ORIGINAL ARTICLE

Rat cytochromes P450 oxidize 2-nitrophenol, a human metabolite of carcinogenic 2-nitroanisole

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Abstract

OBJECTIVES: 2-Nitrophenol (2-NP) is the major detoxification metabolite of an important industrial pollutant and a potent carcinogen, 2-nitroanisole (2-NA). Characterization of the products of 2-NP metabolism by rat hepatic microsomes containing cytochromes P450 (CYPs) and identification of the major CYP enzymes participating in this process are aims of this study.

METHODS: HPLC with UV detection was employed for the separation and characterization of 2-NP metabolites. Inducers and inhibitors of CYPs and rat recombinant CYPs were used to characterize the enzymes participating in 2-NP oxidation.

RESULTS: Rat hepatic microsomes oxidize 2-NP to its hydroxylated metabolite, 2,5-dihydroxynitrobenzene (2,5-DNB). No nitroreductive metabolism leading to the formation of *o*-aminophenol was evident when using rat hepatic microsomes. Selective CYP inhibitors and hepatic microsomes of rats pre-treated with specific CYP inducers were used to characterize CYPs oxidizing 2-NP in rat livers. Based on these studies, we attribute most of 2-NP oxidation in rat liver to CYP2E1 and 3A, followed by CYP2D and 2C. Among recombinant rat CYP enzymes tested in this study, CYP2E1 and 2C11 were the most effective enzymes oxidizing 2-NP. Oxidation of 2-NP by rat CYP2E1 exhibits the Michaelis-Menten kinetics, having the K_m value of 0.35 mM.

CONCLUSION: The results found in this study, the first report on the metabolism of 2-NP by rat hepatic microsomes and rat CYP enzymes, demonstrate that CYP2E1 is the major enzyme oxidizing this compound in rat liver.

INTRODUCTION

Aromatic nitro-compounds are potent toxic or carcinogenic compounds, presenting a considerable danger to the human population (Garner *et al.* 1984, IARC, 1989). 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 (IARC, 1989), 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 (IARC, 1989; Purohit & Basu, 2000). However, the knowledge of the fate of several aromatic nitro compounds and their physiological effects in humans and experimental animals is still

Abbreviations & units

α-NF – α-naphthoflavone
β-NF – naphthoflavone
cDNA – complementary DNA
CYP – cytochrome P450
EtOH – ethanol
DDTC – diethyldithiocarbamic acid
2,5-DNB – 2,5-dihydroxynitrobenzene
2,6-DNB – 2,6-dihydroxynitrobenzene
$HClO_4$ – perchloric acid
HPLC – high performance liquid chromatography
K_m – Michaelis constant
2-NA – 2-nitroanisole
$NADP^+$ – nicotinamidadeninedinucleotide phosphate
2-NP – 2-nitrophenol
PB – phenobarbital
PCN – pregnenolone-16α-carbonitrile
RPM – rotations per minute
U – unit
UV – ultraviolet
XO – xanthine oxidase
V_{max} – maximum reaction rate

scarce (Purohit & Basu, 2000). This is also the case of 2-nitroanisole (2-methoxynitrobenzene, 2-NA).

2-Nitrophenol (2-NP, ***Figure 1***) is one of the major detoxification metabolites of 2-NA, the compound that 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 (NTP, 1978; NTP, 1993). 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 (NTP, 1978; NTP, 1993). 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 (NTP, 1978). 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. Single- and double-strand breaks were induced in DNA of the fire fighters working at the site of the accident (Hengstler *et al.* 1995).

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 (Miksanova *et al.* 2004a; Stiborova *et al.* 2004). 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 as *in vitro* after incubation of 2-NA and DNA with human hepatic cytosols or buttermilk XO (Stiborova *et al.* 1998, 2004). 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), leads to 2-NA detoxification (Miksanova *et al.* 2004b; Dracinska *et al.* 2006; Svobodova *et al.* 2008). This study was undertaken to further investigate oxidation of one of these detoxication metabolites, 2-NP, by CYP enzymes.

