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Induction of biotransformation enzymes by the carcinogenic air pollutant 3-nitrobenzanthrone in liver, kidney and lung after intratracheal instillation in rats*

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Abbreviations: 3-ABA, 3-aminobenzanthrone; Ah, aryl hydrocarbon; COX, cyclooxygenase; CYP, cytochrome P450; dA, deoxyadenosine; dA-N^6-ABA, 2-(2'-deoxyadenosin-N^6-yl)-3-aminobenzanthrone; dG, deoxyguanosine; dG-N^2-ABA, 2-(2'-deoxyguanosin-N^2-yl)-3-aminobenzanthrone; dG-C8-*N*-ABA, *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone; EROD, 7-ethoxyresorufin *O*-deethylation; LPO, lactoperoxidase; MPO, myeloperoxidase; MROD, 7-methoxyresorufin *O*-demethylation; NAT, *N*,*O*-acetyltransferase; 3-NBA, 3-nitrobenzanthrone; *N*-OH-3-ABA, *N*-hydroxy-3-aminobenzanthrone; NQO1, NAD(P)H:quinone oxidoreductase; RAL, relative adduct labelling; SDS, sodium dodecyl sulphate; SULT, sulfotransferase; TLC, thin-layer chromatography;

Abstract

3-Nitrobenzanthrone (3-NBA), a carcinogenic air pollutant, was investigated for its ability to induce cytochrome P450 (CYP) 1A1/2 and NAD(P)H:quinone oxidoreductase (NQO1) in liver, kidney and lung of rats treated by intratracheal instillation. **The organs used were from a previous study performed to determine the persistence of 3-NBA-derived DNA adducts in target and non-target tissues [Bieler *et al.*, Carcinogenesis 28 (2007) 1117-1121].** NQO1 is the enzyme reducing 3-NBA to *N*-hydroxy-3-aminobenzanthrone (*N*-OH-3-ABA) and CYP1A enzymes oxidize human metabolite of 3-NBA, 3-aminobenzanthrone (3-ABA), to the same reactive intermediate. 3-NBA and 3-ABA are both activated to species forming DNA adducts by cytosols and/or microsomes isolated from rat lung, the target organ for 3-NBA carcinogenicity, and from liver and kidney. Each compound generated the same five DNA adducts detectable by ^{32}P-postlabeling. When hepatic cytosols from rats treated with 0.2 or 2 mg/kg bw of 3-NBA were incubated with 3-NBA, DNA adduct formation was 3.2- and 8.6-fold higher, respectively, than in incubations with cytosols from control animals. Likewise, cytosols isolated from lungs and kidneys of rats exposed to 3-NBA more efficiently activated 3-NBA than those of control rats. This increase corresponded to an increase in protein levels and enzymatic activities of NQO1. Incubations of hepatic, pulmonary or renal microsomes of 3-NBA-treated rats **with 3-ABA** led to an up to 9.6-fold increase in DNA adduct formation relative to controls. The highest **induction in DNA adduct levels** was **found in lung**. The stimulation of DNA adduct formation correlated with expression of CYP1A1/2 induced by the intratracheal instillation of 3-NBA. The results demonstrate that 3-NBA induces NQO1 and CYP1A1/2 in livers, lungs and kidneys of rats after intratracheal instillation, thereby enhancing its own genotoxic and carcinogenic potential.

1. Introduction

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 [1-4]. Nitro-aromatic compounds are widely distributed environmental pollutants found in 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 [1-4].

The nitroaromatic 3-nitrobenzanthrone (3-nitro-7*H*-benz[*de*]anthracen-7-one, 3-NBA) **(Fig. 1)** occurs in diesel exhaust and in airborne particulate matter [5-8]. The main metabolite of 3-NBA, 3-aminobenzanthrone (3-ABA) **(Fig. 1)** [9,10], has been found in urine samples of salt mine workers occupationally exposed to diesel emissions [6], demonstrating human exposure to 3-NBA. 3-NBA is carcinogenic in rats, causing lung tumours after intratracheal instillation [7]. It is also an exceptionally potent mutagen in the Ames *Salmonella typhimurium* assay, scoring more than 6 million revertants per nanomole in strain YG1024 overexpressing bacterial nitroreductase and *O*-acetyltransferase [5]. 3-NBA has also been shown to be genotoxic in several other short-term tests and in the transgenic Muta Mouse assay [11,12]. Its genotoxicity has been further documented by the detection of specific DNA adducts formed *in vitro* as well as *in vivo* in rodents in various tissues [11,12,14-22].

In **liver, lung and kidney** most of the metabolic activation of 3-NBA is attributable to cytosolic human and rat NAD(P)H:quinone oxidoreductase **(NQO1). Human** *N*,*O*-acetyltransferase (NAT), NAT2, followed by NAT1, sulfotransferase (SULT), SULT1A1 and, to a lesser extent, SULT1A2 are the major activating phase II enzymes [10,18,23,24]. **Human** hepatic NADPH:cytochrome P450 (CYP) reductase also effectively activates 3-NBA [17], but in mice, 3-NBA is predominantly activated by NQO1 rather than NADPH:CYP reductase [18] (Fig. 1).

