IDENTIFICATION OF HUMAN ENZYMES OXIDIZING A HUMAN METABOLITE OF CARCINOGENIC 2-NITROANISOLE, 2-NITROPHENOL. EVIDENCE FOR ITS OXIDATIVE DETOXIFICATION BY HUMAN CYTOCHROMES P450

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2-Nitrophenol (2-NP) is the major detoxification metabolite of an important industrial pollutant and a potent carcinogen, 2-nitroanisole (2-NA). Here, we characterized the product of 2-NP metabolism catalyzed by human, rat, rabbit and mouse hepatic microsomes containing cytochromes P450 (CYPs) and identified the major human CYP enzymes participating in this process. The 2-NP metabolite was characterized by mass spectrometry and co-chromatography on HPLC with a synthetic standard, 2,5-dihydroxynitrobenzene (2,5-DNB) to be 2,5-DNB. No nitroreductive metabolism leading to the formation of *N*-(2-hydroxyphenyl)hydroxylamine or *o*-aminophenol was evident by all tested hepatic microsomes. Likewise, no DNA binding of 2-NP metabolite(s) measured with the ^{32}P-postlabeling technique was detectable in hepatic microsomes. Therefore, hepatic microsomal CYP enzymes participate in 2-NP metabolism that does not lead to its activation to species binding to DNA. Selective inhibitors of human CYPs were used to characterize CYPs oxidizing 2-NP in human livers. Based on these inhibitory studies, we attribute most of 2-NP oxidation in human liver to CYP2E1, 3A4, 2A6, 2C and 2D6. Among recombinant human CYP enzymes tested in this study, CYP2E1, 2A6 and 2B6 were the most effective enzymes oxidizing 2-NP. Oxidation of 2-NP by human CYP2E1 exhibits the Michaelis-Menten kinetics, having the K_m value of 0.21 mM. The results found in this study, the first report on the metabolism of 2-NP by human hepatic microsomes and human CYP enzymes, demonstrate that CYP2E1 is the major enzyme oxidizing this compound in human.

Keywords: 2-Nitrophenol; 2-Nitroanisole; Cytochrome P450; Oxidation; Detoxification.

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Aromatic nitro compounds are potent toxic or carcinogenic compounds, presenting a considerable danger to the human population[1,2]. They are widely distributed environmental pollutants found in workplaces (e.g. in chemical industry), in emissions from diesel and gasoline engines and on the surface of ambient air particulate matter[2], contributing to local and regional pollution (car exhausts, technological spills). The toxicity and carcinogenicity of these compounds, their metabolic pathways and the persistence of residues of these compounds and/or their metabolites in organisms have been examined[2,3]. However, the knowledge of the fate of several aromatic nitro compounds and their physiological effects in humans is still scarce[3]. This is also the case of 2-nitroanisole (2-methoxynitrobenzene, 2-NA).

2-Nitrophenol (2-NP; Fig. 1) is one of the major detoxification metabolites generated from this carcinogenic nitro compound (2-NA) *in vitro*[4–7] and *in vivo*[8]. 2-NA is used primarily as a precursor in the synthesis of *o*-anisidine (2-methoxyaniline), which is an intermediate in the production of many azo dyes[9,10]. 2-NA and *o*-anisidine exhibit strong carcinogenic activity, causing neoplastic transformation in the urinary bladder and, to a lesser extent, in the spleen, liver and kidneys in rodents[9,10]. 2-NA is also a toxic compound, causing anemia. This type of anemia is characterized by increased levels of methemoglobin and accelerated destruction of erythrocytes[9]. In 1993, an industrial accident in the Hoechst Company in Germany led to a large-scale leakage of 2-NA and subsequent local and regional contamination. Among the toxic effects of chemicals liberated in this accident, single- and double-strand breaks were induced in DNA of the fire fighters working at the site of the accident[11]. In addition, higher frequency of atopic dermatitis and decrease in viral warts were detected among children exposed to chemicals liberated in this accident[12].

Xanthine oxidase (XO) is the principal enzyme responsible for the reductive metabolism of 2-NA, catalyzing the formation of *N*-(2-methoxyphenyl)hydroxylamine and *o*-anisidine[13–15]. Deoxyguanosine adducts derived from *N*-(2-methoxyphenyl)hydroxylamine were found *in vivo* in DNA of several tissues, mainly urinary bladder, of rats treated with 2-NA as well

Abbreviations: α-NF, α-naphthoflavone; CYP, cytochrome P450; DDTC, diethyldithiocarbamic acid; dGp, deoxyguanosine 3′-phosphate; 2,5-DNB, 2,5-dihydroxynitrobenzene; 2,6-DNB, 2,6-dihydroxynitrobenzene; HPLC, high performance liquid chromatography; K_m, Michaelis constant; 2-NA, 2-nitroanisole; $NADP^+$, nicotinamidadeninedinucleotide phosphate; PEI-cellulose, polyethylenimine cellulose; 2-NP, 2-nitrophenol; RAL, relative adduct labeling; rpm, rotations per minute; XO, xanthine oxidase; V_{max}, maximum reaction rate.

as *in vitro* after incubation of 2-NA and DNA with human hepatic cytosols or buttermilk XO [14,15]. On the contrary, 2-NA oxidation by microsomal cytochrome P450 (CYP) enzymes obtained from human, rabbit and rat tissues to 2-nitrophenol (2-NP) and two dihydroxynitrobenzenes, 2,5-dihydroxynitrobenzene (2,5-DNB) and 2,6-dihydroxynitrobenzene (2,6-DNB), were suggested to lead to 2-NA detoxification[4,6,7]. Using rat hepatic microsomes and rat CYP enzymes, we have recently found that one of these metabolites, 2-NP, is a 2-NA metabolic intermediate, which is further oxidized to 2,5-DNB, whereas 2,6-DNB is not formed from 2-NP [16]. Whereas importance of rat CYP enzymes in 2-NP oxidation was established in this former work, showing that rat CYP2E1 is the principal enzyme in oxidative metabolism of 2-NP, similarly to its participation in oxidation of a parent carcinogen, 2-NA [4,6], identification of human CYP enzymes catalyzing this metabolism has not been investigated as yet. Therefore, such a study is the aim of this work. Comparison of efficiencies of human, rat, rabbit and mouse hepatic microsomal enzymes to oxidize 2-NP is another target of this study.