MATERIAL AND METHODS

Enzymes. Microsomes from rat livers were isolated and characterized for CYP activities as described (Miksanova *et al.* 2004b). Supersomes , microsomes

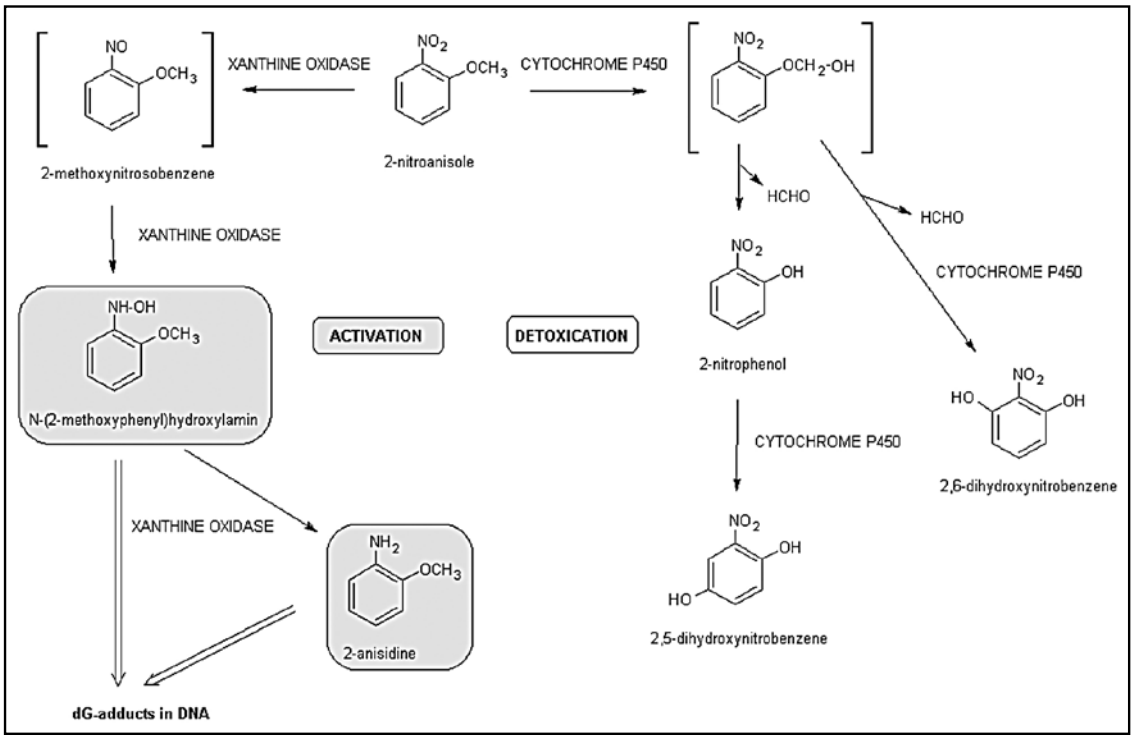

Figure 1. Pathways of 2-nitroanisole metabolism showing the characterized metabolites and those proposed to form DNA adducts. The compounds shown in brackets were not detected under the experimental conditions described previously (Miksanova et al. 2004a;b, Stiborova et al. 2005; Dracinska et al. 2006).

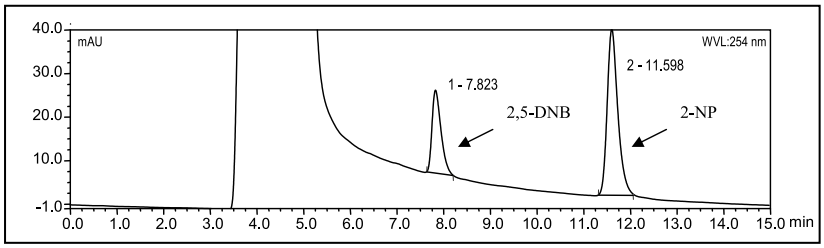

Figure 2. HPLC chromatogram of 2-NP metabolite formed by hepatic microsomes from untreated rats. Incubations (1 mM $NADP^+$, 10 mM D-glucose 6-phosphate, 1 U/mL D-glucose 6-phosphate dehydrogenase, rat hepatic microsomes containing 4 μM CYP and 0.1 mM 2-NP dissolved in methanol in 100 mM sodium phosphate 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 Material and methods).

isolated from insect cells transfected with Baculovirus constructs containing cDNA of one of the following rat CYPs: CYP1A1, 1A2, 2A1, 2A2, 2B1, 2C6, 2C11, 2C12, 2C13, 2D1, 2D2, 2E1, 3A1, 3A2 with cytochrome b_5 and expressing NADPH:CYP reductase were obtained from Gentest Corp. (USA).

