ORIGINAL ARTICLE

Identification of rat cytochromes P450 metabolizing *N*-(2-methoxyphenyl)hydroxylamine, a human metabolite of the environmental pollutants and carcinogens *o*-anisidine and *o*-nitroanisole

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Abstract

OBJECTIVES: *N*-(2-methoxyphenyl)hydroxylamine is a human metabolite of two industrial and environmental pollutants and bladder carcinogens 2-methoxyaniline (*o*-anisidine) and 2-methoxynitrobenzene (*o*-nitroanisole). Metabolism of *N*-(2-methoxyphenyl)hydroxylamine by rat hepatic microsomes and identification of the major microsomal enzymes participating in this process are aims of this study.

METHODS: HPLC with UV detection was employed for the separation of *N*-(2-methoxyphenyl)hydroxylamine metabolites. Inducers and inhibitors of microsomal enzymes and rat recombinant CYPs were used to characterize the enzymes participating in *N*-(2-methoxyphenyl)hydroxylamine metabolism.

RESULTS: *N*-(2-methoxyphenyl)hydroxylamine is metabolized by rat hepatic microsomes predominantly to *o*-anisidine, the parent carcinogen from which *N*-(2-methoxyphenyl)hydroxylamine is formed, while *o*-aminophenol and two *N*-(2-methoxyphenyl)hydroxylamine metabolites, whose exact structures have not been identified as yet, are minor products. Selective inhibitors of microsomal CYPs, NADPH:CYP reductase and NADH:cytochrome b_5 reductase and hepatic microsomes of rats pre-treated with specific inducers of CYPs and NADPH:CYP reductase were used to characterize rat liver microsomal enzymes reducing *N*-(2-methoxyphenyl)hydroxylamine to *o*-anisidine. Based on these studies, we attribute most of *N*-(2-methoxyphenyl)hydroxylamine metabolism to *o*-anisidine in rat liver to CYP2C, followed by CYP2E1, 2D and 2A. Among recombinant rat CYP enzymes tested in this study, rat CYP2C11 and 2E1, followed by CYP2A2, 2D1/2, 2C12, 3A1/2 and 1A1/2 were the most efficient enzymes metabolizing *N*-(2-methoxyphenyl)hydroxylamine to *o*-anisidine.

CONCLUSION: The results found in this study, the first report on the reduction of *N*-(2-methoxyphenyl)hydroxylamine by rat CYP enzymes, demonstrate that CYP2C, followed by CYP2E1, 2D and 2A are the major enzymes participating in this process in rat liver.

Abbreviations:

α-NF	- α-naphthoflavone
β-NF	- β-naphthoflavone
CYP	- cytochrome P450
cDNA	- complementary DNA
CID	- collision-induced dissociation
DDTC	- diethyldithiocarbamic acid
EtOH	- ethanol
HPLC	- high performance liquid chromatography
3-IPMDIA	- isopropenyl-3-methyldiamantane
NADP+	- nicotinamidadeninedinucleotide phosphate
NADPH	- nicotinamidadeninedinucleotide phosphate (reduced)
NMR	- nuclear magnetic resonance
PB	- phenobarbital
PCN	- pregnenolone-16α-carbonitrile
S.E.M.	- standard error medium
U	- unit
UV	- ultraviolet

INTRODUCTION

2-Methoxyaniline (*o*-anisidine) is a potent carcinogen, causing tumors of the urinary bladder in both genders of F344 rats and B6C3F1 mice (NTP 1978; IARC 1982). The International Agency for Research on Cancer has classified *o*-anisidine as a group 2B carcinogen (IARC 1982), which is possibly carcinogenic to humans. Besides its carcinogenicity it exhibits other toxic effects, including hematological changes, anemia and nephrotoxicity (NTP 1978; IARC 1982). *o*-Anisidine is used as an intermediate in the manufacturing of a number of azo and naphthol pigments and dyes, which are used for printing (90%) and for paper (3%) and textile (7%) dyeing (NTP 1978; Garner *et al.* 1984). Such a wide use of this aromatic amine could result in occupational exposure. Furthermore, it may be released from textiles and leather goods colored with these azo dyes and a large part of the population may be exposed. This carcinogen is also a constituent of cigarette smoke (IARC 1982; Stabbert *et al.* 2003). This strongly suggests that *o*-anisidine ranks not only among occupational pollutants produced in the manufacturing of chemicals, but also among environmental pollutants; it can be assumed that human exposure is widespread. Indeed, *o*-anisidine was found in human urine samples in the general population, in concentrations of 0.22 μg/l (median) (Weiss & Angerer 2003). In addition, hemoglobin adducts of *o*-anisidine were detected in blood samples of persons living in urban or rural areas of Germany (Falter *et al.* 1994; Branner *et al.* 1998; Richter *et al.* 2001). The adducts as well as *o*-anisidine in urine might originate not only from the sources mentioned above, but also from a possible *o*-anisidine precursor, 2-methoxynitrobenzene (*o*-nitroanisole). This chemical was released into the environment in the course of an accident in a German chemical plant, causing subsequently local and regional contamination (Falter *et al.* 1994; Hauthal 1993; Traupe *et al.* 1997). *o*-Nitroanisole exhibits strong carcinogenic activity, causing neoplastic transformation in the urinary bladder, and to a lesser extent, in the spleen, liver and kidneys in rodents (NTP 1993). It is also a toxic compound, causing anemia. The anemia is characterized by increased levels of methemoglobin and accelerated destruction of erythrocytes (NTP 1993).

