed in this study, but are planned to be done in future. They might answer the questions whether the transient induction of the mRNAs of CYP1A and NQO1, or the different half-lives for their mRNAs and proteins, and/or the effects of 3-NBA and 3-ABA on the stability of mRNAs and proteins of these enzymes might be the reasons for our observation.

Recently, we have demonstrated that 3-ABA is also oxidatively activated to species binding to DNA by several peroxidases *in vitro* (human MPO, bovine LPO, ovine COX and horseradish peroxidase) (Fig. 1) and *in vivo*, mainly in kidneys and urinary bladders of mice treated with 3-ABA (Arlt et al., 2006a). In contrast to these findings, 3-ABA activation in livers of these mice was predominantly mediated by CYP-dependent reactions, but participation of additional enzymes, including peroxidases in this process could not be excluded (Arlt et al., 2006a). *N*-oxidation of the amino group of 3-ABA is responsible for formation of 3-ABA-derived DNA adducts (Arlt et al., 2004b; 2006a). In the present work we

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showed that hepatic cytosols from rats treated with 3-NBA and 3-ABA in the presence of hydrogen peroxide were capable of activating 3-ABA to form DNA adducts, while hepatic cytosols of control rats were ineffective. The necessity of the presence of hydrogen peroxide for 3-ABA-derived DNA adduct formation by cytosols indicates that 3-ABA activation might be associated with an increase in peroxidase activities in hepatic cytosols of rats treated with 3-NBA and 3-ABA. However, the levels of DNA adducts generated by hydrogen peroxide-mediated 3-ABA activation were one order of magnitude lower than those by hepatic microsomes. Although the expression and activities of peroxidases in hepatocytes or extracellular space in rat livers and the effect of xenobiotics on these enzymes need to be further investigated, the present study suggests that peroxidases might be present in rat hepatic cytosols and are involved, at least partially, in activation of 3-ABA. Moreover, the findings that peroxidases are effective in 3-ABA activation may be of greater significance in extrahepatic tissues rich in peroxidases such as lung, kidneys and urinary bladder, which express MPO and/or COX (Eling et al., 1990; Wiese et al., 2001). In this context, it is noteworthy that 3-ABA is the major metabolite of 3-NBA in human fetal bronchial, rat alveolar type II, rat epithelial bronchial, and rat mesenchymal lung cells (Borlak et al., 2000). Of note, hydrogen peroxide required for MPO-mediated 3-ABA oxidation can also be supplied by xanthine oxidase, a mammalian nitroreductase involved in the bioactivation of the nitroaromatic parent compound, 3-NBA (Borlak et al., 2000; Arlt et al., 2001; 2005). Therefore, a study evaluating the expression of enzymes capable of reducing 3-NBA (NQO1, xanthine oxidase) or oxidizing 3-ABA (CYPs, MPO, COX) and their efficiencies to activate these chemicals in lungs and kidneys of 3-NBA and 3-ABA-pretreated rats is under way in our laboratory.

In conclusion, the results of the present study show for the first time that 3-NBA and 3-ABA are capable of inducing rat hepatic NQO1 and CYP1A1/2, which are the predominant

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biotransformation enzymes involved in their own metabolic activation in both rats and humans. Both compounds increase also the hydrogen peroxide-dependent activation of 3-ABA in hepatic cytosol. By such effects, both 3-NBA and 3-ABA increase their **own** enzymatic activation to reactive DNA adduct forming species, thereby enhancing their own genotoxic potential.

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Footnotes

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Request for reprints: Marie Stiborová, Department of Biochemistry, Faculty of Science, Charles University, Albertov 230, 128 40 Prague 2, Czech Republic. Phone: +420-2-21951285; Fax: +420-2-21951283; E-mail address: stiborov@natur.cuni.cz

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Legends for figures

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; POR, NADPH:cytochrome P450 oxidoreductase; R = -COCH₃ or -SO₃H; 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.

FIG. 2. Induction of CYP1A1 (A,E) and -1A2 (B,F) and their enzymatic activity [EROD activity (C,G) and Sudan I oxidation D,H)] in livers of rats treated with 0.4, 4 or 40 mg/kg of body weight of 3-NBA (A-D) or 3-ABA (E-H). Mean values shown in figure represent results obtained from livers of three rats (n=3). Standard deviations were less than 10%. Inset in A and E: immunoblots of microsomal CYP1A1 and -1A2 from untreated and 3-NBA or 3-ABA-treated rats stained with antibody against rat CYP1A1. Microsomes isolated from rat livers were subjected to SDS-PAGE, proteins transferred to PVDF membranes and probed with antibody as described in Material and Methods. Values significantly different from control: *p<0.05, **p<0.01.