CYP1A1 and 1A2 (CYP1A1/2) are essential for the oxidative activation of 3-ABA in human and rat livers forming the same DNA adducts that are formed *in vivo* by 3-ABA or 3-NBA [25,26]. However, other organs also have the metabolic capacity to activate 3-ABA to form DNA adducts, independent from the CYP-mediated oxidation in the liver [27]. Previous results indicate that besides CYP enzymes expressed in several extra-hepatic tissues, peroxidases may play a role in the oxidative activation of 3-ABA [27]. Mammalian prostaglandin H synthase (cyclooxygenase, COX), lactoperoxidase (LPO) and myeloperoxidase (MPO) were found to be effective in activating 3-ABA [27] (Fig. 1). In animal experiments with Wistar rats the major role of CYP1A1 in 3-ABA activation in lungs and kidneys rather than peroxidases was elucidated [24,28]. In the same rat model we treated rats with single i.p. **doses** of 0.4, 4 or 40 mg/kg body weight (bw) of 3-NBA and saw that 3-NBA induces the major enzymes activating itself and its metabolite 3-ABA in livers [23], lungs and kidneys [24], thereby enhancing its own genotoxic and carcinogenic potential. Since inhalation is the major route by which airborne materials gain access to the body, primary exposure of the lungs to 3-NBA would constitute a better model system. However, experimental inhalation systems, with appropriate generation and characterization of exposure atmospheres, are expensive to acquire and maintain [29]. Therefore, direct instillation of a test material into the lungs *via* the trachea has been employed in many studies as an alternative exposure to inhalation. Instillation has also certain advantages over inhalation, the foremost being that the actual dose delivered to the lungs of each animal can be defined accurately.

Therefore, the objective of the present study was to evaluate whether the major enzymes activating 3-NBA (NQO1) and 3-ABA (CYP1A) are induced after a single intratracheal instillation of 3-NBA in the lungs, livers and kidneys of rats and if so, whether this induction also leads to higher DNA adduct levels, when cytosols and microsomes from these organs are incubated with 3-NBA and 3-ABA. The organs used in this study were from rats treated in an experiment to determine the persistence of 3-NBA-derived DNA adducts in target and non-target tissues [21,22].

2. Material and Methods

2.1. Chemicals

NADPH, deoxyadenosine (dA) 3'-monophospate, deoxyguanosine (dG) 3'-monophosphate, menadione (2-methyl-1,4-naphthoquinone) and calf thymus DNA were from Sigma Chemical Co (St Louis, MO, USA); 7-ethoxyresorufin and 7-methoxyresorufin from Fluka Chemie AG (Buchs, Switzerland). Enzymes and chemicals for the ^{32}P-postlabeling assay were obtained from sources described [30]. All these and other chemicals were reagent grade or better.

2.2. Synthesis of 3-NBA and 3-ABA

3-NBA and 3-ABA were synthesized as described [15] and their authenticity was confirmed by UV spectroscopy, electrospray mass spectra and high field proton NMR spectroscopy.

2.3. Animal experiments

The study was conducted in accordance with the German Regulations for the Care and Use of Laboratory Animals, which is in compliance with Declaration of Helsinki. **Lungs, livers and kidneys used in this study were from rats (female Sprague–Dawley rats, *n* = 3 per group) treated with a single dose of 0.2 or 2 mg/kg body weight (bw) of 3-NBA by intratracheal instillation from a previous experiment to determine the persistence of 3-NBA-derived DNA adducts in target and non-target tissues [21,22]**. Animals were killed 2 days after treatment by cervical dislocation. Lungs, livers and kidneys were removed, immediately after sacrifice, frozen in liquid nitrogen and stored at -80° C until isolation of microsomal and cytosolic fractions.

2.4. Preparation of microsomal and cytosolic fractions

Microsomal and cytosolic fractions were isolated from the livers, lungs and kidneys of **rats, as** described [**31,32**]. Both subcellular preparations were analyzed for the presence of 3-NBA by 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. Eluent was 70% methanol in water, at a flow rate of 0.6 ml min^{-1}, and detection was at 254 nm. The standard of 3-NBA eluted with a retention time of 8.2 min. No 3-

NBA was detectable in microsomal and cytosolic fractions from rats that had been treated with this compound (data not shown).

2.5. 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, cofactor for cytosolic NQO1 (1 mM NADPH), pooled liver, lung and kidney cytosolic sample from 3 rats, treated either with vehicle (control) or with 0.2 or 2 mg/kg bw of 3-NBA (0.5 mg of cytosolic protein), 30 µM 3-NBA (dissolved in 7.5 µl dimethylsulfoxide) and 0.5 mg of calf thymus DNA (2 mM dNp). The reaction was initiated by adding 3-NBA. Incubations with rat cytosols were carried out at 37°C for 3 hr; the cytosol-mediated 3-NBA-derived DNA adduct formation was found to be linear up to 4 hr [18]. Control incubations were carried out either (*i*) without activating system (cytosol), (*ii*) without NADPH, (*iii*) without DNA or (*iv*) without 3-NBA. After extraction with ethyl acetate, DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described [18].

2.6. Microsomal incubations

Incubation mixtures, in a final volume of 750 µl, consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, hepatic, pulmonary and renal microsomal samples from 3 rats **(pooled samples)**, either control or treated with 0.2 or 2 mg/kg bw of 3-NBA (0.5 mg of microsomal protein), 100 µM 3-ABA or 3-NBA (dissolved in 7.5 µl dimethylsulfoxide) and 0.5 mg of calf thymus DNA. The reaction was initiated by adding 3-ABA or 3-NBA and were carried out at 37°C for 2 hr; the microsomal-mediated 3-NBA (3-ABA)-derived DNA adduct formation was found to be linear up to 3 hr [17,25]. Control incubations were carried out either (*i*) without activating system (microsomes), (*ii*) with activating system and 3-ABA or 3-NBA, but without DNA or (*iii*) with activating system and DNA but without 3-ABA or 3-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 [25].