We have found that *o*-anisidine is oxidatively activated by peroxidase and cytochrome P450 (CYP) to species binding to DNA *in vitro* (Stiborova *et al.* 2001; 2002b; 2005b; Rydlova *et al.* 2005; Naiman *et al.* 2008). We also demonstrated that *o*-anisidine forms DNA adducts *in vivo*. The same adducts as found in DNA incubated with *o*-anisidine and human microsomes *in vitro* were detected in urinary bladder, the target organ, and to a lesser extent, in liver, kidney and spleen of rats treated with *o*-anisidine (Stiborova *et al.* 2005b). The *o*-anisidine-derived DNA adducts were identified as deoxyguanosine adducts formed from a metabolite of *o*-anisidine, *N*-(2-methoxyphenyl)hydroxylamine, which is generated by oxidation of *o*-anisidine with human, rabbit and rat hepatic microsomes (Stiborova *et al.* 2005b; Rydlova *et al.* 2005; Naiman *et al.* 2008). The same deoxyguanosine adducts were also detected in DNA of the urinary bladder, kidney, liver and spleen of rats treated with *o*-nitroanisole (Stiborova *et al.* 2004), an oxidized counterpart of *o*-anisidine, and in DNA incubated with *o*-nitroanisole *in vitro* with human and rat hepatic cytosolic enzymes and xanthine oxidase (Stiborova *et al.* 1998, 2004). These enzymatic systems were found to produce *N*-(2-methoxyphenyl)hydroxylamine after *o*-nitroanisole reduction (Miksanova *et al.* 2004). The data indicate that formation of *N*-(2-methoxyphenyl)hydroxylamine, the reactive metabolite of both carcinogens, is critical for generation of DNA lesions in target organs. Therefore, it is clear that *N*-(2-methoxyphenyl)hydroxylamine formation and its further conversion, as well as the enzymes participating in such processes, play a key role in carcinogenic effects of both carcinogens.

Recently, we have found that *o*-anisidine is oxidized by human, rat and rabbit hepatic microsomes not only to *N*-(2-methoxyphenyl)hydroxylamine, but that this compound is a subject of complex redox cycling reactions, forming also *o*-aminophenol, *o*-nitrosoanisole and one additional metabolite, whose exact structure has not been identified as yet (Stiborová *et al.* 2005b; Naiman *et al.* 2008) (Figure 1). *N*-(2-methoxyphenyl) hydroxylamine seems to also be a subject of complex reactions, and its fate is dependent on the environment, in which it occurs. When nucleophiles such as DNA or proteins are present in the incubation mixture, *N*-(2-methoxyphenyl)hydroxylamine forms the adducts (Stiborova *et al.* 2005b). However, the results of our former studies utilizing rat and rabbit hepatic microsomes indicate that *N*-(2-methoxyphenyl)hydroxylamine can be also further metabolized forming three metabolites. Two of them are *o*-aminophenol and *o*-anisidine, the

parent compound from which *N*-(2-methoxyphenyl) hydroxylamine is generated (Naiman *et al.* 2008; 2010). Nevertheless, the enzymes responsible for these reactions have not been identified as yet. Therefore, investigation of participation of microsomal enzymes in *N*-(2-methoxyphenyl)hydroxylamine metabolism is the aim of the present study.

MATERIALS AND METHODS

Chemicals and enzymes

N-(2-methoxyphenyl)hydroxylamine was synthesized by the procedure similar to that described earlier (Balaban *et al.* 1998) and its authenticity was confirmed by electrospray mass and CID spectra and high field proton NMR spectroscopy. 2-Methoxynitrosobenzene (*o*-nitrosoanisole) was synthesized in analogy to the synthesis described earlier (Seidenfaden 1971). *o*-Anisidine and *o*-aminophenol authenticity was confirmed by mass spectra and ^{1}H and ^{13}C NMR spectroscopy (Naiman *et al.* 2008; 2010). Supersomes™, microsomes 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 (Stiborova *et al.* 2003; Krizkova *et al.* 2008; Sistkova *et al.* 2008; Svobodova *et al.* 2009). Protein concentrations in the microsomal fractions were assessed using the bicinchoninic acid protein assay with the bovine serum albumin as a standard (Weichelman *et al.* 1988). The concentration of CYP was estimated according to Omura and Sato (Omura & Sato 1964) based on absorption of the complex of reduced CYP with carbon monoxide. Hepatic microsomes of control (uninduced) rats and rats induced with β-NF, phenolbarbital (PB), ethanol and PCN contained 0.6, 1.3, 2.7, 1.8 and 1.6 nmol CYP/mg protein, respectively. The activity of NADPH:CYP reductase in rat hepatic microsomes was