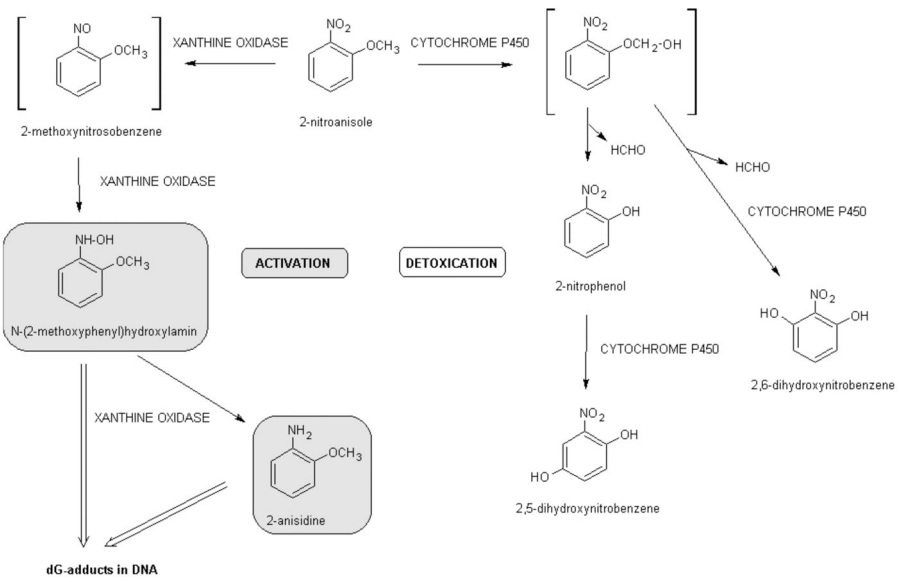

FIG. 1
Pathways of 2-nitroanisole metabolism showing the characterized metabolites and those proposed to form DNA adducts. Compounds shown in brackets were not detected under the experimental conditions described previously[4,6,13,22]

EXPERIMENTAL

Chemicals

Chemicals were obtained from the following sources: α-naphthoflavone (α-NF), $NADP^+$, NADPH, ketoconazole, diethyldithiocarbamate (DDTC), sulfaphenazole, calf thymus DNA, glucose 6-phosphate and chlorzoxazone from Sigma Chemical Co. (St. Louis (MO), USA), 7-pentoxy-, 7-ethoxyresorufin, 2-nitrophenol (2-NP) (>99% based on HPLC) from Fluka Chemie AG (Buchs, Switzerland), diamantane from Pliva–Lachema (Brno, Czech Republic), testosterone, 6β-hydroxytestosterone from Merck (Darmstadt, Germany), bufuralol and its 1′-hydroxyderivative were from Gentest Corp. (Woburn (MA), USA) and bicinchoninic acid from Pierce (Rockford (IL), USA). All these and other chemicals were reagent grade or better. The dihydroxy derivative of nitrobenzene, 2,5-DNB, was synthesized as described[6] and characterized by UV, electrospray mass spectra and high field proton nuclear magnetic resonance (NMR) spectroscopy[6].

Enzymes

Glucose 6-phosphate dehydrogenase was from Sigma Chemical Co. (St. Louis (MO), USA). Supersomes™, microsomes isolated from insect cells transfected with Baculovirus constructs containing cDNA of one of the following human CYPs: CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 with cytochrome b_5 and expressing NADPH:CYP reductase were from Gentest Corp. (USA). Male (pooled sample, Catalog Number 452172), female human hepatic microsomes (pooled sample, Catalog Number 452183) and male human hepatic cytosol (pooled sample, Catalog Number H861) were from Gentest Corp. (USA). Enzymes and chemicals for the ^{32}P-postlabeling assay were obtained from sources described previously[17–19].

Preparation of Microsomes, Isolation of Enzymes 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. Adult male rabbits (2.5–3.0 kg), male Wistar rats (150–200 g) and male C57BL mice (25–30 g) were fed ad libitum on pellet chow and water. Microsomes from livers of ten male rats, three male rabbits and three male mice were prepared by the procedure described previously[6,20–23] and stored in aliquots in liquid nitrogen until use. Protein concentrations in the microsomal fractions were assessed using the bicinchoninic acid protein assay with the bovine serum albumin as a standard[24]. The concentration of CYP was estimated according to Omura and Sato[25] based on absorption of the complex of reduced CYP with carbon monoxide. Rat, rabbit and mouse liver microsomes contained 0.6, 1.37 and 0.4 nmol CYP/mg protein, respectively.

Incubations

Unless stated otherwise, incubation mixtures used to study the 2-NP metabolism by microsomes contained the following in a final volume of 100 μl: 100 mM sodium phosphate buffer (pH 7.4), 1 mM $NADP^+$, 10 mM D-glucose 6-phosphate, 1 U/ml D-glucose 6-phosphate dehydrogenase (NADPH-generating suystem), 10 mM $MgCl_2$, 1 mM ascorbic acid, microsomal fraction containing 0.05–2.4 nmol CYP and 0.1–1.0 mM 2-NP dissolved in 1.0 μl methanol.