2.7. ^{32}P-Postlabeling analysis

^{32}P-Postlabeling analysis using butanol extraction, and thin layer chromatography (TLC) and HPLC were performed as described [19]. Enrichment by butanol extraction has been shown to yield more adduct spots and a better recovery of 3-NBA (3-ABA)-derived DNA adducts than using enrichment by nuclease P1 digestion [15,19]. DNA adduct spots were numbered as reported [15,19]. As reference compounds deoxyadenosine (dAp) and deoxyguanosine (dGp) 3'-monophosphates (4 μmol/ml) (Sigma) were incubated with 3-NBA (300 μM) activated by XO (1 U/ml) (Sigma) in the presence of hypoxanthine and analyzed as **described** [13]. 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 with dAp or dGp and analysed as **described** [19].

2.8. Isolation of CYP1A1 and CYP1A2

Recombinant rat CYP1A1 protein was purified to homogeneity from membranes of *Escherichia coli* transfected with a modified *CYP1A1* cDNA [33,34], in the laboratory of H. W. Strobel (University of Texas, Medical School of Houston, Texas, USA) by P. Hodek (Charles University, Prague, Czech Republic). Rat CYP1A2 was purified to homogeneity from hepatic microsomes of rats treated with β-naphthoflavone **as described** [35].

2.9. Preparation of antibodies

Leghorn chicken were immunized subcutaneously three times a week with rat recombinant CYP1A1 or rat CYP1A2 antigens (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 [33,34].

Rabbit polyclonal antibodies against human NQO1 were from Sigma Chemical Co (St Louis, MO, USA).

2.10. Estimation of CYP1A1, CYP1A2 and NQO1 protein content in microsomes and cytosols of rat liver, lung and kidney

Immunoquantitation of rat hepatic, pulmonary and renal microsomal CYP1A1, CYP1A2 and of cytosolic NQO1 was done by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis. Samples containing 70 μg microsomal or cytosolic proteins were subjected to electrophoresis on SDS/10% polyacrylamide gels [23,24]. After migration, proteins were transferred onto polyvinylidene difluoride membranes. Rat CYP1A1, CYP1A2 and NQO1 proteins were probed with the chicken polyclonal antibodies raised against CYP1A1 or CYP1A2 and rabbit antibodies against human NQO, respectively, as reported elsewhere [23,24,28,33,35]. The antibodies against rat CYP1A1 and those against rat CYP1A2 recognize CYP1A1 and CYP1A2 in liver microsomes always as two protein bands, corresponding to CYP1A1 and CYP1A2. The antibodies against human recombinant NQO1 recognize this enzyme in rat hepatic, pulmonary and renal cytosols as one protein band. Rat recombinant CYP1A1 and CYP1A2 (in Supersomes[TM], 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 CYP1A2 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 chromogenic substrate [23,24,28,33,35]. Band intensities were quantified by Elfoman software.

2.11. CYP1A1, CYP1A2 and NQO1 enzyme activity assays

The microsomal samples were characterized for CYP1A1/2 activity using 7-ethoxyresorufin *O*-deethylation (EROD) activity and for CYP1A2 using 7-methoxyresorufin *O*-demethylation (MROD) activity [33,34,36]. One unit of EROD and MROD activities is defined as the amount of enzyme catalyzing the production of 1 pmol of resorufin in 1 min. The cytosolic samples were characterised for NQO1 activity. NQO1 activity was measured essentially as described by Ernster [37], but because of low activities of this enzyme in rat cytosolic samples, the method was

improved by addition of cytochrome *c*. The standard assay system contained 25 mM Tris-HCl (pH 7.4), 0.07% bovine serum albumin, 200 μM NADH and 10 μM menadione (2-methyl-1,4-naphthoquinone) dissolved in methanol and 77 μM cytochrome *c*. The enzyme activity was determined by following the conversion of cytochrome *c* at 550 nm on a Hewlett-Packard 8453 diode array spectrophotometer. One unit of activity is defined as the amount of enzyme catalyzing the conversion of 1 μmol of cytochrome *c* in 1 min.

3. Results

3.1. The effect of intratracheal instillation of 3-NBA to rats on expression of enzymes activating 3-NBA and 3-ABA

In the present study, Western blots using polyclonal antibodies raised against NQO1, CYP1A1 and CYP1A2 showed that the expression of all these enzymes was induced after 2 days in **liver, lung and kidney of** rats that received 3-NBA by intratracheal instillation (Figs. 2 and 3).

The NQO1 protein was expressed in all organs of control (untreated) rats, being highest in the liver; 3.4- and 6.2-fold higher levels were found in liver than in lung and kidney, respectively (Fig. 2). The levels of NQO1 protein were enhanced **in liver** after intratracheal instillation of rats with 0.2 or 2 mg 3-NBA/kg bw, by 5.8- and 8.7-fold, respectively (Fig. 2). The expression of protein of this enzyme was also induced **dose-dependently** in kidney and lung, but to a lesser extent than in the **liver (Fig**. 2). The increased expression of NQO1 in the liver was paralleled by an increased activity of this enzyme (Table 1). Because of the low activity of NQO1 in kidney and lung, the effect of treating rats with 3-NBA on NQO1 activity in these organs was not evaluated.

The expression of CYP1A1 and CYP1A2 in microsomes was also induced in rats after intratracheal instillation with 3-NBA. Chicken antibodies raised against rat CYP1A1 or CYP1A2 used in this work each recognized both CYP1A isoforms because of a high degree of homology in their proteins [38]. The efficiency of 3-NBA to induce expression of CYP1A1 was higher than that of CYP1A2 in all organs tested in this study. In lung even no induction of CYP1A2 expression was found at all (Fig. 3). The EROD and MROD activities, which were used as markers of CYP1A1/2 and CYP1A2 activities, respectively [38], were induced by 3-NBA in livers (Table 1). **Whereas** the increase in EROD activity was much lower than the increase in CYP1A1 expression, **induction of MROD closely correlated with the increase in CYP1A2 protein expression in rat liver**. Because of the low basal levels of CYP1A enzymes in kidney and lung, the effect of treating rats with 3-NBA on their activities with EROD and MROD was not determined.