Preparation of Microsomes and Assays. The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which complies with the Declaration of Helsinki. Microsomes from livers of ten male untreated Wistar rats and those of ten male rats pre-treated with β-naphthoflavone (β-NF), phenobarbital (PB), ethanol (EtOH) and pregnenolone-16α-carbonitrile (PCN) were prepared by the procedure described previously (Dracinska *et al.* 2006; Stiborova *et al.* 2002b, 2005, 2006; Krizkova *et al.* 2008, Sistkova *et al.* 2008). Protein concentrations in the microsomal fractions were assessed using the bicinchoninic acid protein assay with the bovine serum albumin as a standard (Weichelman *et al.* 1988). The concentration of CYP was estimated according to Omura & Sato (Omura & Sato, 1964) based on absorption of the complex of reduced CYP with carbon monoxide. Untreated rat liver microsomes contained 0.6 nmol CYP/mg of protein. Hepatic microsomes of rats treated with β-NF, PB, EtOH and PCN contained 1.3, 1.5, 0.78 and 1.2 nmol CYP/mg of proteins, respectively.

Incubations. Unless stated otherwise, incubation mixtures used for studying 2-NP metabolism were the same as described previously by Miksanova *et al.* (2004b) and Dracinska *et al.* (2006). Incubations used for the investigation of the time dependence of 2-NP oxidation by rat hepatic microsomes 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 rat 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. Reactions were stopped after 45 minutes by addition of 20 μL of 600 mM perchloric acid and the incubation mixtures centrifuged at 13 000 RPM for 10 minutes. Reactions in the incubation mixtures prepared for investigating the concentration dependence of 2-NP metabolism by rat recombinant CYP2E1 were terminated after 10 minutes. Reactions in the mixtures used to study the effect of the selective CYPs inducers on 2-NP oxidation were stopped after 15 minutes. In all cases, the reactions were terminated by addition of 20 μL of 600 mM perchloric acid and the mixtures were centrifuged at 13 000 RPM for 10 minutes. The following chemicals were used in the inhibition studies of the 2-NP metabolism by rat hepatic microsomes: α-naphthoflavone (α-NF), which inhibits CYP1A1 and 1A2; diamantane, which inhibits CYP2B; sulfaphenazole, which inhibits CYP2C; quinidine, which inhibits CYP2D; diethyldithiocarbamic acid (DDTC), which inhibits CYP2E1 and ketoconazole, which inhibits CYP3A. Inhibitors were dissolved in ethanol, except of α-NF that was dissolved in a mixture of methanol:ethylacetate (3:2, v/v) and DDTC that was dissolved in distilled water, to yield final concentrations of 1-1000 μM in the incubation mixtures. 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 by Miksanova *et al.* (2004b) and Dracinska *et al.* (2006).

RESULTS

When 2-NP was incubated with rat hepatic microsomes in the presence of NADPH-generating system, one product peak was observed by HPLC analysis (***Figure 2***). On the basis of co-chromatography with the synthetic standard, eluted with retention time of 7.8 min, and mass spectrometry (Dracinska *et al.* 2006), this 2-NP metabolite was identified to be 2-NP hydroxylated derivative, 2,5-dihydroxynitrobenzene (2,5-DNB) (Figure 2). No 2-NP oxidation (formation of 2,5-DNB) was observed when microsomes or the NADPH-generating system were omitted from the incubation mixtures (data not shown).

Nitroreduction of 2-NP to *N*-(2-hydroxyphenyl) hydroxylamine or 2-aminophenol was not detected in rat hepatic microsomes.

In order to resolve which rat CYPs are able to oxidize 2-NP, three experimental approaches were employed: (i) induction of specific CYPs, (ii) selective inhibition of CYPs, and (iii) heterologous expression systems (Supersomes™, see Materials and methods).