FIG. 3. Induction of NQO1 (A,C) and its enzymatic activity with menadione as a substrate (B,D) in livers of rats treated with 0.4, 4 or 40 mg/kg of body weight of 3-NBA (A,B) and 3-ABA (CD). Mean values shown in figure represent results obtained from livers of three rats (n=3). Standard deviations were less than 10%. Inset in A and C: immunoblots of cytosolic NQO1 from untreated and 3-NBA or 3-ABA-treated rats stained with antibody against human NQO1. Cytosols isolated from rat livers were subjected to SDS-PAGE, proteins transferred to PVDF membranes and probed with

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antibody as described in Material and Methods. Values significantly different from control: * $p < 0.05$, ** $p < 0.01$.

FIG. 4. Autoradiographic profile of DNA adducts generated (A) by 3-NBA after its activation with cytosols isolated from livers of rats treated with 40 mg/kg body weight of 3-NBA and (B) by 3-ABA after its activation with microsomes isolated from livers of rats treated with 40 mg/kg body weight of 3-NBA by using the n-butanol enrichment version of the ^{32}P -postlabelling assay. Spot 1 = dA- N^6 -ABA, spot 3 = dG- N^2 -ABA, spots 4/5 = dG-C8- N -ABA.

FIG. 5. DNA adduct formation by 3-NBA activated with cytosols isolated from livers of rats, control (uninduced) or pretreated with 40 mg/kg body weight of 3-NBA (A) or 3-ABA (B) and the DNA-adduct levels obtained by adding either acetyl-CoA as a cofactor for NATs or PAPS as a cofactor for SULTs. Mean RAL (relative adduct labelling) shown in the figure represent total levels of DNA adducts of four determinations (duplicate analyses of two independent *in-vitro* incubations).

FIG. 6. DNA adduct formation by 3-ABA activated with microsomes in the presence of NADPH, a cofactor of CYP-dependent enzymatic system (A) and cytosols in the presence of hydrogen peroxide, a cofactor for peroxidases (B) isolated from livers of rats, control (uninduced) or pretreated with 40 mg/kg body weight of 3-NBA or 3-ABA. Mean RAL (relative adduct labeling) shown in the figure represent total levels of DNA adducts of four determinations (duplicate analyses of two independent *in-vitro* incubations).

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TABLE 1
Expression of mRNA of CYP1A1 and -1A2, and NQO1

	CYP1A1		CYP1A2		NQO1	
	Δc_T^a	Fold Change	Δc_T	Fold Change	Δc_T	Fold Change
Control rats	6.37±0.31	-	-5.15±0.32	-	4.77±0.23	-
3-NBA 40 mg/kg body weight	4.12±0.21	4.8	-3.82±0.33	0.4	2.63±0.10	4.4
3-ABA 40 mg/kg body weight	6.96±0.13	0.7	-3.62±0.08	0.4	5.10±0.11	0.8

^aResults shown are mean ± S.D. from data found for three rats.