3.2. The effect of intratracheal instillation of 3-NBA to rats on activation of 3-NBA by hepatic, pulmonary and renal cytosols and microsomes

Cytosolic samples isolated from liver, kidney and lung of rats 2 days **after intratracheal instillation of 3-NBA** were capable of reductively activating 3-NBA to species forming **a cluster of 5 adducts in calf thymus DNA** (see **spots 1−5 in Figure** 4A for the liver **cytosol). Thin**-layer chromatograms of ^{32}P-labeled 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). **Only in DNA from control incubations containing liver cytosols without NADPH 3-NBA-DNA adducts were found (up to 2.6±0.3 adducts per 10^8 deoxynucleotides), formed by cytosolic NQO1 mediated by the basal NADPH level present in hepatic cytosols.** Cochromatographic analysis of individual spots on HPLC confirmed that adduct spots 1−5 that are formed with rat hepatic, pulmonary and renal 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 and 5).

In the presence of NADPH, hepatic cytosol from control rats was 2.6- and 4.7-fold more effective to activate 3-NBA than cytosols from kidney and lung, respectively (**Fig. 5**). 3-NBA was more effectively activated by cytosols from all organs of rats treated with 3-NBA in a dose dependent manner (**Fig. 5**). Up to 8.6-fold higher levels of 3-NBA-derived DNA adducts were found in incubations with hepatic cytosols of rats treated with 2 mg 3-NBA/kg bw relative to those of control rats, whereas only 2.3- and 2-fold higher levels of adducts were found using cytosols of kidney and lung, respectively (**Fig. 5**).

Liver microsomes of control rats and those treated with 3-NBA activated 3-NBA. Only a 1.7-fold increase in 3-NBA-derived DNA adduct levels was found in liver microsomes isolated from rats treated with 2 mg 3-NBA/kg bw (Table **2**). Because CYP1A1/2 were found to also activate 3-

NBA [17], this increase is probably caused by induction of these enzymes. No 3-NBA-derived DNA adducts were however detectable after incubating 3-NBA with any of the pulmonary or renal microsomes and NADPH (data not shown). Hence, it seems that the activities of microsomal NADPH:CYP reductase and/or CYP are not sufficient to activate 3-NBA to an extent that DNA adduct formation is detectable in these extrahepatic organs.

3.3. The effect of intratracheal instillation of 3-NBA to rats on activation of 3-ABA by rat hepatic, pulmonary and renal microsomes

In the presence of NADPH, rat liver, lung and kidney microsomes were capable of oxidizing 3-ABA to form DNA adducts (see Figure 4B). Microsomal activation of 3-ABA generated a pattern of DNA adducts consisting of a cluster of five adducts (spots 1-5 in Figure 4B for the liver **microsomes). No DNA adducts were generated without NADPH a cofactor of CYP-mediated system, (data not shown).** In the presence of NADPH, liver microsomes of control rats 6.4- and 12.4-fold more efficiently activated 3-ABA than microsomes isolated from kidney and lung, respectively (**Fig. 6**). Liver, lung and kidney microsomes isolated from rats after intratracheal instillation with 2 mg 3-NBA/kg bw exhibited 3.9-, 9.7- and 3.3-fold higher efficiencies to activate 3-ABA, respectively, than microsomes from control rats (**Fig. 6). Chromatograms** of DNA digests from control incubations carried out in parallel without microsomes, without NADPH, without DNA, or without 3-ABA were all devoid of adduct spots in the region of interest (data not shown).

4. Discussion

The present study has increased our knowledge on the potential of 3-NBA to induce several biotransformation enzymes after intratracheal instillation that are responsible for metabolic activation of this carcinogen (NQO1) and its human metabolite, 3-ABA (CYP1A1/2), in the lung, the target organ for 3-NBA carcinogenicity, in the liver and kidney of rats. The rat was used as an experimental model on the basis that the same enzymes activate 3-NBA and 3-ABA in livers of rat and human [15,17,18,23,25,27]. Therefore, the results should provide some indication of what might occur in **tissues** of humans **exposed** to this carcinogenic pollutant.

Recently, we have shown that in Wistar rats treated i.p with a single dose of 0.4, 4 or 40 mg/kg bw 3-NBA acts as an inducer of NQO1 and CYP1A1/2 **in liver**, lung and kidney [23,24]. This induction was found to lead to an increase in the activities of these enzymes and in their potential to activate 3-NBA in cytosols (NQO1) and 3-ABA in microsomes (CYP1A) to species forming DNA adducts [23,24]. 3-NBA delivered i.p. is absorbed *via* the mesenteric veins and lymphatic systems, and passes through the liver. Thus, its concentration and effect in this tissue was higher than in the distal tissues such as lung and kidney (induction of NQO1 and CYP1A1 expression in liver of these rats was up to 5- and 2-fold higher than in lungs and kidneys, respectively) [23,24]. Since **human** exposure to 3-NBA occurs primarily *via* the respiratory tract and inhaled particles, we here evaluated the potential of 3-NBA administered by intratracheal instillation on enzymes capable of reducing 3-NBA or oxidizing 3-ABA. Sprague-Dawley rats were used for this study, because this animal model was successfully utilized to evaluate the long-term persistence of 3-NBA-derived DNA adducts after treatment by instillation [22]. **The present results confirm that hepatic, renal and pulmonary cytosols and microsomes from Sprague-Dawley rats activate 3-NBA and 3-ABA to a similar extent as Wistar rats, producing the same pattern of DNA adducts [23,24,28].**

The results of this work show that 3-NBA **induces** NQO1 and CYP1A1/2 also **after intratracheal** instillation to rats. The highest increase in expression levels of the enzymes studied

was found again in the liver, followed by kidney and lung, with exception of CYP1A1 expression in the kidney. **However,** expression of this protein in this organ in control rats was hardly detectable by Western blotting, which might overestimate its **relative** increase after 3-NBA treatment. The increase in expression of CYP1A1/2 and particularly NQO1 in the liver correlated with increased enzymatic activities and the activation of 3-NBA and its metabolite, 3-ABA, by hepatic cytosols and microsomes. The induction of CYP1A1/2 and NQO1 by 3-NBA is probably caused by binding **of** this planar molecule to the aryl hydrocarbon (Ah) receptor, which is known to be responsible for induction of CYP1A [38,39] and is one of the mechanisms responsible for induction of NQO1 (see [40] for a review).

