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# CARCINOGENIC POLLUTANTS *o*-NITROANISOLE AND *o*-ANISIDINE ARE SUBSTRATES AND INDUCERS OF CYTOCHROMES P450

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2-Methoxyaniline (*o*-anisidine) and 2-methoxynitrobenzene (*o*-nitroanisole) are important pollutants and potent carcinogens for rodents. *o*-Anisidine is oxidized by microsomes of rats and rabbits to *N*-(2-methoxyphenyl)hydroxylamine that is also formed as the reduction metabolite of *o*-nitroanisole. *o*-Anisidine is a promiscuity substrate of rat and rabbit cytochrome P450 (CYP) enzymes, because CYPs of 1A, 2B, 2E and 3A subfamilies oxidize *o*-anisidine. Using purified CYP enzymes, reconstituted with NADPH: CYP reductase, rabbit CYP2E1 was the most efficient enzyme oxidizing *o*-anisidine, but the ability of CYP1A1, 1A2, 2B2, 2B4 and 3A6 to participate in *o*-anisidine oxidation was also proved. Utilizing Western blotting and consecutive immunoquantification employing chicken polyclonal antibodies raised against various CYPs, the effect of *o*-anisidine and *o*-nitroanisole on the expression of the CYP enzymes was investigated. The expression of CYP1A1/2 was found to be strongly induced in rats treated with either compounds. In addition, 7-ethoxyresorufin *O*-deethylation, a marker activity for both CYP1A1 and 1A2, was significantly increased in rats treated with either carcinogen. The data demonstrate the participation of different rat and rabbit CYP enzymes in *o*-anisidine oxidation and indicate that both experimental animal species might serve as suitable models to mimic the *o*-anisidine oxidation in human. Furthermore, by induction of rat hepatic and renal CYP1A1/2, both *o*-nitroanisole and *o*-anisidine influence their carcinogenic effects, modifying their detoxification and/or activation pathways.

## INTRODUCTION

Arylamines and nitroarenes are important intermediates used in the industrial manufacture of dyes, pesticides and plastics, and are significant environmental pollutants (*e.g.* from car exhausts and technical spills). They rank among potent toxic or carcinogenic compounds, presenting a considerable danger for human population[1]. *o*-Nitroanisole (2-methoxynitrobenzene) is used primarily as a precursor in the synthesis of o-anisidine (2-methoxyaniline), an intermediate in the manufacture of many azo dyes. Both chemicals exhibit strong carcinogenic activity, causing neoplastic transformation in the urinary bladder and, to a lesser extent, in spleen, liver and kidney of rats and mice[2,3]. In 1993, an industrial accident in the Hoechst Company in Germany led to a large-scale leakage of o-nitroanisole and subsequent local and regional contamination with this compound.

Xanthine oxidase is the principal enzyme responsible for the reduction metabolism of *o*-nitroanisole, catalyzing formation of *N*-(2-methoxyphenyl)hydroxylamine and *o*-anisidine[4,5]. Deoxyguanosine adducts derived from *N*-(2-methoxyphenyl)hydroxylamine were found *in vivo* in tissues, mainly urinary bladder, of rats treated with *o*-nitroanisole as well as *in vitro* after incubation of *o*-nitroanisole with human hepatic cytosols or purified buttermilk xanthine oxidase[4]. In contrast, human hepatic microsomal cytochrome P450 (CYP) enzymes as well as these of experimental animals participate in the detoxification metabolism of *o*-nitroanisole, leading to its demethylation, which enables its excretion from the organism[6].

*o*-Anisidine is oxidized by human hepatic microsomes to *N*-(2-methoxyphenyl)hydroxylamine, which is the same active intermediate as that formed from *o*-nitroanisole by nitroreduction[4–7]. The major enzyme participating in this reaction is CYP2E1, followed by CYP1A and 2B6 (ref. [7]). Such an *o*-anisidine activation leads to the formation of DNA adducts. The *in vitro* DNA adducts formed in this reaction are identical with those formed by *N*-(2-methoxyphenyl)hydroxylamine and dGp (ref.[4,7]). Furthermore, similar DNA adduct patterns were obtained also *in vivo* in urinary bladder, the target organ and, to a lesser extent, in liver, kidney and spleen of rats treated with *o*-anisidine or *o*-nitroanisole[4,7].