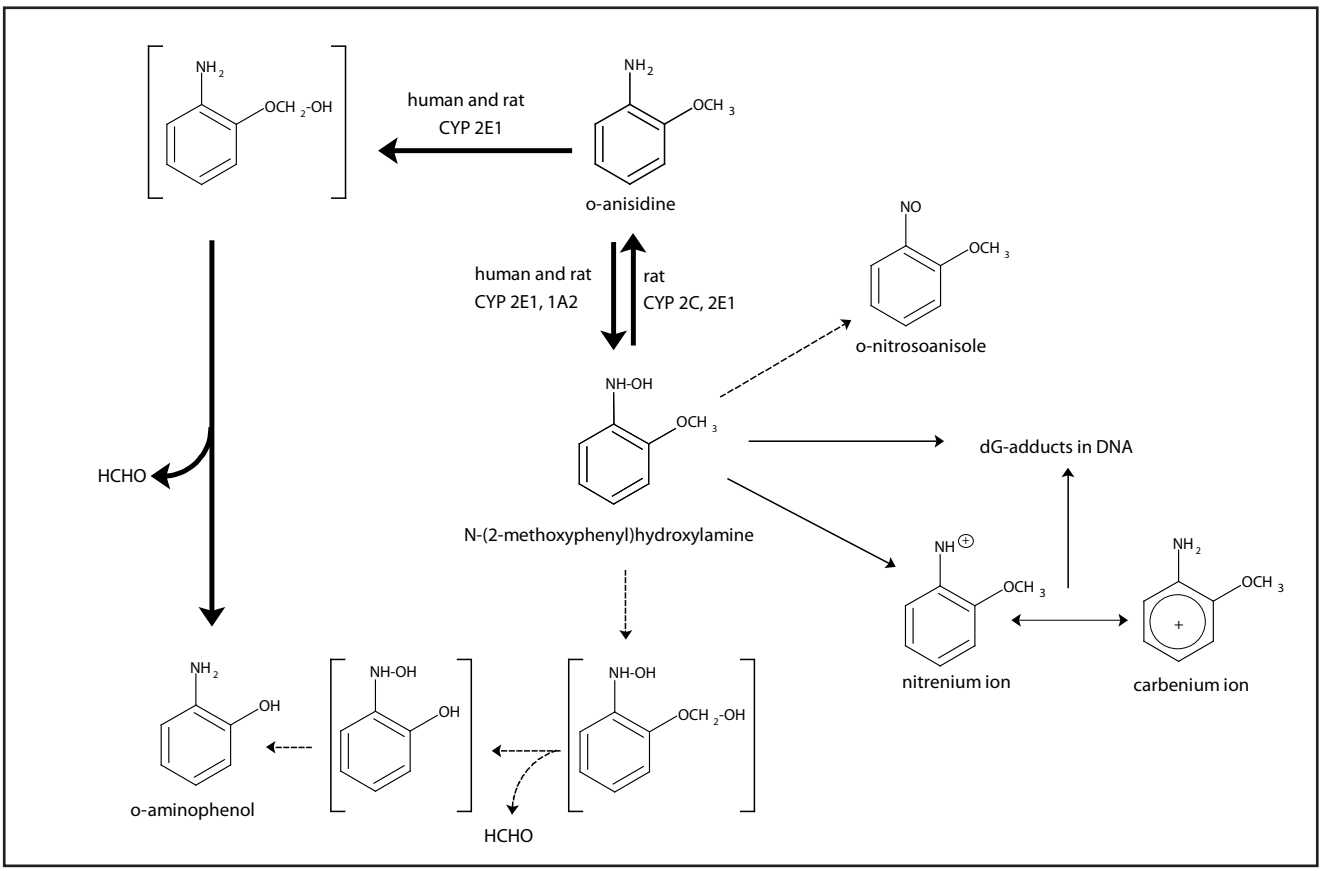

Fig. 1. Pathways of *N*-(2-methoxyphenyl)hydroxylamine and *o*-anisidine metabolism by the cytochrome P450 system showing the characterized metabolites and those proposed to form DNA adducts. The compounds shown in brackets were not detected under the experimental conditions. The reactions that are highly populated are indicated by bold arrows, those not as frequent by general arrows and those that are rare are indicated by dashed arrows.

measured according to Sottocasa *et al.* (1967) using cytochrome c as substrate (i.e., as NADPH:cytochrome c reductase). NADPH:CYP reductase activities in hepatic microsomes of control (uninduced) rats and rats induced with β-NF, PB, ethanol and PCN were 0.210, 0.199, 0.325, 0.201 and 0.210 μmol/min/mg protein, respectively.

NADPH:CYP reductase was isolated from liver microsomes of rats pre-treated with PB by a procedure described earlier (Strobel & Dignam 1978).

Incubations

Incubation mixtures used for study of the metabolism of *N*-(2-methoxyphenyl)hydroxylamine contained the following concentrations 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-generation system), a rat hepatic microsomal fraction containing 0.7 nmol CYP, and 1.0 mM *N*-(2-methoxyphenyl) hydroxylamine dissolved in 10 μl distilled water. The reaction was initiated by adding the substrate. After incubation in open glass tubes (37 °C, 30 min), the reactions were terminated by adding 100 μl of methanol and centrifuged at 5,000 g for 5 min. Conversion of *N*-(2-methoxyphenyl)hydroxylamine with rat CYP enzymatic systems to its metabolites was linear until 40 min. The supernatants were collected and 30 l aliquots applied onto a high-performance liquid chromatography (HPLC) column, where metabolites of *N*-(2-methoxyphenyl)hydroxylamine were separated. Compositions of incubation mixtures used for study of the metabolism of *N*-(2-methoxyphenyl)hydroxylamine by rabbit NADPH:P450 reductase were analogous to those described above, but 2 μM NADPH:CYP reductase instead of microsomal enzymes was present. NADPH:CYP reductase was active with its substrate, cytochrome c. The HPLC was performed on a C-18 high density reversed-phase column (250 × 4.6 mm, 5 μm, Nucleosil 100-5HD, Macherey-Nagel, Duren, Germany). Metabolites were eluted with 20% methanol, 80% 7.18 μM aqueous ammonia, pH 8.0 (v/v), at a flow rate of 0.6 ml/min and monitored at 234 nm. The *N*-(2-methoxyphenyl)hydroxylamine metabolites were separated from parent *N*-(2-methoxyphenyl)hydroxylamine by HPLC with UV detection and characterized by mass spectrometry and c*o*-chromatography with a synthetic standard as described (Naiman *et al.* 2008, 2010). 2-Methoxynitrosobenzene, *o*-aminophenol, *N*-(2-methoxyphenyl)hydroxylamine and *o*-anisidine standards, were eluted at retention times (r.t.) of 9.8, 10.8, 24.8 and 35.0 min, respectively.

Inhibition studies

The following chemicals were used to inhibit the metabolism of *N*-(2-methoxyphenyl)hydroxylamine in rat hepatic microsomes and rat recombinant CYP enzymes: α-naphthoflavone (α-NF), which inhibits CYP1A1 and 1A2; isopropenyl-3-methyldiamantane (3-IPMDIA), which inhibits CYP2B; sulfaphenazole, which inhibits CYP2C; quinidine, which inhibits CYP2D; diethyldithiocarbamic acid (DDTC), which inhibits CYP2E1 and 2A and ketoconazole and troleandomycin, which inhibit CYP3A (Rendic & DiCarlo 1997; Stiborova *et al.* 2002a; 2005a). α-Lipoic acid and 6-propyl-2-thouracil, which inhibit NADPH:CYP reductase (Slepneva *et al.* 1995) and NADH:cytochrome b_5 reductase (Okabe *et al.* 1983), respectively, were also used in the experiments. 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 0.001–10 mM in the incubation mixtures.