The induction of NQO1 and CYP1A1 in the livers by 3-NBA administered by intratracheal instillation was higher than in the liver of rats treated by intraperitoneal injection **[23]**; a more than one order of magnitude lower 3-NBA dose produced similar expression levels. This finding underlines the risk for humans when exposed to this carcinogenic air pollutant by inhalation. All the more so, if one considers that the influence of 3-NBA instillation on the activation of its metabolite 3-ABA was highest in lungs. Indeed, the levels of 3-ABA-derived DNA adducts generated by lung microsomes isolated from 3-NBA-treated rats were almost 10 times higher than those formed by pulmonary microsomes from controls. This is the highest value of enzyme induction determined in this study.

As it is known that both NQO1 and CYP1A1/2 are expressed in **liver, lung and kidney not only in** rats but also **in humans** [35,40,42], **these enzymes** could contribute significantly and specifically to the metabolic activation of 3-NBA and/or 3-ABA in the human lung, thereby mediating 3-NBA carcinogenicity in this organ.

Inter-individual variations in susceptibility and variations in drug-metabolizing enzyme activities in target tissues appear to be important determinants of cancer risk [41,42]. Expression levels and activities of NQO1 and CYP1A1/2 differ considerably among individuals, because the enzymes are influenced by several factors, including smoking, drugs and environmental chemicals

[38,41,43]. It should be noted that besides induction of these enzymes by several compounds including 3-NBA [23,,24,38-40,44 and present paper], different activities of NQO1 and **CYP1A1/2** are also determined by genetic polymorphisms. Thus, genetic polymorphisms in **both enzymes could** be important determinants of a possible lung cancer risk from 3-NBA. So far two polymorphisms in the human *NQO1* gene have been found in the general population, one of them being associated with an increased risk of urothelial tumours [45] and paediatric leukaemia [46]. The polymorphic expression of CYP1A1 has been attributed to altered expression of the aryl hydrocarbon (Ah) receptor, the transcription factor that modulates its regulation, or the Ah receptor nuclear translocator (Arnt) protein, its associated transcription factor [40,44,47]. Moreover, the *CYP1A1* **and** *CYP1A2* **genes are** polymorphic [38,42,**48,49**]. So far, CYP1A1*2A, CYP1A1*2B and CYP1A1*4 polymophisms have been found that might be associated with cancers of lung, oesophagus or breast and with acute myeloid leukaemia [50-53], **while** the **CYP1A2*1F polymorphism might be associated with an increased risk of colorectal cancer [48]. In addition, the combination of NAT2 plus CYP1A1*2A, CYP1A1*2C, or CYP1A2*1F genotypes, and that of CYP1A2*1F plus CYP1A2*1C may define a group of persons who are genetically susceptible to colorectal cancer [49].**

In conclusion, the results of the present study show for the first time that 3-NBA is capable of inducing NQO1 and CYP1A1/2 enzymes in rat liver, lung and kidney after intratracheal instillation. **The result of such an exposure is both an enhanced reductive activation of 3-NBA and an oxidative activation of its metabolite 3-ABA to species forming DNA adducts, because 3-NBA induces both NQO1 and CYP1A.** Because 3-NBA is a potent genotoxin and a suspected human carcinogen [17-19,22], the results found in this work support our efforts in determining the risk of 3-NBA to human health.

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Table 1

Rat hepatic cytosolic NQO1 and microsomal CYP1A1/2 and CYP1A2 specific enzyme activities

Enzyme activity	Control rat	3-NBA-treated rat	
		0.2 mg/kg bw	2 mg/kg bw
NQO1^a [μmo/min.mg]	237.6 ± 20.1	1136.7 ± 25.6 (**4.8**)***	1742.5 ± 315.7 (**7.3**)***
CYP1A1/2^b (EROD) [pmo/min.mg]	10.5 ± 1.0	12.1 ± 1.2 (**1.2**)	20.7 ± 1.8 (**2.0**)***
CYP1A2^c (MROD) [pmo/min.mg]	10.1 ± 1.1	12.4 ± 1.3 (**1.2**)	18.8 ± 1.8 (**1.9**)***

^{a}NQO1 enzyme activities were assayed using menadione as a substrate as described in Materials and Methods. ^{b}EROD and ^{c}MROD activities were used to measure CYP1A1/2 and CYP1A2 enzyme activities (see Materials and Methods). **Numbers are** averages and SEs of **four measurements, fold increase over control is shown in parenthesis**.