The reaction was initiated by adding the substrate. The incubations were performed under aerobic (open tubes) and/or anaerobic conditions. The deaerated and argon-purged reaction mixtures were incubated in the second case. The incubations used for the investigation of the time dependence of 2-NP oxidation by male and female human hepatic microsomes and human CYP2E1 in Supersomes™ contained 0.1 mM 2-NP dissolved in methanol. In all other incubations, 1 mM 2-NP was used. Incubation mixtures for testing the efficiency of Supersomes™ expressing human CYP enzymes were the same except the fact that only 10 pmol of CYPs per incubation were used. Control incubations were carried out either without CYP enzymes (microsomes, Supersomes™) or without the NADPH-generating system. Incubation mixtures used to study the 2-NP metabolism by human hepatic cytosol under aerobic and anaerobic conditions (see above) contained the following in a final volume of 200 μl: 50 mM Tris-HCl buffer (pH 7.4) containing 0.2% Tween 20, cofactors for cytosolic enzymes (1 mM NADPH or hypoxanthine), 0.25 mg of male human hepatic cytosolic protein, 1 mM 2-NP dissolved in 1 ml methanol. The reaction was initiated by adding the substrate. Incubations were carried out at 37 °C for 60 min. Control incubations were carried out (i) without cytosol, (ii) without cofactors (NADPH, hypoxanthine) and (iii) without 2-NP. Reactions were stopped by addition of 20 μl of 600 mM perchloric acid and the incubation mixtures centrifuged at 13 000 rpm for 10 min. The supernatants were collected and 20 μl aliquots applied onto HPLC column, where metabolites of 2-NP were separated. The 2-NP metabolite was separated from parent 2-NP by HPLC with UV detection and characterized by mass spectrometry and co-chromatography with a synthetic standard, 2,5-DNB, as described previously[4,6,16]. Recoveries of 2-NP and 2,5-DNP were around 95%.

Kinetic analyses were carried out using the non-linear least-squares method described by Cleland[26]. Incubation mixtures were the same as those described above (incubations containing microsomes) except that they contained 0.05–0.5 mM 2-NP and human recombinant CYP2E1. Mixtures were incubated at 37 °C for 10 min.

Mass Spectroscopy

ESI mass spectra were recorded on a Bruker Esquire quadrupole ion trap mass spectrometer (Bruker GmbH, Bremen, Germany). Metabolites (final concentration 1 pmol/μl) dissolved in methanol–water (1:1, v/v) were continuously infused into the ion source via a linear syringe pump at a rate of 1 μl/min (Harvard Apparatus 22). The ionizer and ion transfer optics parameters of the ion trap were as follows: capillary voltage –3500 V, end plate –3000 V, capillary exit 100 V, skimmer I 35 V, skimmer II 7 V, octopole I offset 8 V, octopole II offset 8 V, octopole r.f. 100 V peak-to-peak (pp), lens I –4 V, lens II –45 V. A flow of nitrogen (drying gas at 125 °C) was used to stabilize the spray. The spectra were scanned in the range *m/z* 50–2000 and the gating time was set to accumulate and trap 1×10^5 ions.

Inhibition Studies

The following chemicals were used in the inhibition studies of the 2-NP metabolism by human hepatic microsomes: α-naphthoflavone (α-NF), which inhibits CYP1A1 and 1A2; sulfaphenazole, which inhibits CYP2C; quinidine, which inhibits CYP2D6; diethyldithiocarbamic acid (DDTC), which inhibits CYP2E1; 2A6 and ketoconazole, which inhibit CYP3A4 [27]. Inhibitors were dissolved in ethanol, except of α-NF, which was dissolved in a mixture of methanol–ethylacetate (3:2, v/v) and DDTC, which was dissolved in distilled water, to yield final concentrations of 1–1000 μM in the incubation mixtures. The complete mixtures with-

out 2-NP were then incubated at 37 °C for 5 min, 2-NP was added and incubation continued for further 45 min at 37 °C. An equal volume of methanol alone was added to the control incubations. In order to evaluate the inhibition of 2-NP oxidation by human recombinant CYP enzymes, the same experimental conditions were used except that human recombinant CYP1A2, 2A6, 2B6, 2C19, 2D6, 2E1 and 3A4 in Supersomes™ were used instead of human hepatic microsomes. In these experiments, the inhibition of human recombinant CYP2B6 with its inhibitor (diamantane)[4] was also tested.

Covalent DNA Binding

Incubation mixtures used to study the binding of 2-NP to DNA after its metabolism with human hepatic microsomes contained in a final volume of 0.75 ml: 50 mM sodium phosphate (50 mM, pH 7.4) containing 0.5 mM 2-NP dissolved in methanol (10 µl/0.75 ml incubation), 1 mM NADPH, male human hepatic microsomes containing 1 mg protein and 1 mg of calf thymus DNA. The reaction was initiated by adding 2-NP. The incubation mixtures used to study the binding of 2-NP to DNA after its metabolism with human hepatic cytosol contained in a final volume of 0.75 ml: 50 mM Tris-HCl buffer (pH 7.4) containing 0.2% Tween 20, cofactors for cytosolic enzymes (1 mM NADPH or hypoxanthine), 1 mg of male human hepatic cytosolic protein, 1 mM 2-NP dissolved in methanol (10 µl/0.75 ml incubation) and 1 mg of calf thymus DNA. The reaction was initiated by adding 2-NP. All incubations were carried out for 60 min at 37 °C under aerobic (open tubes) and/or anaerobic conditions. The deaerated and argon-purged reaction mixtures were incubated in the second case. Control incubations were carried out either without microsomes or without cytosol or without DNA or without 2-NP. DNA was isolated from the water phase after extraction of incubations with ethyl acetate by the phenol/chloroform extraction method as described earlier[17–19]. DNA was dissolved in 0.2 ml of distilled water and its content was determined spectrophotometrically[28].