RESULTS

When *N*-(2-methoxyphenyl)hydroxylamine was incubated with rat hepatic microsomes in the presence of NADPH, four product peaks with r.t. of 8.2, 10.8, 12.8 and 35.0 min, were separated by HPLC with UV monitoring at 234 nm (see peaks in Figure 2A for the profile obtained with hepatic microsomes of rats pretreated with ethanol) (Naiman *et al.* 2010). Two of them were indentified as *o*-aminophenol and *o*-anisidine (Naiman *et al.* 2008; 2010), while structure of other two metabolites, M1 and M2, remains to be investigated. Whereas *o*-aminophenol and metabolites M1 and M2 are the minor products, *o*-anisidine is formed as the major metabolite (Table 1). These results indicate that *N*-(2-methoxyphenyl)hydroxylamine is metabolized in microsomes by oxidative and mainly by reductive reactions.

When *N*-(2-methoxyphenyl)hydroxylamine was incubated without hepatic microsomal enzymes or without NADPH, metabolite M1, M2, *o*-aminophenol and *o*-anisidine peaks were also detectable by HPLC, but only under acidic conditions (pH 4.5 for 60 min). At pH 7.4, used for microsomal incubations, their spontaneous formation was negligible; only a low but detectable amount of *o*-anisidine was found (Table 1). In the presence of NADPH in the incubation mixture, the amount of this metabolite was increased, by 2.4-fold (Table 1). These findings indicate that conversion of *N*-(2-methoxyphenyl) hydroxylamine in microsomes is mediated mainly by enzymatic reactions, but participation of NADPH in formation of *o*-anisidine cannot be excluded.

When *N*-(2-methoxyphenyl)hydroxylamine was incubated with NADPH and NADPH:CYP reductase, the enzyme that is besides CYPs the prominent component of the microsomal enzymatic system, formation of metabolite M1 and *o*-anisidine was increased, but to a low extent (Figure 2, Table 1). This result suggests that NADPH:CYP reductase plays a minor role in *N*-(2-methoxyphenyl)hydroxylamine metabolism in microsomes. Indeed, an inhibitor of NADPH:CYP reductase,

Tab. 1. Metabolism of *N*-(2-methoxyphenyl)hydroxylamine by rat hepatic microsomes and rabbit NADPH:CYP reductase

	***N*-(2-methoxyphenyl)hydroxylamine metabolites[a]**			
	M1	**M2**	***o*-aminophenol**	***o*-anisidine**
Hepatic microsomes from rats pre-treated with[b]				
None - control microsomes	2.0 ± 0.7	0.9 ± 0.8	0.6 ± 0.6	12.3 ± 6.9
β-naphthoflavone (CYP1A1/2)	5.1 ± 2.8	4.0 ± 2.8	0.8 ± 0.2	13.3 ± 5.2
Phenobarbital (CYP2B1/2)	2.8 ± 0.9	0.7 ± 0.5	0.1 ± 0.1	40.2 ± 1.1***
Ethanol (CYP2E1)	2.8 ± 0.5	n.d.[c]	0.1 ± 0.1	55.5 ± 4.1***
PCN (CYP3A)	2.9 ± 1.2	1.4 ± 0.5	1.1 ± 0.1	25.0 ± 10.6*
Without microsomes and NADPH	n.d.	n.d.	n.d.	0.7 ± 0.3
NADPH without microsomes	n.d.	n.d.	n.d.	1.7 ± 0.4§§
NADPH:CYP reductase	3.5 ± 0.6	n.d.	n.d.	2.2 ± 0.5§§§

[a]The numbers are the peak area/min/nmol CYP (or NADPH:CYP reductase) for each metabolite; averages ± S.E.M of three determinations in separate experiments. [b]Isoforms of CYP induced are shown in brackets. [c]n.d. - not detectable. Values significantly different from control microsomes: *$p<0.05$, ***$p<0.001$. Values significantly different from incubations without microsomes and without NADPH: §§$p<0.01$, §§§$p<0.001$ (Student's t-test).

Tab. 2. The effects of CYP inhibitors on *N*-(2-methoxyphenyl) hydroxylamine metabolism in rat hepatic microsomes.

Hepatic microsomes from rats pre-treated with[a]	**Inhibitor[b]**	**IC_{50} (μM)[c] for formation of *o*-anisidine**
β-NF (CYP1A1/2)	α-NF(CYP1A1/2)	1200
β-NF (CYP1A1/2)	Furafylline (CYP1A2)	1300
PB (CYP2B1/2)	3-IPMDIA (CYP2B)	n.i.[d]
Control	Sulfaphenazole (CYP2C)	50
Control	Quinidine (CYP2D)	300
Ethanol (CYP2E1)	DDTC (CYP2E1, 2A)	5
PCN (CYP3A)	Ketoconazole (CYP3A)	3500
PCN (CYP3A)	Troleandomycin (CYP3A)	1500
PB (CYP2B, POR)	α-lipoic acid (POR)[e]	5000
PB (CYP2B, POR)	6-propyl-2-thouracil (NADH:b_5 reductase)	3000

[a]Isoforms of CYP induced are shown in brackets.
[b]Isoforms of CYP inhibited are shown in brackets.
[c]Estimated from concentration-dependent inhibition of formation of *N*-(2-methoxyphenyl)hydroxylamine metabolites by interpolation (inhibitors were 0.001–10 mM depending on the chemical). 1 mM *N*-(2-methoxyphenyl)hydroxylamine and 0.4 nmol CYP were present in the incubation medium.
[d]n.i., no inhibition, which is IC_{50} greater than 10 mM.
[e]POR, NADPH:CYP reductase
The numbers in the table are averages of three determinations in separate experiments.

α-lipoic acid (Slepneva *et al.* 1995), showed only a negligible effect on *o*-anisidine formation in hepatic microsomes (Table 2).