***Significantly different from controls: $p < 0.001$ (Student's t-test).

Table 2

DNA adduct formation by 3-NBA activated by liver microsomes of rats either control or treated with 3-NBA

| | RAL[a] (mean/10^8 nucleotides) | | | | | |
	Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Total
control + NADPH	0.3±0.02	0.3±0.03	0.9±0.07	0.2±0.02	0.1±0.01	1.8±0.1
3-NBA (0.2 mg/kg bw) + NADPH	0.4±0.03	0.3±0.04	1.4±0.1[*]	0.2±0.02	0.2±0.02	2.5±0.2
3-NBA (2 mg/kg bw) + NADPH	0.4±0.03	0.4±0.03	1.7 ± 0.1[*]	0.5±0.05[*]	0.2±0.02	3.1±0.2[*]

[a] Mean RAL (relative adduct labeling) ± SE of triplicate *in-vitro* incubations; spot 1 = dA-N^6-ABA, spot 3 = dG-N^2-ABA, spots 4/5 = dG-C8-*N*-ABA.

*p<0.05.Values significantly different from control (Student *t*-test).

Legends to 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, sulfotransferases; COX-1, cyclooxygenase 1; CYP, cytochrome P450; LPO, lactoperoxidase; MPO, myeloperoxidase; POR, NADPH:cytochrome P450 oxidoreductase; R = -$COCH_3$ or -SO_3H; 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 NQO1 in the cytosol of livers, lungs and kidneys of rats after intratracheal instillation of 0.2 or 2 mg/kg bw of 3-NBA. Mean values **± SD ($n = 3$) in percent of control values from rats given vehicle only.** Inset: immunoblots of NQO1 from untreated and 3-NBA-treated rats stained with antibody against human NQO1. Bar graphs show the relative intensities of the stained bands. Hepatic, pulmonary and renal cytosols 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: *$p<0.05$, ***$p<0.01$ (Student t-test).

Fig. 3. Induction of CYP1A1 (A) and CYP1A2 (B) in livers, lungs and kidneys of rats after intratracheal instillation of 0.2 or 2 mg/kg bw of 3-NBA. Mean **± SD ($n = 3$) in percent of control values from rats given vehicle only.** Inset: immunoblots of microsomal CYP1A1 (A) and CYP1A2 (B) from untreated and 3-NBA-treated rats stained with anti rat CYP1A1 and CYP1A2 antibodies. Bar graphs show the relative intensities of the stained bands. 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: *$p<0.05$, ***$p<0.01$ (Student t-test).

Fig. 4: Autoradiographic profiles of DNA adducts generated (A) by 3-NBA after activation with cytosols isolated from livers of rats treated with 2 mg/kg bw of 3-NBA, and (B) by 3-ABA after activation with microsomes isolated from livers of rats treated with 2 mg/kg bw of 3-NBA using the butanol enrichment version of the ^{32}P-postlabeling 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, lungs and kidneys of rats, control or treated with 0.2 or 2 mg/kg bw of 3-NBA by intratracheal instillation. Mean RAL (relative adduct labeling) ± SE of triplicate *in-vitro* incubations are shown. *$p<0.05$, ***$p<0.01$ (Student *t*-test).