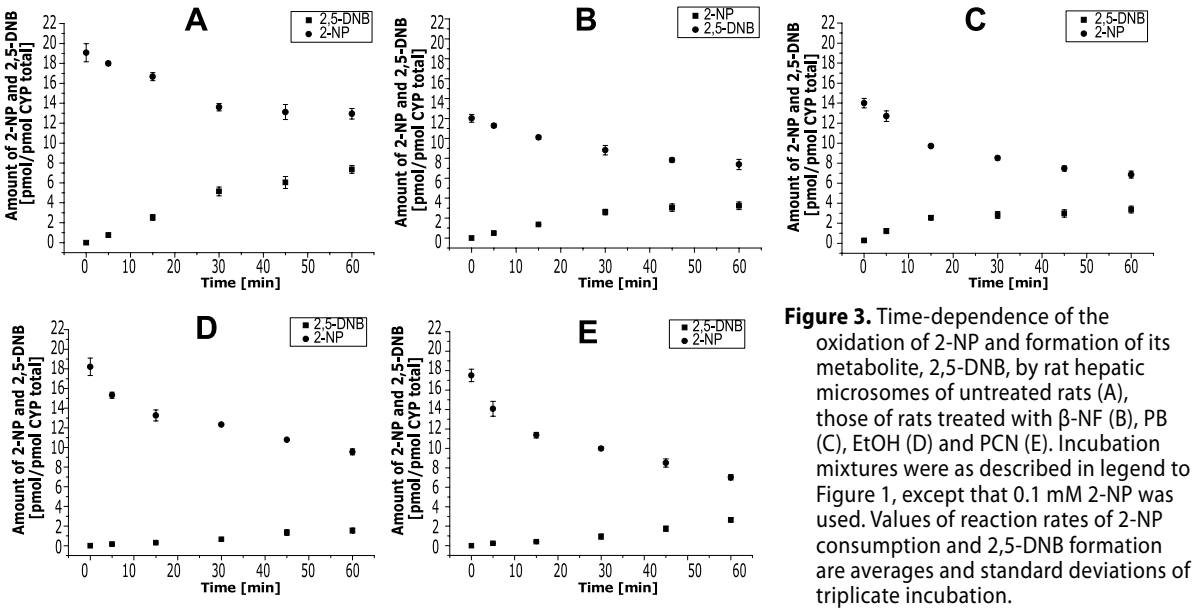

Figure 3. Time-dependence of the oxidation of 2-NP and formation of its metabolite, 2,5-DNB, by rat hepatic microsomes of untreated rats (A), those of rats treated with β-NF (B), PB (C), EtOH (D) and PCN (E). Incubation mixtures were as described in legend to Figure 1, except that 0.1 mM 2-NP was used. Values of reaction rates of 2-NP consumption and 2,5-DNB formation are averages and standard deviations of triplicate incubation.

Microsomes isolated from livers of uninduced rats and those of rats pre-treated with β-NF (enriched with CYP1A1/2), PB (enriched with CYP2B1/2), ethanol (enriched with CYP2E1) and PCN (enriched with CYP3A1/2) were used in the experiments. The consumption of 2-NP (0.1 mM) and formation of 2,5-DNB with the rat microsomal systems were time-dependent, being linear early in the incubation (15 min) but appeared to deviate significantly from linearity at later incubation times (***Figure 3***). Incubations of 2-NP with ethanol- and PCN-microsomes led to an increase in 2-NP oxidation (measured as formation of 2,5-DNB) (Figure 3). On the other hand, incubations of 2-NP with PB- and β-NF-microsomes led to a decrease in the formation of this metabolite (2,5-DNB) (***Figure 4***).