In order to evaluate whether another reductase present in the microsomes, NADH:cytochrome b_5 reductase, is involved in reduction of *N*-(2-methoxyphenyl) hydroxylamine to *o*-anisidine, the effect of its inhibitor, 6-propyl-2-thouracil (Okabe *et al.* 1983), was also investigated. As shown in Table 2, the IC_{50} value of this inhibitor suggests only a slight effect (if any) on *N*-(2-methoxyphenyl)hydroxylamine reduction to *o*-anisidine, excluding participation of this enzyme in the reaction.

To evaluate the participation of microsomal CYP enzymes in *N*-(2-methoxyphenyl)hydroxylamine metabolism, hepatic microsomes of control (untreated) rats and rats treated with inducers of individual CYPs were utilized. Microsomes isolated from livers of untreated rats and rats pre-treated with β-NF (enriched with CYP1A1/2), PB (enriched with CYP2B1/2 and/or 2C), ethanol (enriched with CYP2E1) and PCN (enriched with CYP3A) were all capable of metabolizing *N*-(2-methoxyphenyl)hydroxylamine, participating differently in this reaction process (Table 1). While incubations of *N*-(2-methoxyphenyl)hydroxylamine with the microsomes of rats pre-treated with ethanol, PB and PCN led to a 4.5-, 3.3- and 2-fold increase ($p<0.05$) in *o*-anisidine formation, respectively (Table 1), inducers of other CYP enzymes had essentially no effect. Microsomes of rats treated with β-NF were the most effective system to form other metabolites, *o*-aminophenol and metabolites M1 and M2 (Table 1).

To investigate the role of individual CYPs in *N*-(2-methoxyphenyl)hydroxylamine metabolism further, the effect of CYP inhibitors was also performed (Table 2). Only the effect of inhibitors on *o*-anisidine formation was carried out, because formation of other metabolites was very low. An inhibitor of CYP2E1 and 2A, DDTC, was highly effective in inhibiting *o*-anisidine formation by microsomes of rats treated with ethanol, with the IC_{50} value of 5 μM (Table 2). Another efficient inhibitor decreasing *o*-anisidine formation in microsomes

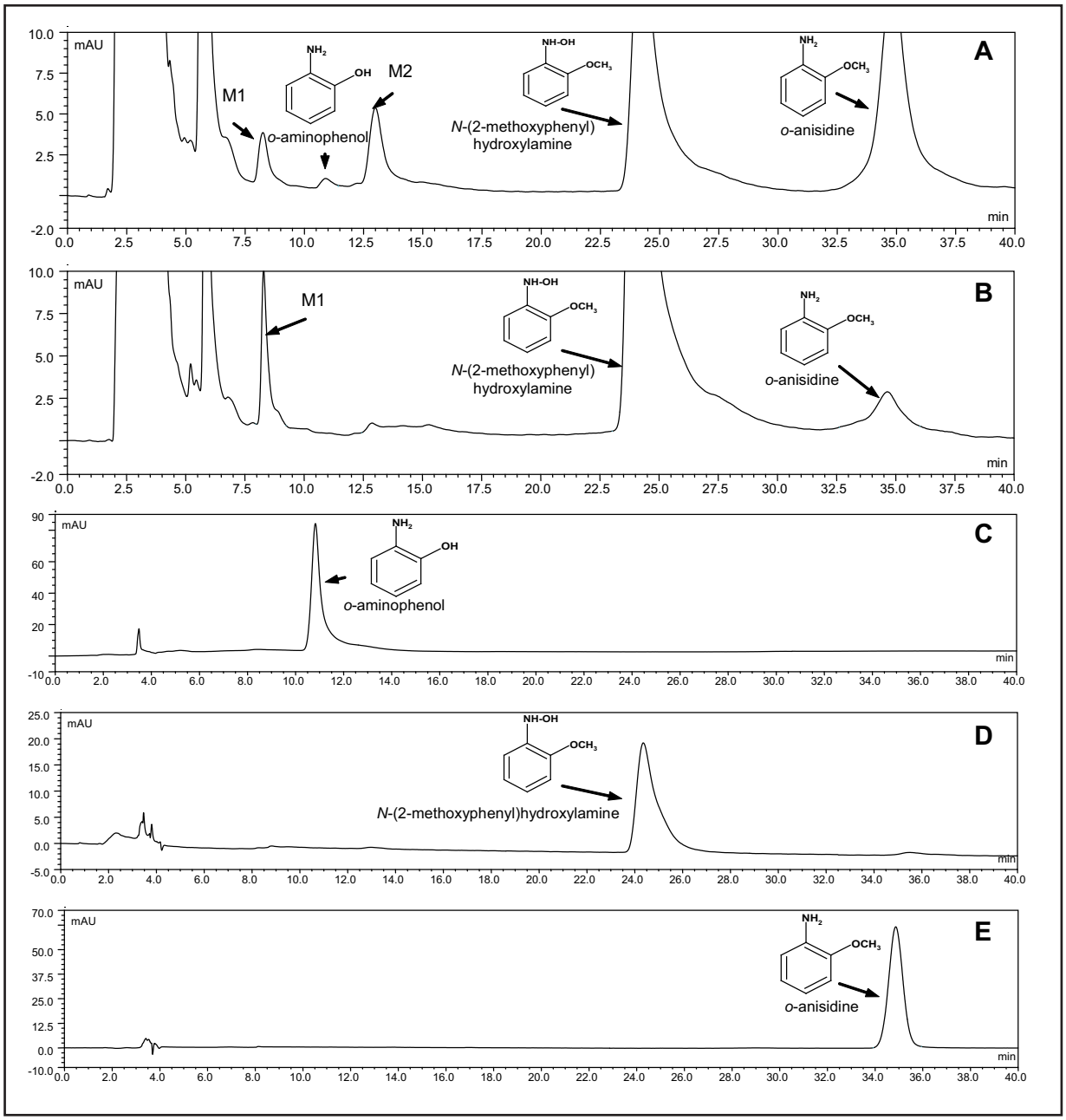

Fig. 2. HPLC elution profiles of metabolites of 1 mM *N*-(2-methoxyphenyl)hydroxylamine incubated with hepatic microsomes of rats pre-treated with ethanol (A), of 1 mM *N*-(2-methoxyphenyl)hydroxylamine incubated with NADPH:CYP reductase and NADPH (B). (C) synthetic *o*-aminophenol (D) *N*-(2-methoxyphenyl)hydroxylamine, (E) *o*-anisidine. For incubation conditions see the Experimental section. Peaks eluting between 2.0 and 5.5 min, solvent front, NADPH and protein components of microsomes and NADPH-generation system.