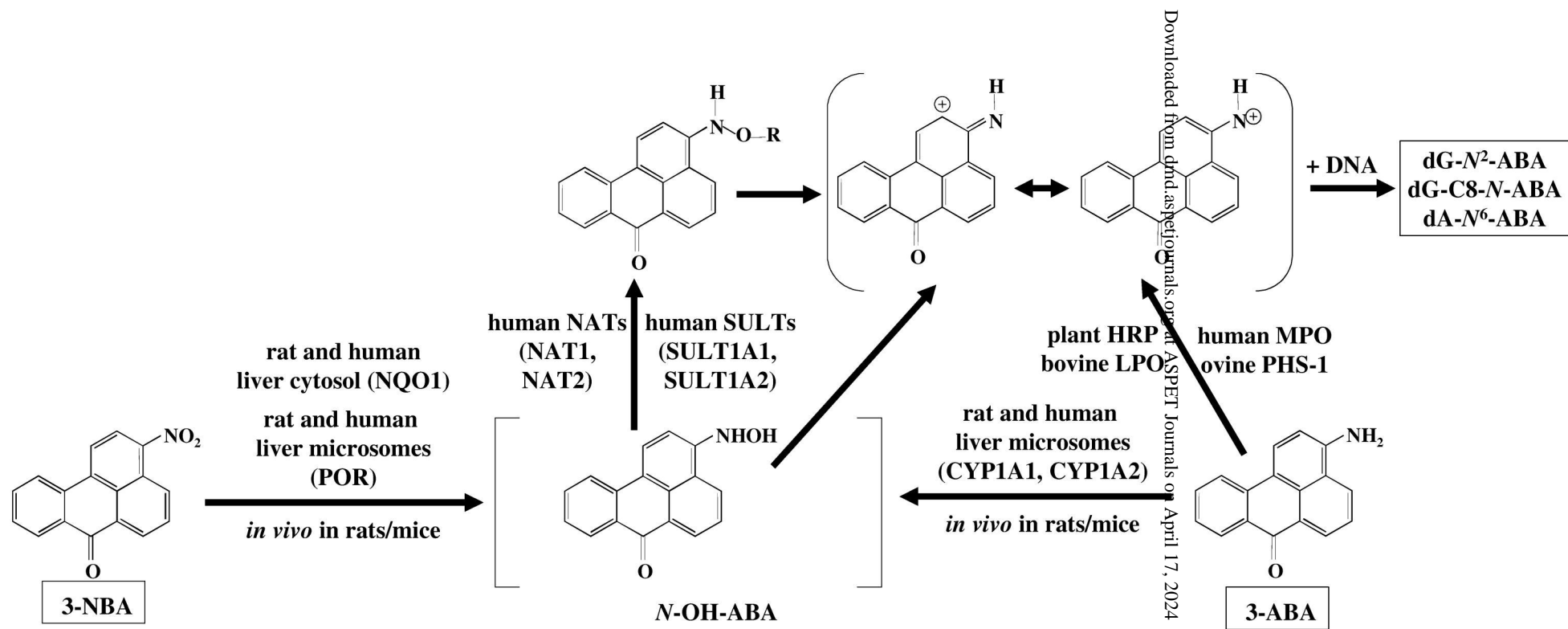


Figure 1

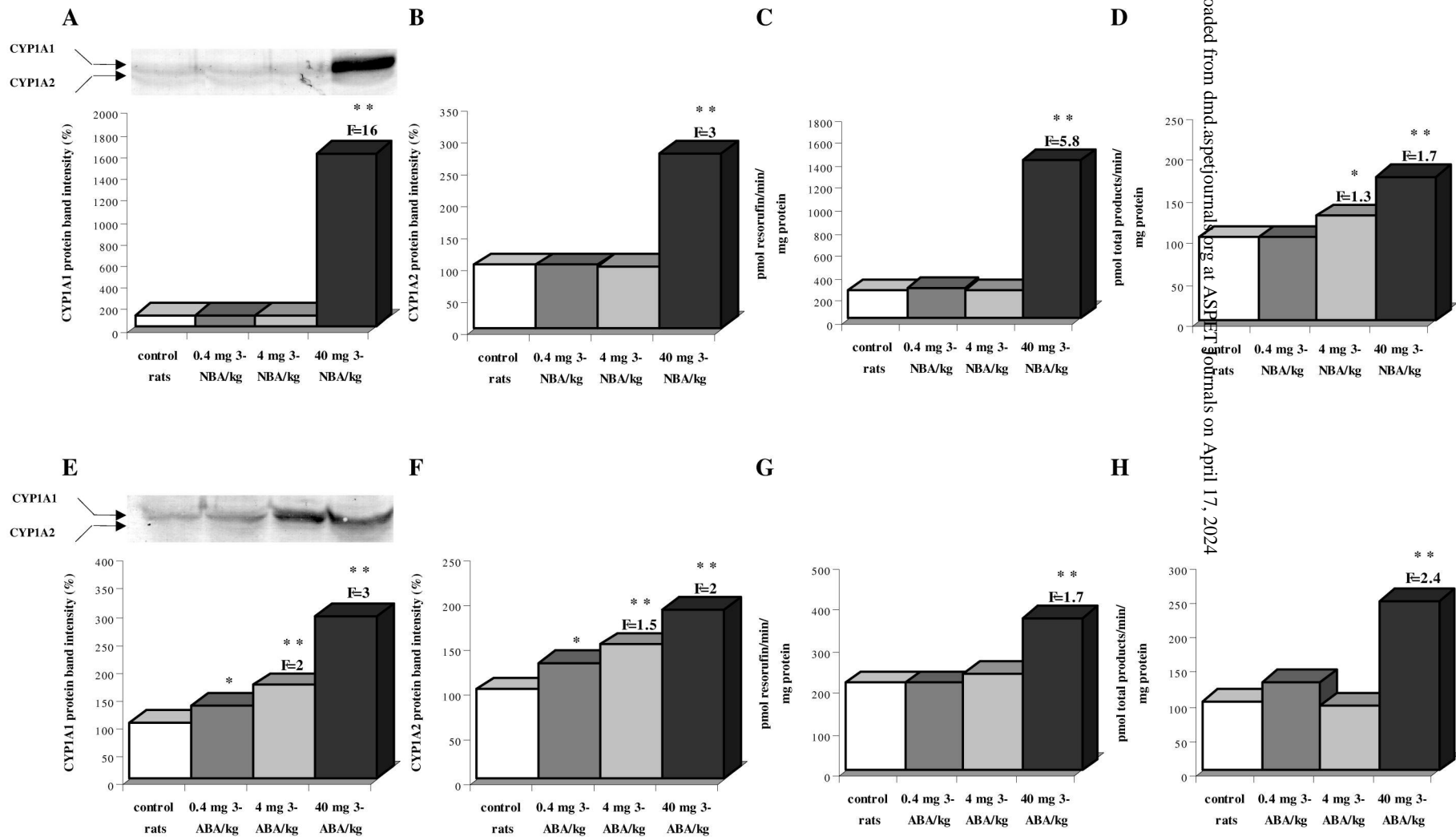