Table 1. The effects of CYP inhibitors on 2-NP oxidation to 2,5-DNB in rat hepatic microsomes

Hepatic microsomes from rats pretreated with[a]	Inhibitor[b]	IC_{50} (μM)[c] for formation 2,5-DNB
β-NF (CYP1A1/2)	α-NF (CYP 1A1/2)	NI[d]
PB (CYP2B)	Diamantane (CYP2B)	16[e] ± 1.0
untreated	Sulfaphenazole (CYP2C)	40 ± 3.6
untreated	Quinidine (CYP2D)	10.8 ± 0.8
untreated	DDTC (CYP2E1)	9.3 ± 0.8
PCN (CYP3A)	Ketoconazole (CYP3A)	8.8 ± 0.6

[a] Isoforms of CYPs induced by inducers are shown in brackets. [b] Isoforms of CYP inhibited by selective inhibitors are shown in brackets. [c] Estimated from concentration-dependent inhibition of formation of 2-NP metabolite by interpolation (inhibitors were 1 – 1000 μM depending on the chemical). 2-NP (1mM) and 0.4 nmol of CYP were present in the incubation mixture. [d] NI, no inhibition, which is IC_{50} greater than 1000 μM. [e] Averages and standard deviations of three determinations.

An inhibitor of CYP2E1, DDTC, was highly effective in inhibiting 2,5-DNB formation by microsomes of control (untreated) rats with the IC_{50} value of 9.3 μM (***Table 1***). Another efficient inhibitor decreasing 2,5-DNB formation in microsomes of control (untreated) rats was an inhibitor of CYP2D, quinidine, while sulfaphenazole, an inhibitor of CYP2C, was less effective (Table 1). An inhibitor of CYP3A, ketoconazole, was the most efficient to inhibit the 2,5-DNB formation in rat hepatic microsomes, namely in microsomes of rats treated with PCN (Table 1). A strong inhibitor of CYP2B, diamantane (Stiborova *et al.* 2002a), was also effective in inhibiting 2,5-DNB formation by PB-microsomes with the IC_{50} value of 16 μM (Table 1). An inhibitor of CYP1A1/2 enzymes, *α*-NF, caused no inhibition of 2,5-DNB formation (Table 1). All the above results indicate that rat CYP2E1 and 3A, followed by CYP2D and 2C seem to be the most efficient enzymes catalyzing hydroxylation of 2-NP in rat liver microsomes.

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 rat CYPs and NADPH:CYP reductase. The recombinant rat CYPs used in the experiments efficiently oxidized their typical substrates (results not shown). Rat CYP2E1 followed by CYP2C11 were the most efficient to metabolize 2-NP to 2,5-DNB. Among other CYPs tested in this study, CYP2B1, 1A1/2 and 3A1, were also capable to oxidize 2-NP, but to a lesser extent (***Figure 5***).

In additional part of our study, kinetics of oxidation of 2-NP by the most efficient enzyme catalyzing this

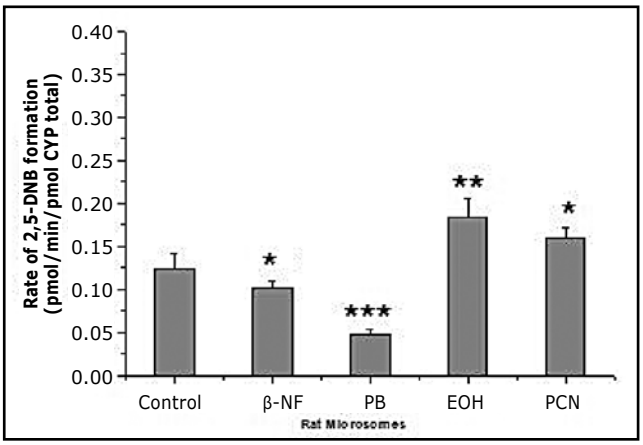

Figure 4. Rate of 2,5-DNB formation from 2-NP (1 mM) by rat hepatic microsomes. Reaction mixtures were incubated for 15 minutes. Values of reaction rates of 2,5-DNB formation are averages and standard deviations of triplicate incubation. The data were analyzed statistically by Student's *t*-test. Values significantly different from microsomes of control (untreated) rats: *P<0.05, **P<0.01, ***P<0.001.