of control (untreated) rats was an inhibitor of CYP2C, sulfaphenazole, while quinidine, an inhibitor of CYP2D, was less effective (Table 2). Inhibitors of other CYP enzymes such as α-NF (inhibitor of CYP1A1/2), furafylline (inhibitor of CYP1A2), ketoconazole (inhibitor of CYP3A), troleandomycin (inhibitor of CYP3A), and 3-IPMDIA (inhibitor of CYP2B) caused either low or no inhibition of *o*-anisidine formation (Table 2). All these results indicate that rat CYP2E1 and/or 2A, followed by CYP2C and 2D seem to be the most efficient enzymes catalyzing reduction of *N*-(2-methoxyphenyl) hydroxylamine to *o*-anisidine in rat 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.

Therefore, to identify and prove the role of individual CYPs in *N*-(2-methoxyphenyl)hydroxylamine metabolism, we utilized microsomes of Baculovirus transfected insect cells (Supersomes™) containing recombinantly expressed rat CYPs and NADPH:CYP reductase (Figure 3). Because of importance of another

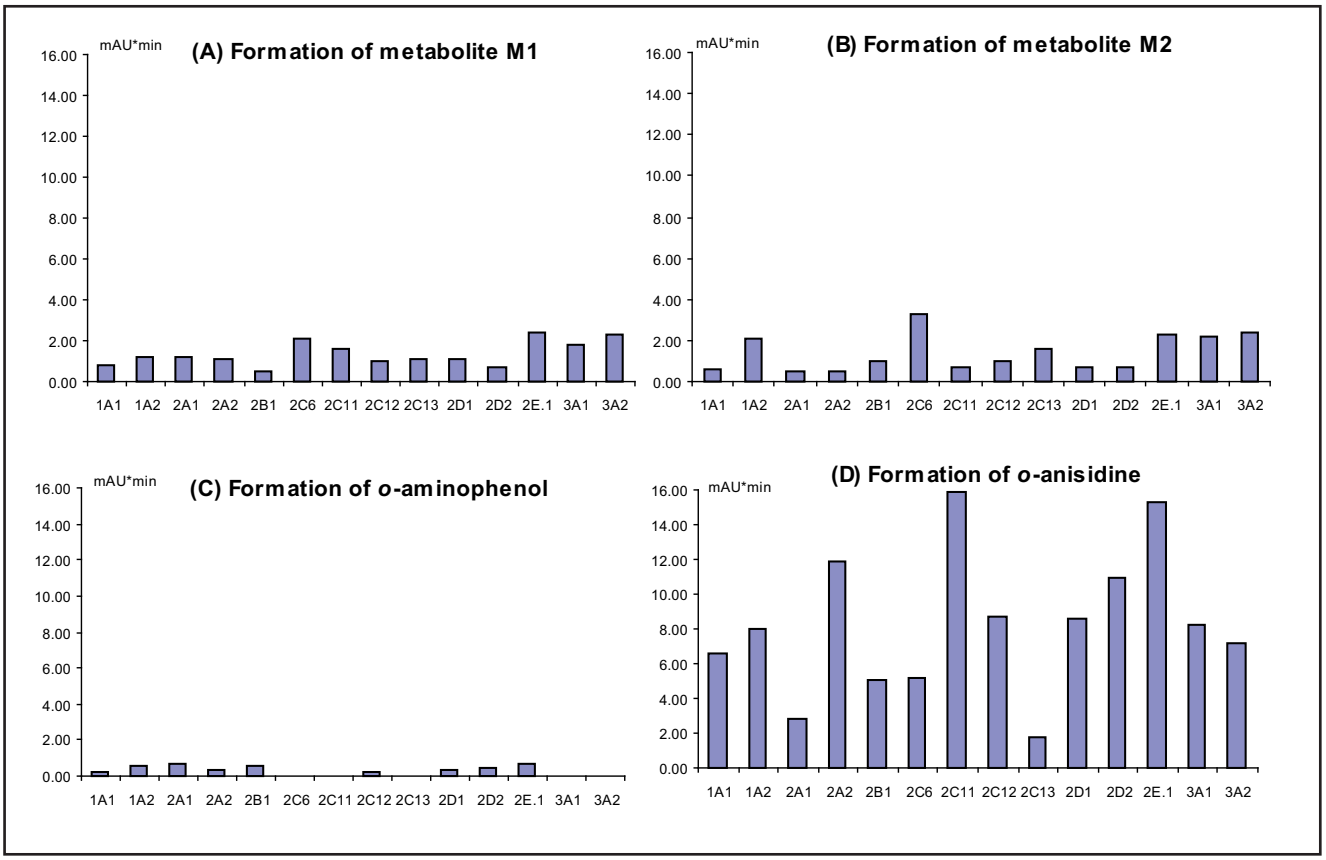

Fig. 3. Formation of metabolite M1 (A), metabolite M2 (B), *o*-aminophenol (C), *o*-anisidine (D) from *N*-(2-methoxyphenyl)hydroxylamine by rat recombinant CYPs. The values in the figure are means of two parallel experiments.

Tab. 3. The effects of CYP inhibitors on *N*-(2-methoxyphenyl) hydroxylamine metabolism by rat recombinant CYPs.