^{32}P-Postlabeling Analysis

The standard procedure[29], this procedure under the ATP-deficient conditions[30,31] and the nuclease P1 enrichment version[32,33] of the ^{32}P-postlabeling assay were performed as described earlier[14,15,21,30,31]. Labeled DNA digests were separated by two chromatographic methods on polyethylenimine (PEI)-cellulose plates. (i) Essentially as described previously[21], except that D3 solvent was 3.5 M lithium formate, 8.5 M urea (pH 3.5); D4 solvent was 0.8 M lithium chloride, 0.5 M Tris-HCl, 8.5 M urea (pH 8.0), followed by a final wash with 1.7 M sodium phosphate (pH 6.0). D2 was omitted (method *A*). (ii) ^{32}P-labeled adducts were also resolved by the modification described by Reddy et al.[33]. This procedure has been shown to be suitable for resolution of DNA adducts formed by *N*-(2-methoxyphenyl)hydroxylamine or by 2-NA, reductively activated *in vitro* and *in vivo*[14,15]. The solvents used in this case were: D1, 2.3 M sodium phosphate (pH 5.77); D2 was omitted; D3, 2.7 M lithium formate, 5.1 M urea (pH 3.5); D4, 0.36 M sodium phosphate, 0.23 M Tris-HCl, 3.8 M urea (pH 8.0). After D4 development and brief water wash, the sheets were developed (along D4) in 1.7 M sodium phosphate (pH 6.0) (D5), to the top of the plate, followed by an additional 30–40 min development with the TLC tank partially opened, to allow the radioactive impurities to concentrate in a band close to the top edge (method *B*)[14,15]. Adduct levels were calculated in units of relative adduct labeling (RAL), which is the ratio of cpm of adducted nucleotides to cpm of total nucleotides in the assay.

RESULTS

Metabolism of 2-NP by Human, Rat, Rabbit and Mouse Hepatic Microsomes

When 2-NP was incubated with human, rat, rabbit and mouse hepatic microsomes in the presence of NADPH-generating system, one product peak was observed by HPLC analysis (see Fig. 2 for male human hepatic microsomes). On the basis of co-chromatography with the synthetic standard, eluted with retention time of 7.8 min, and mass spectrometry (Fig. 3), this 2-NP metabolite was identified to be 2,5-DNB (Fig. 2a). Essentially no 2-NP oxidation (formation of 2,5-DNB) was observed when the NADPH-generating system was omitted from the incubation mixtures (Fig. 2b). A time-dependent decrease in 2-NP in incubation mixture containing all used hepatic microsomes corresponded to an increase in 2,5-DNB formation (see Fig. 4 for male and female human hepatic microsomes).

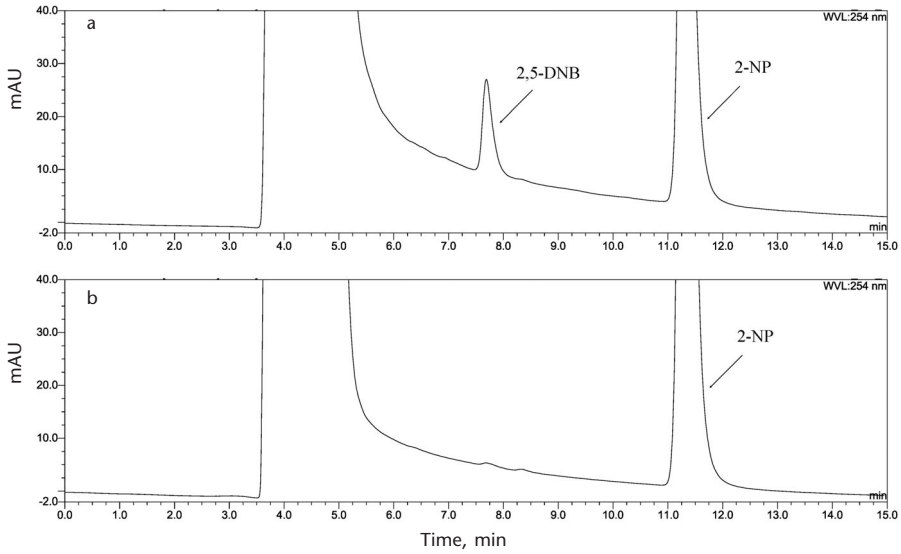

FIG. 2

a HPLC chromatogram of 2-NP metabolite formed by male human hepatic microsomes. Incubations (1 mM $NADP^+$, 10 mM D-glucose 6-phosphate, 1 U/ml D-glucose 6-phosphate dehydrogenase (NADPH-generating system), human hepatic microsomes containing 4 μM CYP and 1 mM 2-NP dissolved in methanol (2 μl) in 100 mM sodium phosphate buffer, pH 7.4, a final volume of 100 μl) were stopped after 15 min by addition of 20 μl of $HClO_4$ and analyzed by HPLC (see Experimental). b HPLC chromatogram of the control (the incubation mixture was of the same composition, but did not contain the NADPH-generating system)

Human hepatic microsomes were much more effective to oxidize 2-NP than rat, rabbit and mouse hepatic microsomes; more than 40-, 8- and 4-fold higher efficiency to oxidize 2-NP were found for human male hepatic microsomes than that for mouse, rabbit and rat hepatic microsomes, respectively (Fig. 5).