Figure 2

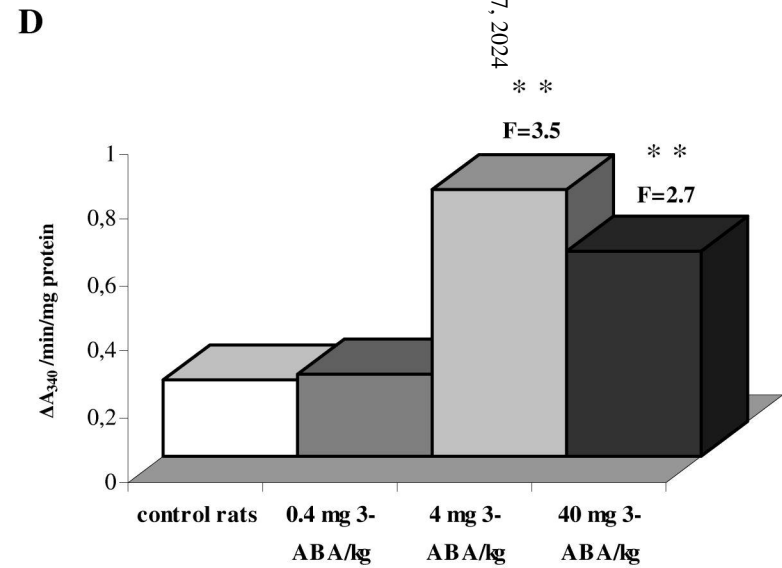
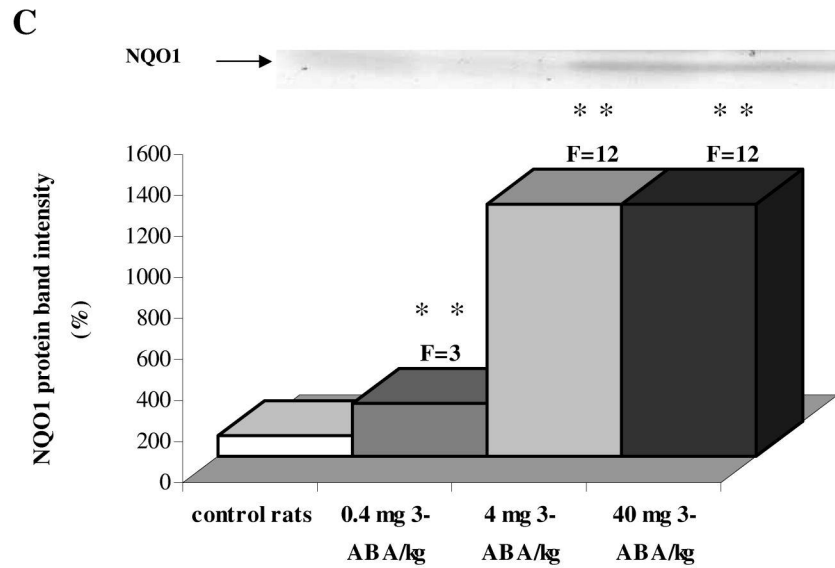