Inhibitor[a]	IC_{50} (μM)[c] for formation of *o*-anisidine
DDTC (CYP2A2)	35
Sulfaphenazole (CYP2C11)	10
Quinidine (CYP2D2)	40
DDTC (CYP2E1)	20
Ketoconazole (CYP3A1)	1500

[c]Estimated from concentration-dependent inhibition of formation of *N*-(2-methoxyphenyl)hydroxylamine metabolites by interpolation (inhibitors were 0.001–10 mM depending on the chemical). 1 mM *N*-(2-methoxyphenyl)hydroxylamine and 0.4 nmol CYP were present in the incubation medium. The numbers in the table are averages of two determinations in separate experiments.

component of the microsomal system, cytochrome b_5, for activities of several CYPs (Yamazaki *et al.* 2001; McLaughlin *et al.* 2010), the Supersomes™ used in the experiments contained also this protein. The recombinant rat CYPs used in the experiments efficiently oxidized their typical substrates (results not shown). Rat CYP2C11 and 2E1, followed by CYP2A2, 2D1/2, 2C12, 3A1/2 and 1A1/2 were the most efficient to reduce *N*-(2-methoxyphenyl)hydroxylamine to *o*-anisidine. Among other CYPs tested in this study, CYP2B1, 2C6 and 2A1, were also capable to metabolize *N*-(2-methoxyphenyl) hydroxylamine, but to a lesser extent (Figure 3).

Metabolites M1, M2 and *o*-aminophenol are also formed by individual rat recombinant enzymes, but in low quantities (Figure 3).

In order to investigate the contribution of individual CYP enzymes to metabolism *N*-(2-methoxyphenyl) hydroxylamine to *o*-anisidine in rat hepatic microsomes, the effect of inhibitors of the most effective recombinant CYP enzymes catalyzing *o*-anisidine formation, CYP2A2, 2C11, 2D2, 2E1 and 3A1, on this reaction was also analyzed (Table 3). The IC_{50} values for inhibition of *N*-(2-methoxyphenyl)hydroxylamine metabolism to *o*-anisidine, catalyzed by CYP2A2, 2C11, 2D2, 2E1 and 3A1 (Table 3), were similar to those found for inhibition of this reaction in rat hepatic microsomes (Table 2). Therefore, inhibition of *N*-(2-methoxyphenyl)hydroxylamine metabolism by these inhibitors in microsomes corresponds to inhibition of these enzymes. Based on the data showing the reduction of *N*-(2-methoxyphenyl)hydroxylamine to *o*-anisidine by recombinant CYPs (Figure 3), the IC_{50} values for inhibition of this reaction catalyzed by microsomes

or recombinant CYPs (Tables 2 and 3) and the expression levels of CYP enzymes in rat hepatic microsomes (55, 15, 10, 7, 7, 5 and 2% of CYP2C, 2E1, 3A, 2D, 2A, 2B and 1A, respectively) (Nedelcheva & Gut 1994; Rolsted & KIssmeyer 2008), contributions of individual CYP enzymes to *N*-(2-methoxyphenyl)hydroxylamine reduction in rat hepatic microsomes were estimated. The highest contribution to *N*-(2-methoxyphenyl) hydroxylamine reduction in rat hepatic microsomes is attributed to CYP2C (~74%), followed by CYP2E1 (~12%), CYP2D (~8%), and CYP2A (~6%). Even though the activity of rat recombinant CYP3A and 1A to reduce *N*-(2-methoxyphenyl)hydroxylamine is relatively high (Figure 3), because of a low degree of inhibition of reaction catalyzed by CYP1A and 3A (Table 2) and low expression of CYP1A enzymes in rat livers (Nedelcheva & Gut 1994; Rolsted & Kissmeyer 2008), their contribution to this reaction in rat hepatic microsomes is negligible.

DISCUSSION

The results of this study show that rat hepatic microsomes can metabolize *N*-(2-methoxyphenyl)hydroxylamine, a reactive metabolite of carcinogenic *o*-anisidine and *o*-nitroanisole. This compound is responsible for genotoxic effects of both carcinogens, because it is easily decomposed to the nitrenium/carbenium ion forming DNA adducts (Figure 1) (Stiborova *et al.* 2004; 2005b; Naiman *et al.* 2008; 2010). As shown previously (Naiman *et al.* 2008; 2010) and in the present work, microsomal enzymes also catalyze its further oxidative and reductive metabolism to produce four metabolites. Among them, *o*-anisidine, which is the parent carcinogen from which *N*-(2-methoxyphenyl)hydroxylamine is formed, is the major one**.** Minor products are *o*-aminophenol and two metabolites, whose structures remain to be resolved. Therefore, reduction of *N*-(2-methoxyphenyl)hydroxylamine is the predominant reaction catalyzed by microsomal enzymes. Recently, redox cycling reactions similar to those we found with *N*-(2-methoxyphenyl)hydroxylamine were observed by Kim *et al.* (2004), who studied metabolism of several aromatic and heterocyclic amines by a CYP1A2/NADPH:CYP reductase enzymatic system. They reported that the CYP system catalyzes oxidation of the *N*-hydroxylated intermediate formed from the carcinogenic heterocyclic amine 2-amino-3-methylimidazo[4,5-*f*] quinoline (IQ), to a nitrosoderivative. They demonstrated that NADPH:CYP reductase can catalyze the reduction of the IQ oxidation products, *N*-nitroso-IQ and *N*-hydroxyl-IQ, to *N*-hydroxyl-IQ and the parent amine, IQ (Kim *et al.* 2004). *N*-hydroxylation products of two other aromatic amines investigated by Kim *et al.* (2004), 2-aminofluorene and 4-aminobiphenyl, are, however, reduced non-enzymatically by NADPH.