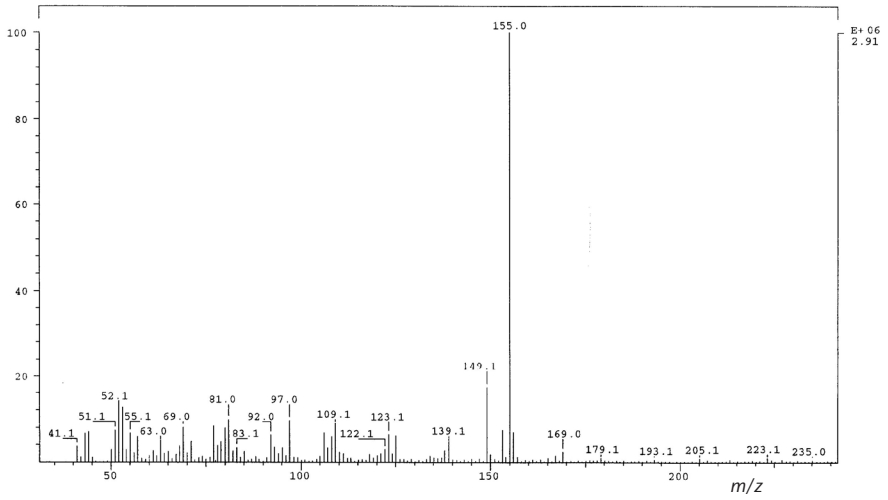

FIG. 3

Mass spectrum of 2-NP oxidation metabolite, 2,5-DNB. The ion at *m*/*z* 155 indicates the molecular mass of dihydroxynitrobenzene

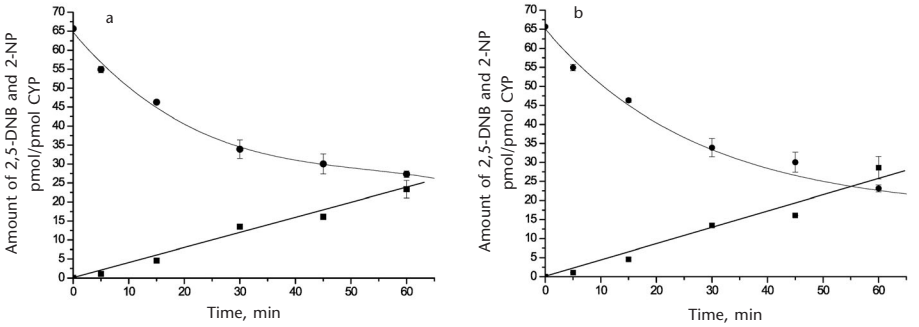

FIG. 4

Time-dependence of the oxidation of 2-NP (●) and formation of its metabolite, 2,5-DNB (■), by male (a) and female (b) human hepatic microsomes. Incubation mixtures were as described in legend to Fig. 2, but 0.1 mM 2-NP was used. Values of reaction rates of 2-NP consumption and 2,5-DNB formation are averages and standard deviations of triplicate incubation

Involvement of Human CYP Enzymes in Oxidation of 2-NP

In order to resolve which human CYPs are able to oxidize 2-NP, two experimental approaches were employed: (i) selective inhibition of CYPs and (ii) heterologous expression systems (Supersomes™, see Experimental).

Inhibitors of CYP enzymes that are expressed at higher levels in human livers (CYP3A4, 1A, 2C, 2A6, 2D6 and 2E1)[27] were used for analysis of their effects on 2-NP oxidation by human hepatic microsomes. Because of low expression of CYP2B6 in human livers (0.2%)[27], inhibitors of this enzyme were not tested. Quinidine, an inhibitor of CYP2D6, ketoconazole, an inhibitor of CYP3A4, sulfaphenazole, an inhibitor of CYP2C and DDTC, an inhibitor of CYP2E1 and 2A6, were effective in inhibiting 2,5-DNB formation by human hepatic microsomes, while an inhibitor of CYP1A enzymes, α-NF, was without such an effect (Table I). These results indicate that human CYP2A6, 2C, 2D6, 2E1 and 3A4 seem to be the enzymes participating in hydroxylation of 2-NP in human liver microsomes. It should be noted, however, that the interpretation of the results from the inhibitory studies is sometimes difficult, because one inhibitor may be more effective with one substrate than another.

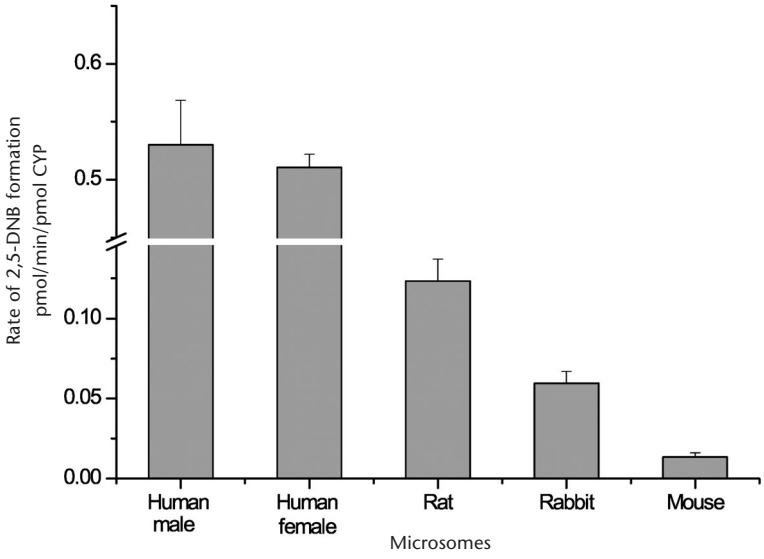

FIG. 5
Rate of 2,5-DNB formation from 1 mM 2-NP by human, rat, rabbit and mouse hepatic microsomes. Reaction mixtures were incubated for 15 min. Values of reaction rates of 2,5-DNB formation are averages and standard deviations of triplicate incubation

Therefore, to identify and prove the role of individual CYPs in oxidation of 2-NP further, we utilized microsomes of Baculovirus transfected insect cells (Supersomes™) containing recombinantly expressed human CYPs and NADPH:CYP reductase (Fig. 6). The recombinant human CYPs used in the experiments oxidized their typical substrates (results not shown). Human CYP2E1 followed by CYP2A6 and 2B6 were the most effective human recombinant enzymes metabolizing 2-NP to 2,5-DNB. Among other CYPs tested in this study, CYP3A4, 2C19 and 2D6 were also capable to oxidize 2-NP, but to a lesser extent (Fig. 6).