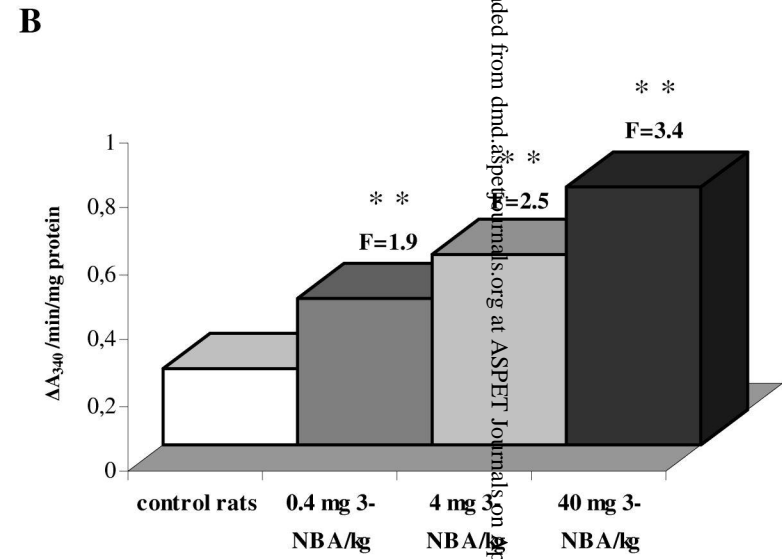
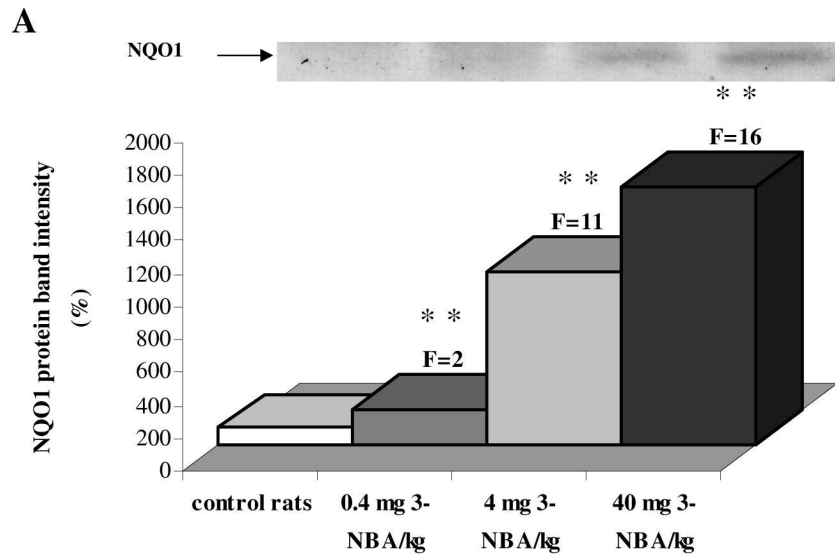