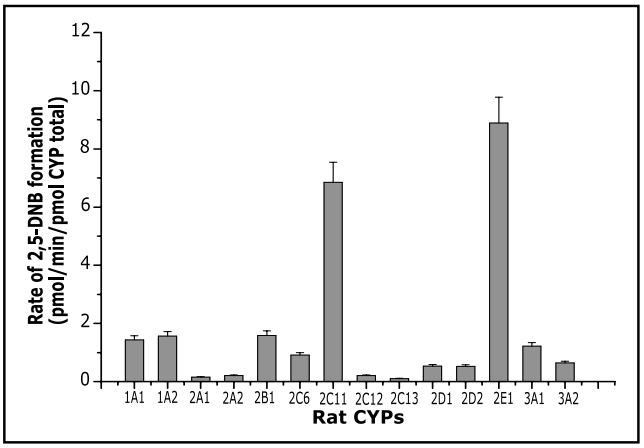

Figure 5. Oxidation of 2-NP (1 mM) to 2,5-DNB by recombinant CYPs of rat. A 10 pmol amount of rat recombinant CYP/ incubation and 1 mM 2-NP were used in this experiment. Values of reaction rates of 2,5-DNB formation are averages and standard deviations of triplicate incubation.

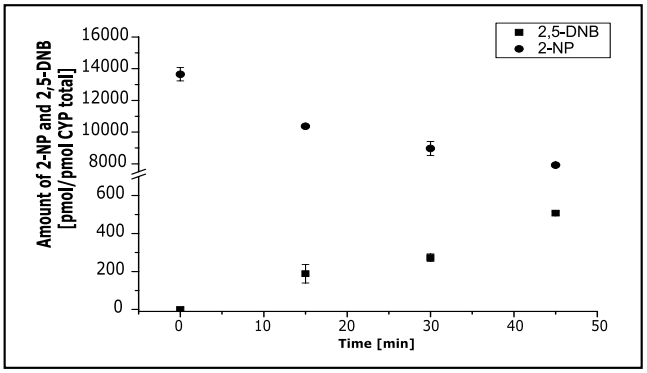

Figure 6. Time-dependence of the oxidation of 2-NP to 2,5-DNB by recombinant rat CYP2E1. A 10 pmol amount of rat recombinant CYP/incubation and 1 mM 2-NP were used in this experiment. Values of 2,5-DNB are average and standard deviations of triplicate incubation.

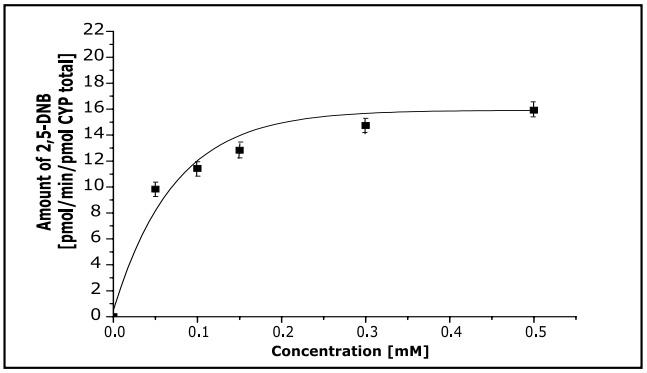

Figure 7. Dependence of production of 2,5-DNB by recombinant rat CYP2E1 on 2-NP concentration. A 10 pmol amount of rat recombinant CYP/incubation were used in this experiment and incubation mixtures were stopped after 10 minutes. Values of 2,5-DNB are average and standard deviations of triplicate incubation.

reaction, CYP2E1, was investigated. Oxidation of 2-NP (measured as disappearance of this substrate and production of 2,5-DNB) by rat CYP2E1 was time-dependent, being linear up to 45 min (***Figure 6***). Oxidation of 2-NP to 2,5-DNB by rat CYP2E1 (***Figure 7***) exhibits the Michaelis-Menten kinetics; double reciprocal plots of initial velocities of 2-NP oxidation *versus* concentrations of 2-NP were linear (data not shown). The values of maximum reaction rate (V_{max}) and Michaelis constant (K_m) for 2-NP oxidation (measured as 2,5-DNB production) by rat CYP2E1 are 16.2 pmol/min per nmol CYP2E1 and 0.35 mM, respectively.