In order to evaluate the contribution of individual CYP enzymes to oxidation of 2-NP to 2,5-DNB in human hepatic microsomes, the effect of CYP inhibitors on 2-NP oxidation catalyzed with these recombinant human CYP enzymes were also analyzed (Table I). The IC_{50} values for inhibition of 2-NP hydroxylation to 2,5-DNB, catalyzed by CYP2C19, 2D6, 2E1 and 3A4, were comparable with those found for inhibition of this reaction in human hepatic microsomes. However, DDTC, which is declared as an inhibitor of both CYPE1 and CYP2A6 [27] were without the inhibitiory effect on 2-NP oxidation catalyzed by human CYP2A6 (Table I). Therefore, inhibition of 2-NP

TABLE I
The effects of CYP inhibitors on 2-NP oxidation to 2,5-DNB with human female hepatic microsomes and human recombinant CYP enzymes

Inhibitor[a]	IC_{50} (μM)[b] for formation of 2,5-DNB by	
	Human microsomes	Human CYPs
α-NF (CYP 1A2)	NI[c]	5[d] ± 1
DDTC (CYP2A6)	150 ± 10	NI
Diamantane (CYP2B6)	ND[e]	2 ± 0.2
Sulfaphenazole (CYP2C)	100 ± 8	50[f] ± 7
Quinidine (CYP2D6)	7 ± 1	5 ± 1
DDTC (CYP2E1)	150 ± 10	130 ± 10
Ketoconazole (CYP3A4)	11 ± 1	4 ± 1

[a] Isoforms of CYP inhibited by selective inhibitors are shown in brackets. [b] Estimated from concentration-dependent inhibition of formation of 2-NP metabolite by interpolation (inhibitors were 1–1000 μM depending on the chemical). 2-NP (1 mM) and 0.1 nmol of CYP were present in the incubation mixture. [c] NI, no inhibition, which IC_{50} is greater than 1000 μM. [d] Averages and standard deviations from three determinations. [e] Not determined (not measured). [f] Measured with CYP2C19.

oxidation by DDTC in human hepatic microsomes should be attributed to only inhibition of CYP2E1. Based on the data showing the velocities of 2-NP oxidation to 2,5-DNB by recombinant CYPs (Fig. 6), the IC_{50} values for inhibition of this reaction catalyzed by recombinant CYPs or microsomes (Table I) and the expression levels of human CYP enzymes in hepatic microsomes[27], contributions of individual CYP enzymes to 2-NP oxidation in human hepatic microsomes were estimated. The highest contribution to 2-NP oxidation in human hepatic microsomes is attributed to CYP2E1 (~45%), followed by CYP3A4 (~27%), CYP2A6 (~13%), CYP2C (~8%) and CYP2D6 (~2%). Even though the activity of human recombinant CYP2B6 to oxidize 2-NP is high (Fig. 6), because of low expression of this enzyme in human livers (0.2%)[27], its contribution to this reaction in human hepatic microsomes is low (~0.4%). Other CYP enzymes expressed in human liver[27] have essentially no 2-NP oxidation activity in human hepatic microsomes.

In additional part of our study, kinetics of oxidation of 2-NP by the most effective human enzyme catalyzing this reaction, CYP2E1, was investigated. Oxidation of 2-NP (measured as disappearance of this substrate and production of 2,5-DNB) by CYP2E1 was time-dependent, being linear up to 15 min

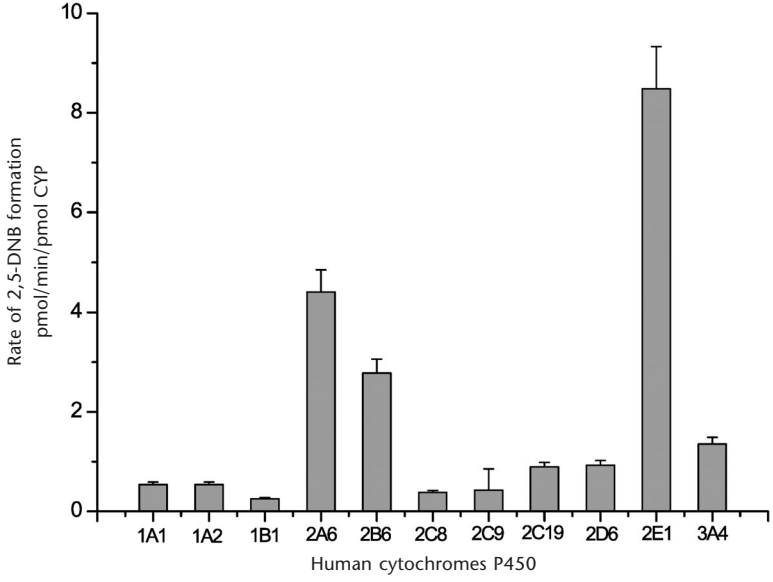

FIG. 6
Oxidation of 2-NP to 2,5-DNB by human recombinant CYPs. A 10-pmol amount of human recombinant CYP/incubation and 1 mM 2-NP were used in this experiment. Values of reaction rates of 2,5-DNB formation are averages and standard deviations of triplicate incubation

(Fig. 7). Oxidation of 2-NP to 2,5-DNB by CYP2E1 exhibits the Michaelis–Menten kinetics; double reciprocal plots of initial velocities of 2-NP oxidation versus concentrations of 2-NP were linear (Fig. 8). The values of maximum reaction rate (V_{max}) and Michaelis constant (K_m) for 2-NP oxidation (measured as 2,5-DNB production) by human CYP2E1 are 29.5 pmol/min per nmol CYP2E1 and 0.21 mM, respectively.

Metabolism of 2-NP by Hepatic Microsomes is a Detoxication Pathway for This Compound

Nitroreduction of 2-NP to *N*-(2-hydroxyphenyl)hydroxylamine or 2-aminophenol was not detected in human, rat, rabbit and mouse hepatic microsomes even under anaerobic conditions (deareated and argon-purged incubations). No metabolites of 2-NP were also found to be formed by human hepatic cytosols under aerobic and/or anaerobic conditions (deareated and argon-purged incubations).