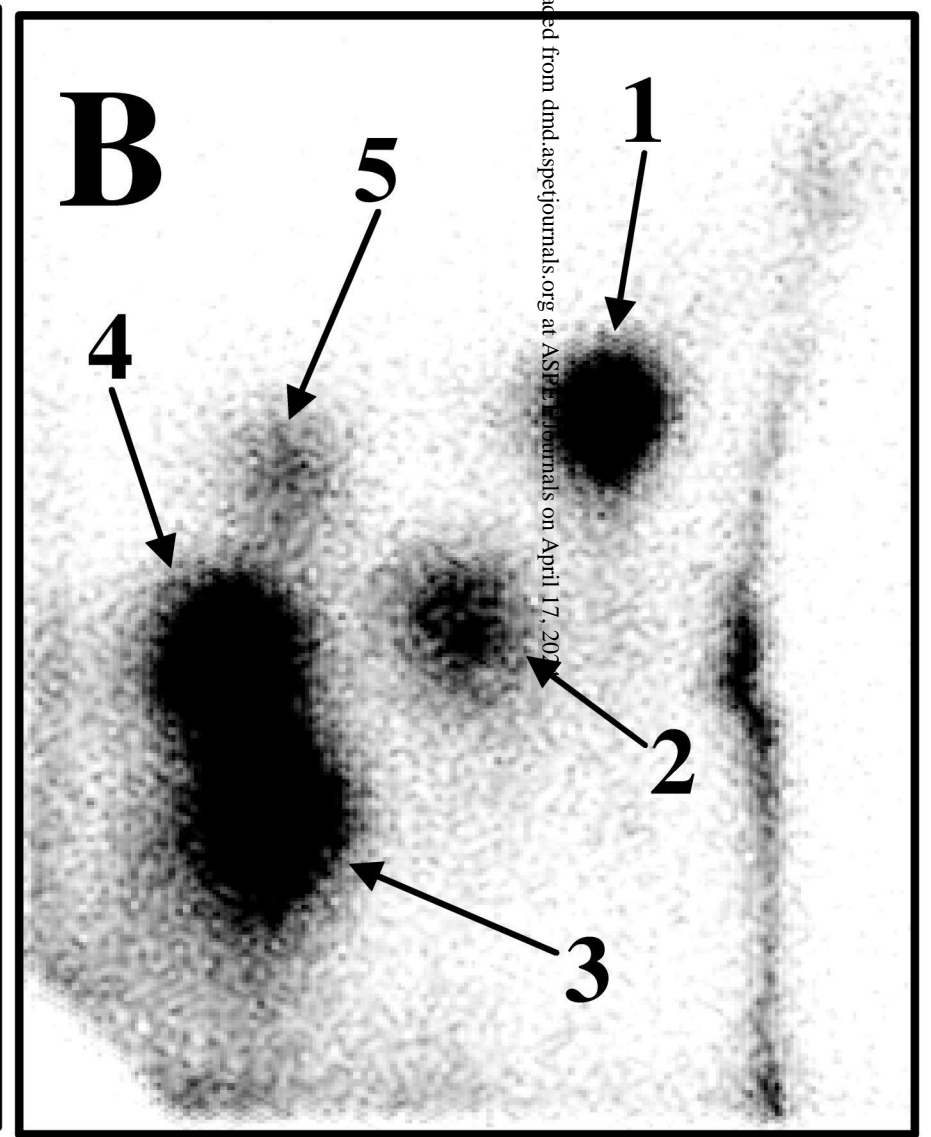
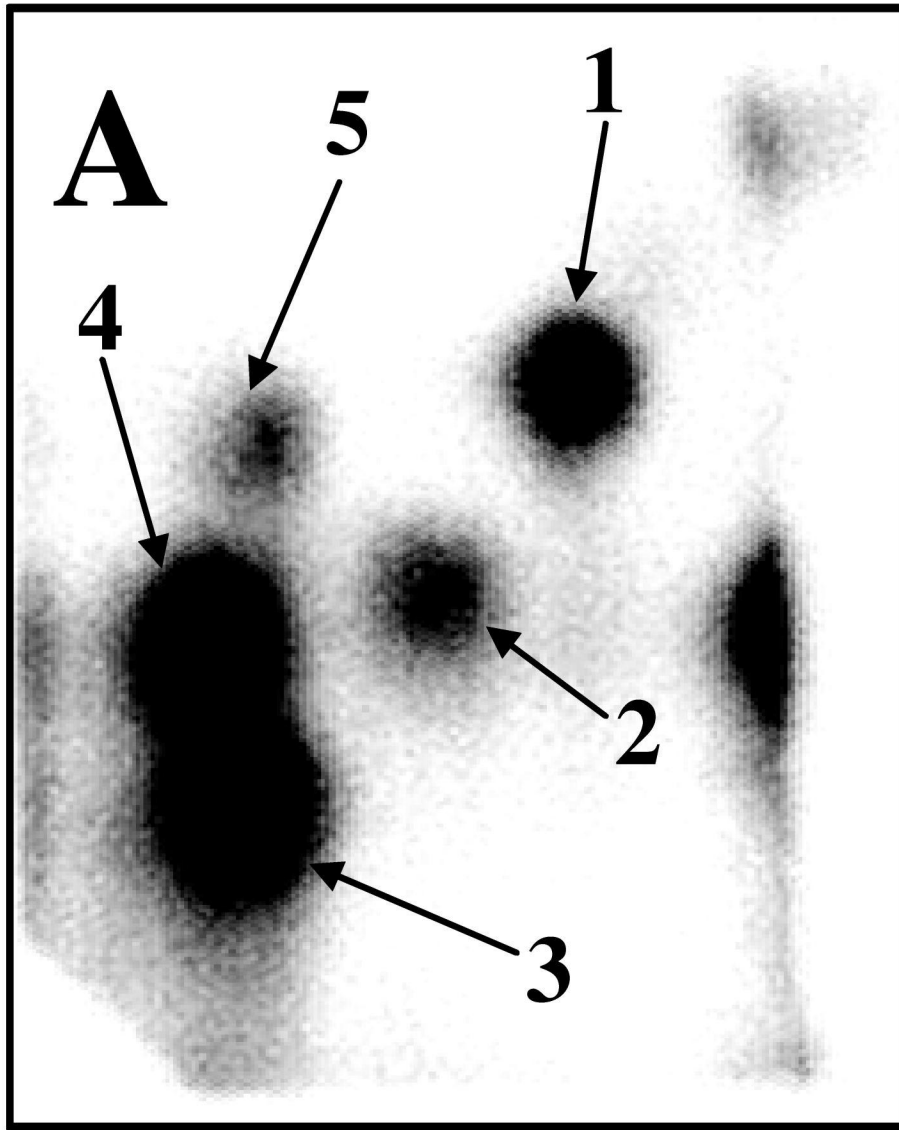