DISCUSSION

The results of the presented study demonstrate that rat hepatic microsomes metabolize 2-NP. We increased our knowledge on oxidative metabolism of 2-NP and the compound from which is 2-NP generated, a carcinogenic substance, 2-NA. As follows from our former studies, three detoxification metabolites were generated from 2-NA, namely, 2-NP and its two hydroxylated metabolites, 2,5-DNB and 2,6-DNB (Miksanova *et al.* 2004b; Rydlova *et al.* 2005; Dracinska *et al.* 2006). 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 proceeds by reactions shown in Figure 1. Whereas α-C-hydroxylation metabolic intermediate of 2-NA can be further converted to 2-NP, 2,5-DNB and 2,6-DNB, from 2-NP only 2,5-DNB is generated (Figure 1). No reductive metabolite of 2-NP are generated by rat hepatic microsomal systems.

Rat CYP2E1 is the principal enzyme responsible for the metabolism of 2-NP in rat livers as proved by an inhibition of 2-NP oxidation by DDTC, a specific inhibitor of CYP2E1. Inhibitors of CYP3A, 2D and 2C were also very effective to inhibit 2-NP oxidation by rat hepatic microsomes, indicating participation of these CYPs in the reaction. It should be noted that the interpretation of the results of inhibitors is sometimes dif-

ficult, because one inhibitor may be more effective with one substrate than another. To further evaluate the role of microsomal CYPs in 2-NP oxidation, rats were therefore treated with specific inducers of CYPs of 1A, 2B, 2E and 3A subfamilies and hepatic microsomes isolated from such animals were used in evaluating participation of these enzymes in 2-NP oxidation. Results of experiments utilizing hepatic microsomes of rats treated with these CYP inducers supported the results of experiments employing CYP inhibitors. Pre-treatment of rats with ethanol, which is a compound increasing levels of CYP2E1 in livers, lead to an increase in 2-NP oxidation by liver microsomes. Likewise, an inducer of CYP3A stimulated efficiency of rat hepatic microsomes to oxidize 2-NP. A high efficiency of inhibitor of CYP2D to inhibit 2-NP oxidation in microsomes of uninduced rats as well as a high efficiency of these microsomes to oxidize 2-NP corresponds to high CYP2D expression in rat livers (Nedelcheva & Gut, 1994).

Utilizing microsomes containing rat recombinant CYP2E1 fully corroborated the role of CYP2E1 in the metabolism of 2-NP. Besides the CYP2E1 and other liver microsomal enzymes found in the studies utilizing CYP inducers and inhibitors (CYP3A and 2D), the CYP2C11 enzyme might also participate in 2-NP oxidation in rats. Namely, the rat recombinant CYP2C11 enzyme in Supersomes™ also effectively oxidizes 2-NP and sulfaphenazole, an inhibitor of CYP2C, decreased 2-NP oxidation in rat liver microsomes.

In summary, the presented study shows that rat hepatic enzymes metabolize 2-NP to 2,5-DNB. Whereas importance of rat CYP enzymes in 2-NP oxidation was established in this 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 (Miksanova *et al.* 2004b; Dracinska *et al.* 2006), identification of human CYP enzymes catalyzing this metabolism remains to be investigated. Therefore, such a study is under way in our laboratory. It should bring information whether rats might be a suitable model to predict human susceptibility to 2-NP, the major metabolite formed from carcinogenic 2-NA.

Acknowledgements

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REFERENCES

1 Dracinska H, Miksanova M, Svobodova M, Smrcek S, Frei E, Schmeiser HH *et al.* (2006). Oxidative detoxication of carcinogenic 2-nitroanisole by human, rat and rabbit cytochrome P450. Neuro Endocrinol Lett. **27 Suppl 2**: 9–13.

2 Garner RC, Martin CN, Clayson DB (1984). Carcinogenic Aromatic Amines and Related Compounds. In: Searle C, editor. Chemical Carcinogens. 2nd ed. Washington, DC: American Chemical Society. p. 175–302.

3 Hengstler JG, Fuchs J, Bolm-Audorff U, Meyer S, Oesch F (1995). Single-strand breaks in deoxyribonucleic acid in fire fighters accidentally exposed to o-nitroanisole and other chemicals. Scand J Work Environ Health. **21**: 36–42.

4 International Agency for Research in Cancer (IARC). (1989) Diesel and Gasoline Engine Exhausts and Some Nitroarenes. IARC Monographs on the Evaluation of Carcinogenic Risk to Humans; Lyon, France

5 Krizkova J, Burdova K, Hudecek J, Stiborova M, Hodek P (2008). Induction of cytochromes P450 in small intestine by chemopreventive compounds. Neuroendocrinol. Lett. **29**, 717-721.