Using the ^{32}P-postlabeling technique, we tested whether during the 2-NP metabolism by human hepatic microsomes and cytosols reactive species binding to DNA are generated. Male human hepatic microsomes and

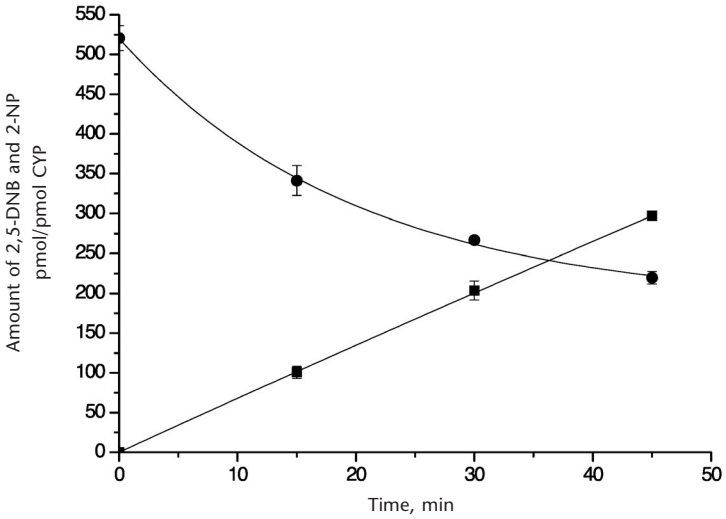

FIG. 7

Time-dependence of the oxidation of 2-NP (●) to 2,5-DNB (■) by human CYP2E1. A 10-pmol amount of human recombinant CYP/incubation and 0.1 mM 2-NP were used in this experiment. Values of amounts (in pmol) of 2-NP and 2,5-DNB are averages and standard deviations of triplicate incubation

cytosols were incubated with DNA, 2-NP and NADPH under aerobic or anaerobic (argon) conditions. Using the ^{32}P-postlabeling analysis, the binding of 2-NP to DNA was evaluated. The standard procedure under the ATP-deficient conditions and the nuclease P1 version of the ^{32}P-postlabeling assay, suitable for detection of adducts mediated in deoxyguanosine 3′-phos-

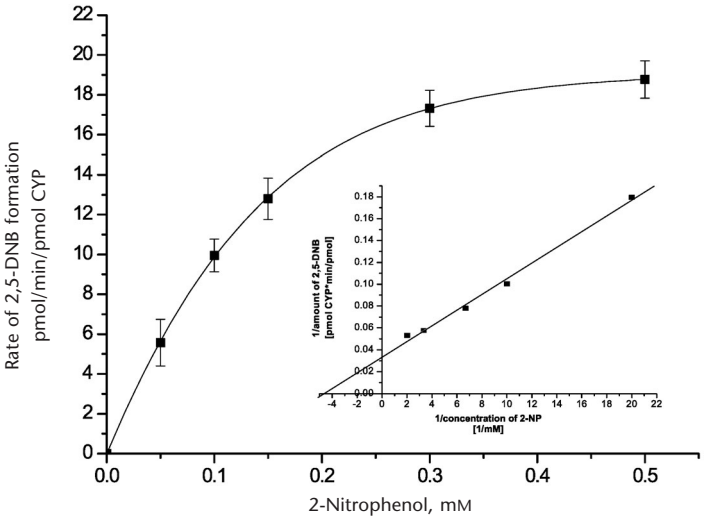

FIG. 8
Dependence of production of 2,5-DNB by recombinant human CYP2E1 on 2-NP concentration. Insert: double reciprocal plots. A 10-pmol amount of human recombinant CYP/incubation was used in this experiment and reactions were stopped after 10 min. Values of reaction rates of 2,5-DNB are averages and standard deviations of triplicate incubations

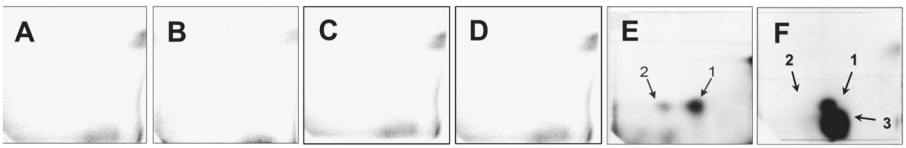

FIG. 9
Autoradiographic profiles of ^{32}P-labeled DNA adducts in calf thymus DNA formed by 2-NP activated with human hepatic microsomes under anaerobic conditions (see Experimental) (A, B), with human hepatic cytosol under the same conditions (C, D), in DNA of urinary bladder of rats treated with 2-NA (E) and in dGp reacted with *N*-(2-methoxyphenyl)- hydroxylamine (F). Samples E and F were used as positive controls. The nuclease P1 version of the ^{32}P-postlabeling assay was used for analysis shown in panels (A), (C) and (E), the standard procedure under ATP-deficient conditions for that in panels (B) and (D) and the standard procedure for that in the panel (F). Method *B* (see Experimental) was utilized for resolution of adducts

phate (dGp) by *N*-(2-methoxyphenyl)hydroxylamine or adducts in DNA of the urinary bladder of rats pretreated with 2-NA (Fig. 9) were used. Using these methods, no DNA adducts mediated by 2-NP metabolism in human hepatic microsomes and cytosols were detectable (Fig. 9).