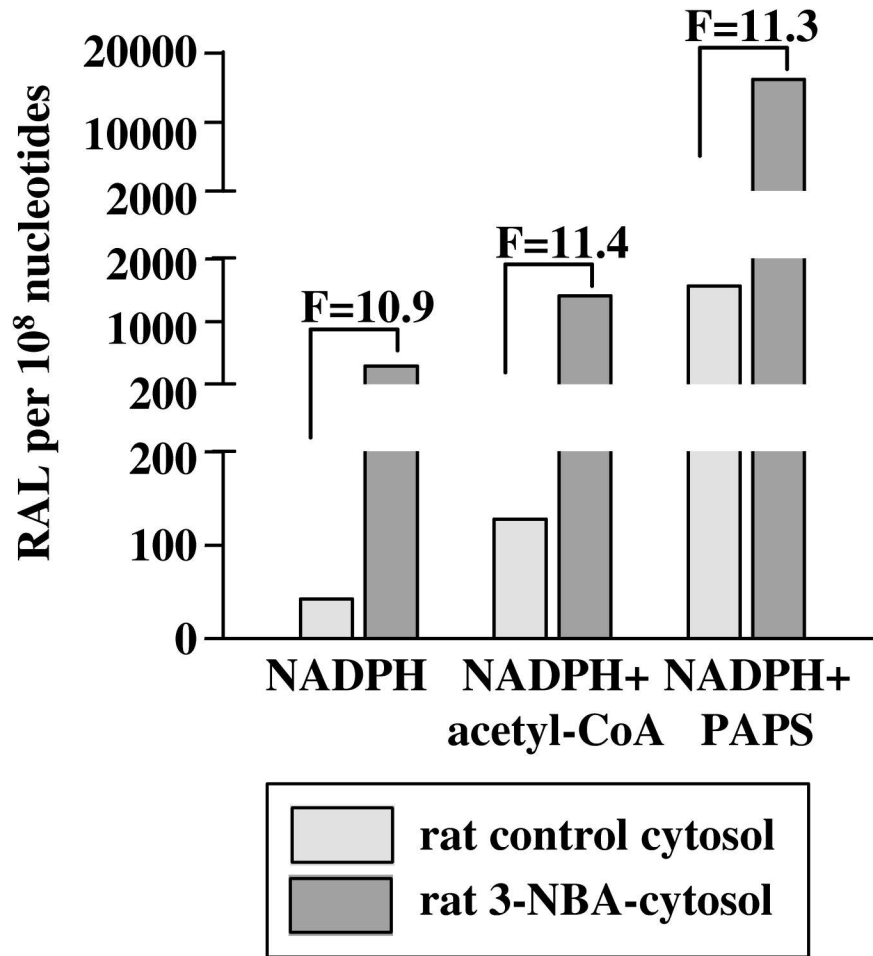
Figure 3



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Figure 4