The present study was undertaken to identify the *o*-anisidine metabolism by rat and rabbit CYP enzymes, to compare the data with those found in human enzymatic systems[7] and to determine the effect of *o*-nitroanisole and *o*-anisidine on the expression of major biotransformation enzymes in rats.

## MATERIAL AND METHODS

*Chemicals*

Chemicals were obtained from the following sources: $NADP^+$, NADPH, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), menadione, dilauroyl phosphatidylcholine, dioleyl phosphatidylcholine, phosphatidylserine and glucose 6-phosphate from Sigma Chemical Co. (St. Louis, MO, USA); 7-ethoxyresorufin, *o*-nitroanisole, *o*-anisidine, *o*-aminophenol (>99% based on HPLC) from Fluka Chemie AG (Buchs, Switzerland), glucose 6-phosphate dehydrogenase from Serva (Heidelberg, Germany), glutathione from Roche Diagnostics Mannheim (Germany), bicinchoninic acid from Pierce (Rockford, IL, USA) and Sudan I (1-phenylazo-2-naphthol) from British Drug Houses (London, UK). All these and other chemicals were of analytical purity or better. *N*-(2-methoxyphenyl)hydroxylamine was synthesized by the procedure similar to that described earlier[8]. Briefly, to a solution of 2 g ammonium chloride and 90 mmol *o*-nitroanisole in 60% ethanol/water, 180 mmol zinc powder was added in small portions. After addition of the first portion at room temperature, the reaction starts; this can be monitored by the rising temperature in the flask. The reaction mixture was kept at 10–15 °C using a cooling bath (ice/sodium chloride mixture) and slowly adding additional doses of zinc powder. After 1 h, excess zinc was removed by filtration and ethanol was removed under reduced pressure. The product was extracted into 100 ml ethyl acetate and crystallized by adding hexane. The yield was 60%. *N*-(2-Methoxyphenyl)hydroxylamine authenticity was confirmed by electrospray mass and CID spectra and high field proton NMR spectroscopy. The positive-ion electrospray mass-spectrum exhibited the protonated molecule at *m*/*z* 140.1, while the CID of its ion fragments at *m*/*z* 125.2 108.1 and 109.1. The $^1$H-NMR spectra were recorded at 400 MHz in dimethyl sulfoxide-$d_6$. The central line of dimethyl sulfoxide at 2.500 ppm was used as reference line. The spectra showed the presence of the following protons: 8.28 (1H, d, J = 2.3 Hz, exchanged with $CD_3OD$), 7.64 (1H, d, J = 1.5 Hz, exchanged with $CD_3OD$), 7.01 (1H, m, Σ J = 9.6 Hz), 6.84 (2H, m, Σ J = 15.0 Hz), 6.75 (1H, m, Σ J = 16.9 Hz), 3.75 (3H, s).

*Preparation of microsomes and assays*

Microsomes from rat and rabbit livers were prepared by the procedure described previously[9]. Protein concentrations in the microsomal fractions were assessed using the bicinchoninic acid protein assay with bovine serum albumin as a standard[10]. The concentration of CYP was estimated according to Omura and Sato[11] by measuring the absorption of the complex of reduced CYP with carbon monoxide. Rat and rabbit liver microsomes contained 0.6 and 1.8 nmol CYP/mg protein, respectively.

*Isolation of individual CYPs*

CYP1A2, 2B4, 2C3 and 2E1 enzymes were isolated from liver microsomes of rabbits induced with β-naphthoflavone (CYP1A2), phenobarbital, (CYP2B4, 2C3) or ethanol (CYP2E1), by procedures described by Haugen and Coon[12] and Yang *et al*.[13] CYP3A1 and 3A6 were isolated from hepatic microsomes of rats and rabbits induced with pregnenolone-16α-carbonitrile[14] and rifampicin[15], respectively. The procedure was analogous to that used for isolation of CYP2B4. Rat CYP2B2 was isolated from liver microsomes of rats pretreated with phenobarbital by the procedure as described[16]. Recombinant rat CYP1A1 protein was purified to homogeneity by the procedure described previously[17] from membranes of *Escherichia coli* transfected with a modified *CYP1A1* cDNA, in the laboratory of H. W. Strobel (University of Texas, Medical School of Houston, Texas, USA) by P. Hodek (Charles University, Prague, Czech Republic). Rabbit liver NADPH:CYP reductase was purified as described[18]. Rabbit liver cytochrome $b_5$ was prepared as described elsewhere[19].