DISCUSSION

The results of this study demonstrate that CYP enzymes present in human hepatic microsomes are capable of oxidizing 2-NP, generating its hydroxylated derivative, 2,5-NP. The same reaction is also catalyzed by hepatic microsomes of rats, rabbits and mice. Interestingly, 2-NP and its two hydroxylated derivatives, 2,5-DNB and 2,6-DNB, are metabolites we found to be formed from a parent compound from which 2-NP is generated, 2-NA [4–6]. However, as shown in this study, 2-NP is only oxidized to 2,5-DNB. Therefore, we can suggest that oxidative pathways of 2-NA catalyzed by human, rat, rabbit and mouse CYP enzymes proceed by reactions shown in Fig. 1. When once 2-NA is demethylated by α-C-hydroxylation to 2-NP, only 2,5-DNB is generated (Fig. 1). On the contrary, α-C-hydroxylation metabolic intermediate of 2-NA can be further converted to 2-NP, 2,5-DNB and 2,6-DNB (Fig. 1). Nitroreduction of 2-NP was not detectable in human, rat, rabbit or mouse hepatic microsomal subcellular fractions used in our study; no formation of *N*-(2-hydroxyphenyl)hydroxylamine or 2-aminophenol from 2-NP was found both under aerobic and anaerobic conditions. In addition, no DNA binding of 2-NP metabolized by hepatic microsomal samples (male human hepatic microsomes were used in this case) was detectable. Therefore, the microsomal CYPs mediate the detoxification pathways of 2-NP. Among hepatic microsomes, human microsomes were the most efficient system catalyzing this detoxification reaction. No DNA-binding was also detected when 2-NP was metabolized with human hepatic cytosols. These results indicate that not only the 2-NP is a detoxification metabolite of carcinogenic 2-NA, but its further conversion is also the detoxification metabolism, which does not lead to formation of species generating DNA adducts. This finding corresponds to the results found *in vivo*; only 2-NA-derived DNA adducts mediated by its reduction were found in DNA of tissues of rats treated with this carcinogens[14]. Likewise, only 2-NP and no its reductive metabolites such as 2-aminophenol were found in rats exposed to 2-NA [8].

Inhibitors of CYP2E1, 2D6, 2C and 3A4 were effective to inhibit 2-NP oxidation to 2,5-DNB by human hepatic microsomes, indicating participation of these CYPs in the reaction. Likewise, CYP2E1, 2D, 2C and 3A were found

to be the active enzymes oxidizing 2-NP in livers of rats that were used as a model in our former work[16]. Among these CYPs, the CYP2E1 was the predominant enzyme catalyzing 2-NP hydroxylation[16]. Utilizing microsomes containing human recombinant CYP enzymes (Supersomes™), we found that the CYP2E1 enzyme is also the principal human CYP enzyme oxidizing 2-NP. This finding is consistent with the results found in our previous study, concerning the oxidation of a parent carcinogenic compound of 2-NP, 2-NA [4,6]. These former data fully corroborated a major role of CYP2E1 not only in α-C-hydroxylation of 2-NA to 2-NP, but also in 2-NP oxidation in human livers. Using correlations between the CYP catalytic activities provided with the set of human hepatic microsomes from nine different human donors and the rates of formation of each of the 2-NA metabolites (2-NP, 2,5-DNB and 2,6-DNB) in the same set of human hepatic microsomes, we found highly sigificant correlation coefficients of 0.858 ($p < 0.01$), 0.951 ($p < 0.001$) and 0.705 ($p < 0.01$) between the rates of chlorzoxazone 6-hydroxylase, a marker for CYP2E1, and the levels of 2-NP, 2,5-DNB and 2,6-DNB, respectively[6]. Among them, the highest correlation coefficient was found for 2,5-DNB formation. This finding demonstrates a major role of CYP2E1 mainly in formation 2,5-DNB from 2-NA or 2-NP, and confirms the results obtained from experiments utilizing human recombinant CYP2E1 carried out in the present work. Besides the CYP2E1, human recombinant CYP2A6 and 2B6 followed by CYP3A4, 2C19 and 2D6 also effectively oxidize 2-NP, indicating their participation in 2-NP metabolism in human. The CYP2E1, 3A4, 2A6 and 2C enzymes, which are expressed in human liver at higher levels[27], are crucial for contribution to 2-NP oxidation in this tissue. However, even though the efficacy of CYP2B6 to oxidize 2-NP to 2,5-DNB is high (Fig. 6), its contribution to this reaction in human liver microsomes is low (0.4%). Such a low contribution of this enzyme might be caused by its low expression in human liver (0.2%)[27]. Indeed, the relative involvement of particular CYP enzymes in oxidations of xenobiotics was found to depend, to some extent, on the levels of the CYP present in human liver[34]. Likewise, even though a parent carcinogen, 2-NA, generating 2-NP, is effectively oxidized by human CYP2B6, its impact in 2-NA oxidation in human liver is low[4,6]. Nevertheless, this enzyme is expressed at higher levels in some human extrahepatic tissues such as lung[27]. Therefore, its participation in 2-NA and 2-NP detoxification in this tissue should be evaluated to be important. This is also the case of CYP2E1, 2A6 and 2D6, which are expressed at moderate levels in human lung[27]. These findings can, at least partially, explain the absence of a carcinogenic potency of a parent compound, 2-NA, from which 2-NP is formed to induce

tumors in lung. Even though 2-NA is inhaled by humans and introduces easily to pulmonary cells, its CYP2E1-, 2A6-, 2B6- and 2D6-mediated detoxification metabolism to 2-NP and additional oxidation products in this tissue is high, thereby protecting this organ from the carcinogenic effect of 2-NA.

In summary, the present study shows that human hepatic CYP enzymes detoxicate 2-NP by its oxidation to 2,5-DNB. Here, we clearly demonstrate similarities in an oxidation metabolism of 2-NP catalyzed by human CYP enzymes with that by the same enzymes in rats[16]. The human and rat CYP2E1 are the principal enzymes in oxidative metabolism of 2-NP, similarly to their participation in oxidation of a parent carcinogen, 2-NA [4,6,7]. These results suggest that rats might, to some extent, be a suitable model to predict human susceptibility to 2-NP, the major metabolite formed from carcinogenic 2-NA.

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