Fig. 6: DNA adduct formation by 3-ABA activated with microsomes in the presence of NADPH isolated from livers, lungs and kidneys of rats, control or treated with 0.2 or 2 mg/kg bw of 3-NBA by intratracheal instillation. Mean RAL (relative adduct labeling) ± SE of triplicate *in-vitro* incubations are shown. ***$p<0.001$ (Student *t*-test).
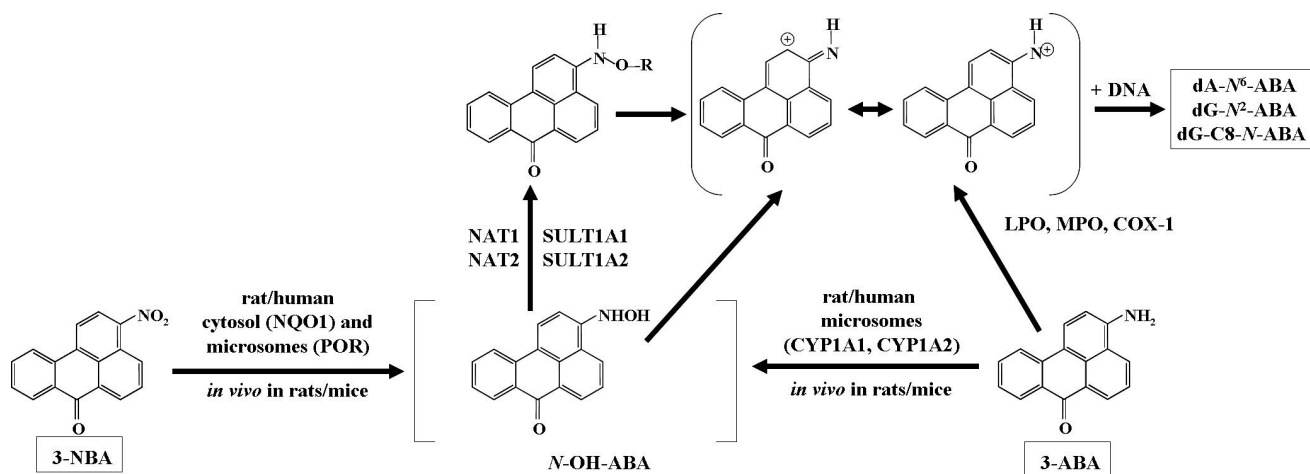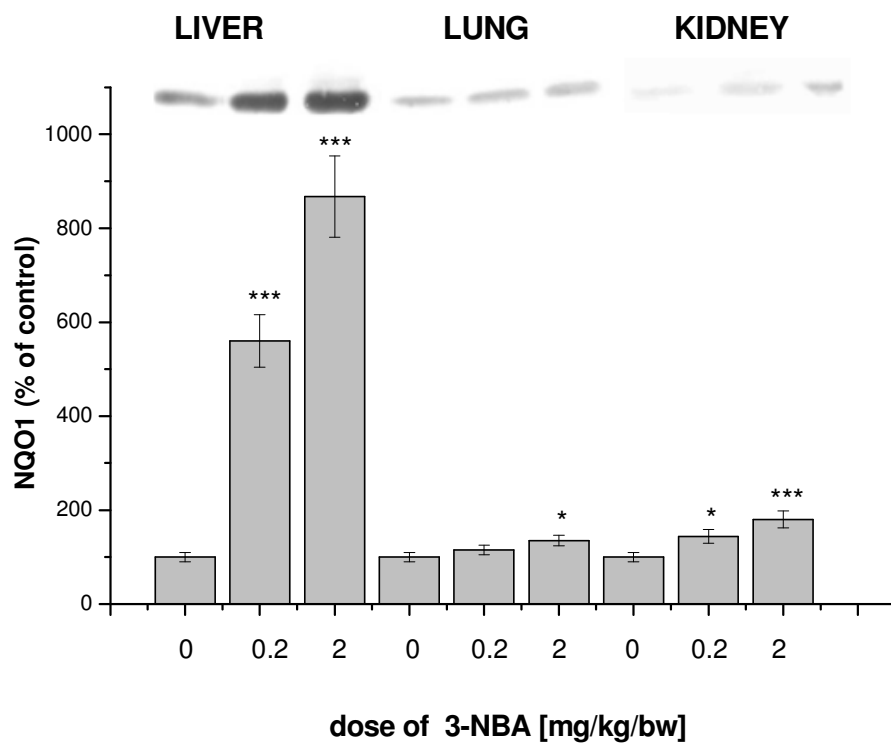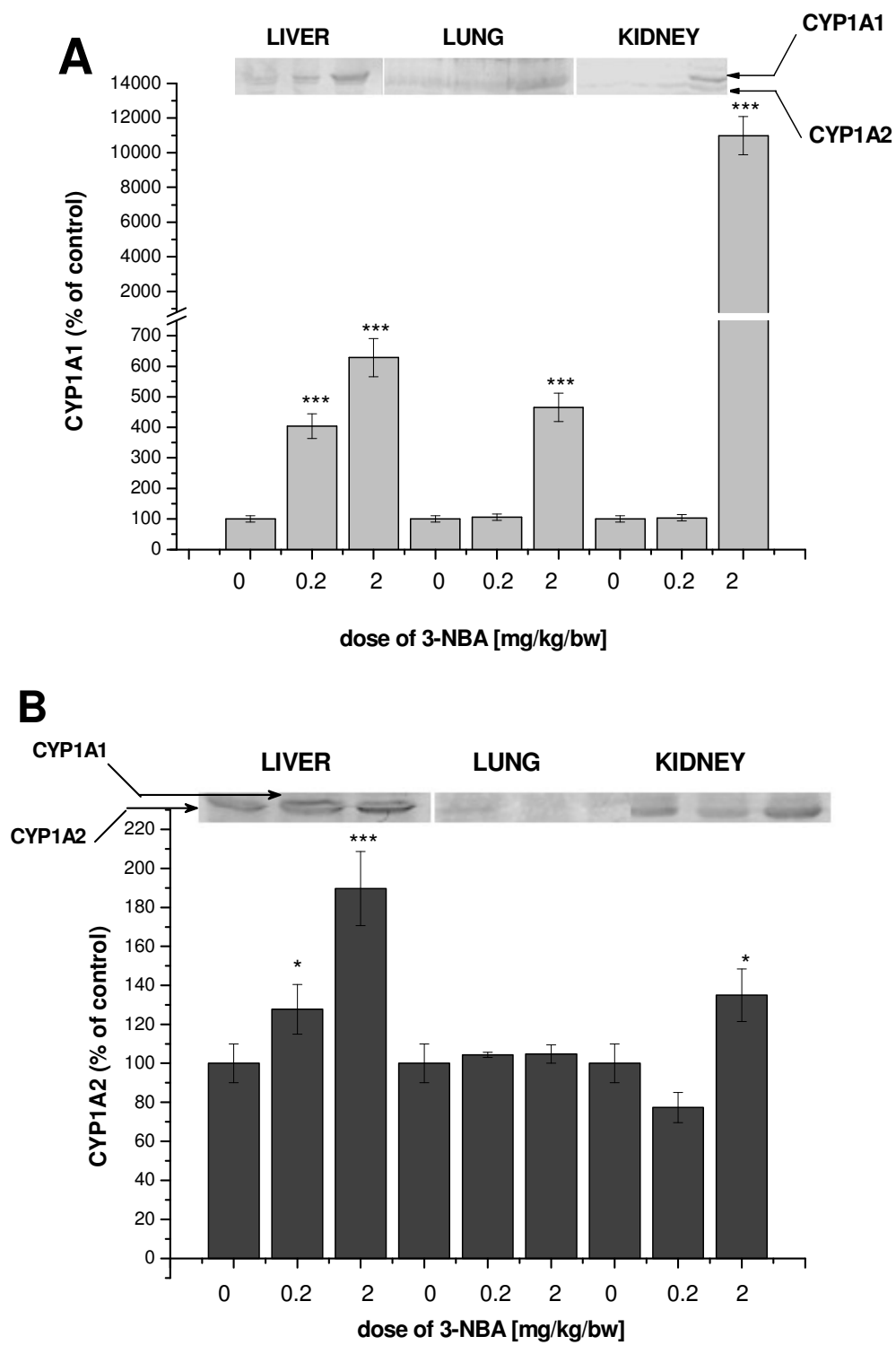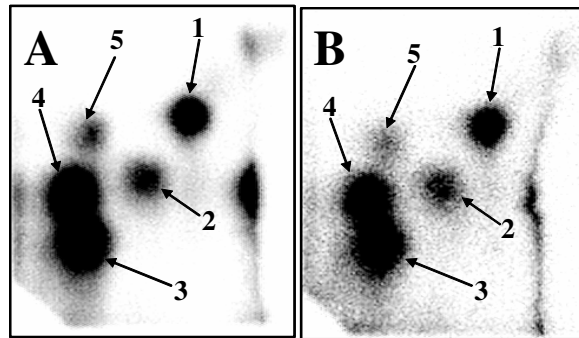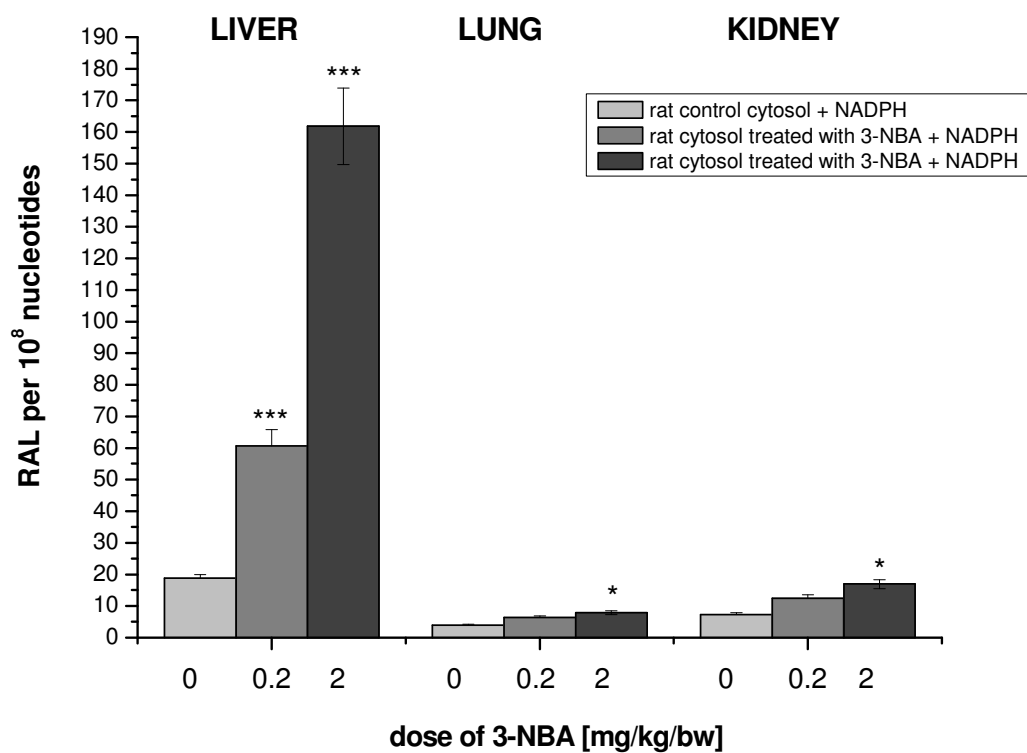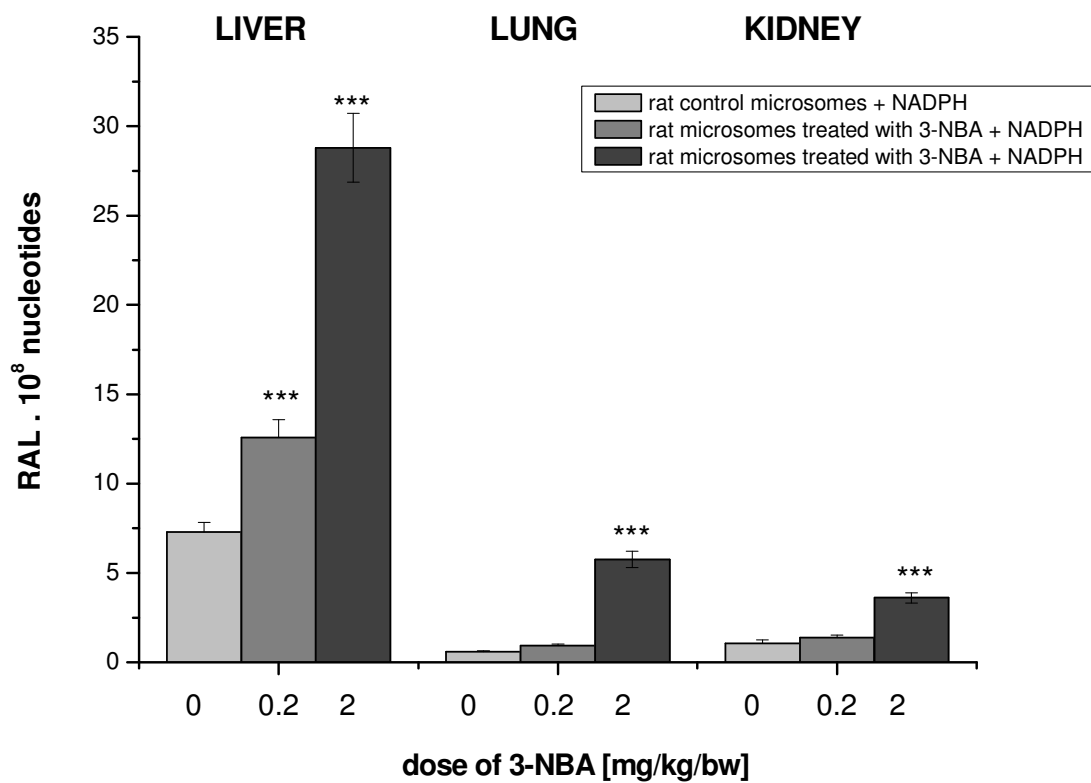
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6