A



B

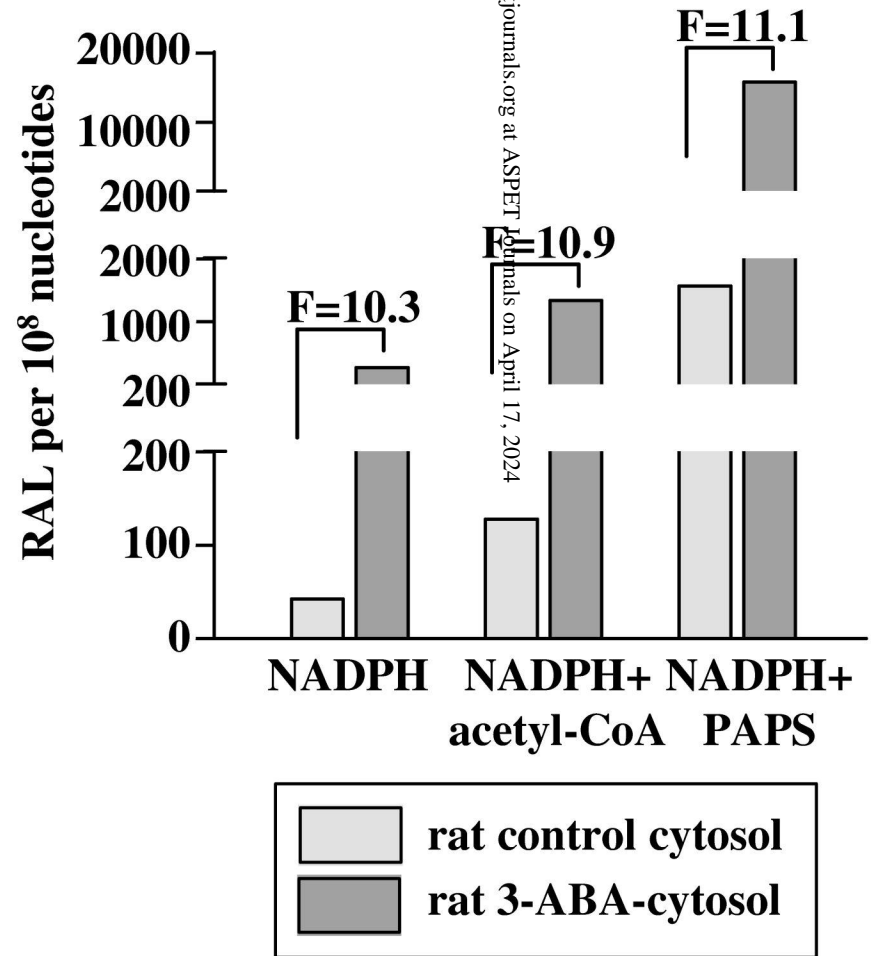


Figure 5

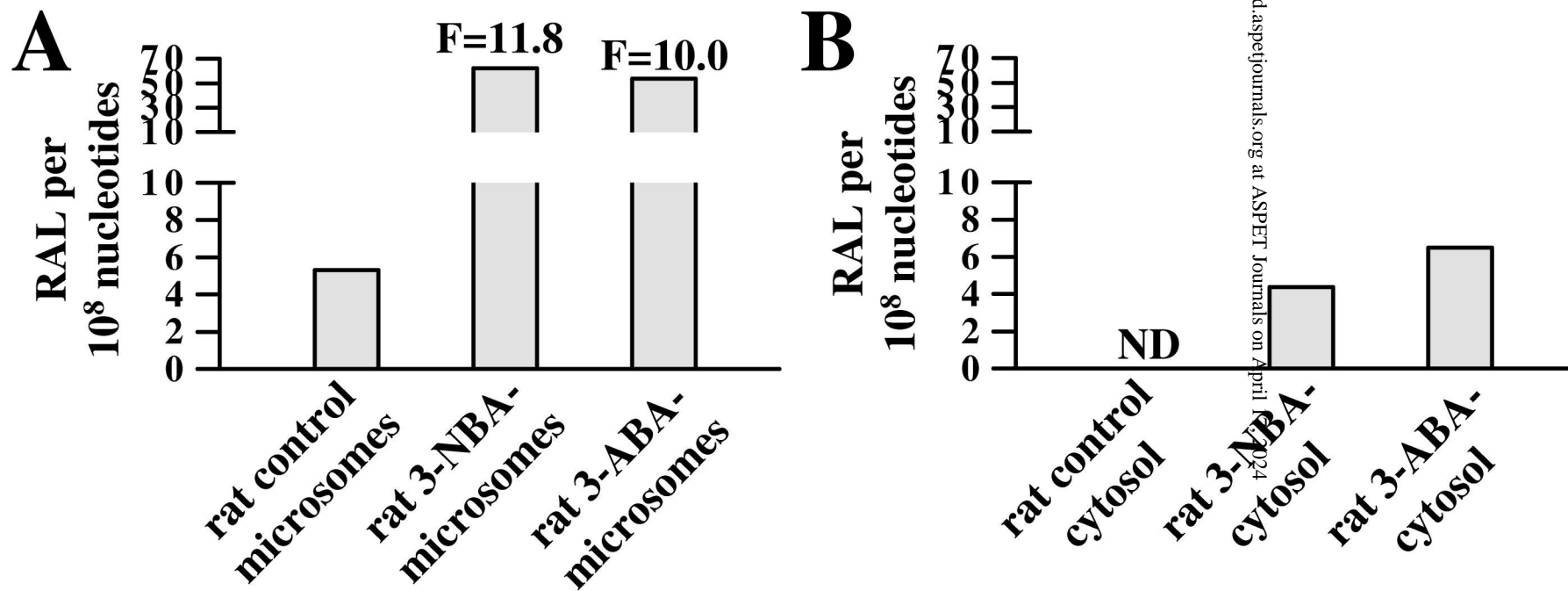


Figure 6