6 Miksanova M, Novak P, Frei E, Stiborova M (2004a). Metabolism of carcinogenic 2-nitroanisole by rat, rabbit, porcine and human hepatic cytosol. Collect Czech Chem Commun. **69**: 589–602.

7 Miksanova M, Sulc M, Rydlova H, Schmeiser HH, Frei E, Stiborova M (2004b). Enzymes involved in the metabolism of the carcinogen 2-nitroanisole: evidence for its oxidative detoxication by human cytochromes P450. Chem Res Toxicol. **17**: 663–671.

8 Nedelcheva V, Gut I (1994). P450 in the rat and man: methods of investigation, substrate specificities and relevance to cancer. Xenobiotica. **24**: 1151–1175.

9 NTP (1978). Bioassay of o-anisidine Hydrochloride for Possible Carcinogenicity. NTP Technical Report 89, National Institute of Health, US Department of Health and Human Services; Bethesda, MD

10 NTP (1993). Toxicology and Carcinogenesis. Studies of 2-nitroanisole. NTP Technical Report 89, National Institute of Health, US Department of Health and Human Services; Bethesda, MD

11 Omura T, Sato R (1964). The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J Biol Chem. **239**: 2370-8.

12 Purohit V, Basu AK (2000). Mutagenicity of nitroaromatic compounds. Chem Res Toxicol. **13**: 673–692.

13 Rydlova H, Miksanova M, Ryslava H, Stiborova M (2005). Carcinogenic pollutants o-nitroanisole and o-anisidine are substrates and inducers of cytochromes P450. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. **149**: 441–447.

14 Sistkova J, Hudecek J, Hodek P, Frei E, Schmeiser HH, Stiborova M (2008). Human cytochromes P450 1A1 and 1A2 participate in detoxication of carcinogenic aristolochic acid. Neuroendocrinol Lett. **29**: 733–737.

15 Stiborova M, Schmeiser HH, Frei E (1998). To the mechanism of 2-nitroanisole carcinogenity: *in vitro* metabolism 2-nitroanisole mediated by peroxidases and xanthine oxidase. Collect Czech Chem Commun. **63**: 857–869.

16 Stiborova M, Borek-Dohalska L, Hodek P, Mraz J, Frei E (2002a). New selective inhibitors of cytochromes P450 2B and their application to antimutagenesis of tamoxifen. Arch Biochem Biophys. **403**: 41-49.

17 Stiborova M, Martinek V, Rydlova H, Hodek P, Frei E (2002b). Sudan I is a potential carcinogen for humans: evidence for its metabolic activation and detoxication by human recombinant cytochrome P450 1A1 and liver microsomes. Cancer Res. **62**: 5678–5684.

18 Stiborova M, Miksanova M, Smrcek S, Bieler CA, Breuer AK, Schmeiser HH *et al.* (2004). Identification of a genotoxic mechanism for 2-nitroanisole carcinogenicity andof its carcinogenic potential for humans. Carcinogenesis. **25**: 833-840.

19 Stiborova M, Miksanova M, Sulc M, Rydlova H, Schmeiser HH, Frei E (2005). Identification of a genotoxic mechanism for the carcinogenicity of the environmental pollutant and suspected human carcinogen o-anisidine. Int J Cancer. **116**: 667–678.

20 Stiborova M, Martinek V, Schmeiser HH, Frei E (2006). Modulation of CYP1A1-mediated oxidation of carcinogenic azo dye Sudan I and its binding to DNA by cytochrome b_5. Neuro Endocrinol Lett. **27 Suppl 2**: 35–39.

21 Svobodova M, Dracinska H, Martinkova M, Hudecek J, Hodek P, Frei E et al (2008). Oxidation of carcinogenic 2-nitroanisole by rat cytochromes P450 - similarity between human and rat enzymes. Interdisc Toxicol. **1**: 182–185.

22 Weichelman KJ, Braun RD, Fitzpatrick JD (1988). Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation. Anal Biochem. **175**: 231–237.