The results of this study demonstrate that reduction of *N*-(2-methoxyphenyl)hydroxylamine is mediated mainly by enzymatic reactions. They also identify, which of microsomal enzymes are responsible for this reduction. Here, we found that NADPH:CYP reductase and NADH:cytochrome b_5 reductase play a minor role (if any) in conversion of *N*-(2-methoxyphenyl) hydroxylamine to *o*-anisidine. In contrast to these results, microsomal CYP enzymes are capable of catalyzing this reaction. When compared the efficiency of pure NADPH:CYP reductase with microsomes rich in CYP2E1, an up to 25-fold increase in *o*-anisidine formation was detected.

N-(2-methoxyphenyl)hydroxylamine was found to be a promiscuity substrate of rat CYP enzymes. The CYP2C, 2E1, 2D and 2A enzymes, which are expressed in rat liver, are crucial for *N*-(2-methoxyphenyl) hydroxylamine reduction in this tissue. These results indicate that *N*-(2-methoxyphenyl)hydroxylamine acts as ligand of heme iron of these CYPs, being reduced instead of molecular oxygen during the CYP-mediated reaction cycle. Even though the efficacy of rat recombinant CYP3A and 1A to reduce *N*-(2-methoxyphenyl)hydroxylamine to *o*-anisidine is relatively high (Figure 3), its contribution to this reaction in rat liver microsomes was found to be low. Namely, the relative involvement of particular CYP enzymes in *N*-(2-methoxyphenyl)hydroxylamine reduction in liver depends on the levels of the CYP present in this organ. Indeed, the metabolism of several xenobiotics in human livers was found to also depend, to some extent, on the levels of the CYP expression in this tissue (Lewis 2003).

While the formation of *N*-(2-methoxyphenyl) hydroxylamine was clearly identified to be the activation pathway of *o*-anisidine and *o*-nitroanisole metabolism (Stiborova *et al.* 2004; 2005b), biological significance of reverse reduction of this compound to *o*-anisidine for detoxication/activation metabolism is a matter of debate. The *N*-(2-methoxyphenyl)hydroxylamine metabolism decreases its levels, thereby decreasing formation of nitrenium/carbenium ions, its ultimate carcinogenic species binding to DNA (Naiman *et al.* 2010). However, *o*-anisidine generated as the major metabolite is easily oxidized by redox cyclic reactions back to *N*-(2-methoxyphenyl)hydroxylamine. Besides several CYP enzymes, CYP2E1 is the major enzyme catalyzing this reaction (Stiborova *et al.* 2005b). Nevertheless, the CYP enzymes also oxidize *o*-anisidine to *o*-aminophenol, which was supposed as a detoxication metabolite of *o*-anisidine (Naiman *et al.* 2008; 2010). Genotoxicity of *o*-anisidine and *N*-(2-methoxyphenyl)hydroxylamine is thus dictated by the efficiency of CYP enzymes to catalyze either of the reactions. In other words, on a type of binding of these compounds to the CYP active center that determines preferences of individual reactions.

Because a role of *o*-aminophenol and its metabolism in detoxication and/or activation of *o*-anisidine were not known, these subjects were studied in our recent work (Naiman *et al.* 2010). We have found that metabolism of *o*-aminophenol by hepatic microsomal

CYP enzymes or peroxidases do not lead to formation of covalent DNA adducts. No metabolism of and DNA adduct formation by this compound were found in the microsomal system. No DNA adducts were also generated during its oxidation with peroxidases. These findings suggest that *o*-aminophenol and its metabolism by peroxidases are not included into genotoxic processes occurring during carcinogenesis caused by *o*-anisidine. Nevertheless, *o*-aminophenol might be considered to be mutagenic, it induces sister chromatid exchanges in a dose-dependent manner in cultured human lymphocytes *in vitro* and in Chinese hamster bone marrow cells *in vivo* (Kirchner & Bayer 1992). In addition, Brennan and Schiestl (1997; 1999) reported that *o*-aminophenol is positive in the deletion recombination assay in *Saccharomyces cerevisiae*. Even though *o*-aminophenol has not been found to form covalent DNA adducts (Naiman *et al.* 2010), it was demonstrated in *in vitro* experiments to cause DNA damage, forming 8-oxy- 7,8-dihydro-2'-deoxyguanosine in the presence of metal ions such as Cu(II) (Okhuma & Kawanishi 2001). Hence, due to such processes, *o*-aminophenol may contribute to initiation of the *o*-anisidine- and/or *o*-nitroanisole-mediated carcinogenesis in the urinary bladder, and in a tumor development induced by other bladder carcinogenic aromatic amines, which produce this compound as one of the metabolites (Brennan & Schiestl 1997). Furthermore, *O*-demethylation reactions produce formaldehyde (Figure 1), which is known to modify DNA, generating several products including hydroxymethyl adducts and cross-links (Beland *et al.* 1984; Huang & Hopkins 1993; Cheng *et al.* 2003). Formaldehyde is mutagenic in a variety of different test systems and carcinogenic in laboratory animals (IARC 2006) and has been described as "carcinogenic to human" by the IARC and "reasonably anticipated to be a human carcinogen" by the U.S. Department of Health and Human Services (2004). Therefore, on the one hand, it is plausible that formaldehyde-DNA adducts could also play a role in carcinogenesis by *o*-anisidine and *o*-nitroanisole. On the other hand, however, formaldehyde produced in the cell is also detoxified by conjugation to glutathione and oxidized (Dhareshwar & Stella 2008); therefore, it is not likely a strong contributor to carcinogenicity caused by *o*-anisidine and *o*-nitroanisole.

Collectively, the results found in the present study show for the first time capability of rat hepatic CYP enzymes to catalyze reduction of *N*-(2-methoxyphenyl)hydroxylamine to *o*-anisidine. Among them, the CYP2C, 2E1, 2D and 2A are the most important enzymes catalyzing this reaction in rat liver. The question however arises, whether the human CYP enzymatic system is also capable of catalyzing this *N*-(2-methoxyphenyl)hydroxylamine conversion. Therefore, such a study is planned to be carried out in our future work.

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