*Incubations*

Unless stated otherwise, incubation mixtures used for study of the *o*-anisidine metabolism 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, rat or rabbit hepatic microsomal fraction containing 0.04–1.0 nmol CYP, and 0.1–1.0 mM *o*-anisidine dissolved in 1.0 µl methanol. The reaction was initiated by adding the substrate. Incubations containing purified CYP reconstituted with NADPH:CYP reductase and cytochrome $b_5$ contained 50–200 pmol of each CYP (ref.[6,7]). Briefly, CYP was reconstituted as follows (0.5 µM CYP, 0.5 µM NADPH:CYP reductase, 0.5 µM cytochrome $b_5$, 0.5 µg/µl CHAPS, 2.0 µg/µl liposomes [dilauroyl phosphatidylcholine, dioleyl phosphatidylcholine, phosphatidylserine (1:1:1)], 3 mM reduced glutathione and 50 mM HEPES/KOH, pH 7.4)[6,7]. An aliquot of CYP of the reconstitution mixture was then added to incubation mixtures. After incubation in open glass tubes (37 °C, 30 min) the residual *o*-anisidine and its metabolites were extracted from the incubation mixtures with ethyl acetate (2 × 100 µl). Ethyl acetate extracts were evaporated to dryness, dissolved in 50 µl of methanol and applied onto the plates of thin layer of silica gel. Thin layer chromatography (TLC) was carried out using the solvent system consisting of ethyl acetate: petrol ether (2 : 3, v/v). A major product spot (UV detection at 254 nm) was scraped from the plates, extracted with ethanol, concentrated on a speed-vac evaporator and analyzed by mass spectrometry. In parallel, the incubation mixtures were terminated by addition of 100 µl of methanol and centrifuged at 5,000 g for 5 min. The supernatants were collected and 20 µl aliquots applied onto a high-performance liquid chromatography (HPLC) column, where metabolites of *o*-anisidine were separated. HPLC was also used to characterize chromatographic properties of potential *o*-anisidine metabolites, *o*-aminophenol, 2-methoxynitrosobenzene, *N*-(2-methoxyphenyl)hydroxylamine and 2-methoxynitrobenzene, which eluted at retention times (r.t.) of 8.4, 8.8, 18.7 and 57.5 min, respectively. In the control incubation, the CYP was omitted from the reconstitution mixture. To study of

metabolism of *N*-(2-methoxyphenyl)hydroxylamine, incubation mixtures 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, rabbit hepatic microsomal fraction containing 0.04–1.0 nmol CYP, and 0.1–1.0 mM *N*-(2-methoxyphenyl)hydroxylamine dissolved in 1.0 µl methanol. The reaction was initiated by adding the substrate.

*HPLC*

The HPLC was performed with a Bishoff HPLC pump with a LDC/Milton spectrophotometric detector set at 254 nm, and peaks were integrated with a Waters QA 1 integrator. The column used was a Nucleosil 100-5 $C_{18}$ (Macherey-Nagel, Duren, Germany, 25 cm × 4.6 mm, 5 µm) proceeded by a C-18 guard column. Chromatography was under isocratic conditions of 20 % methanol in 50 mM ammonium carbonate, pH 8.0, with a flow rate of 0.7 ml/min. Two product peaks with r.t. of 6.4 and 10.0 min (peaks 1 and 2 in Figure 1) were separated by HPLC.

*Mass spectroscopy*

Positive-ion ESI mass spectra were recorded on a Finnigan LCQ-DECA quadrupole ion trap mass spectrometer (FinniganMAT, San Jose, CA, USA). Metabolites (final concentration 1 pmol/µl) dissolved in methanol/water (1 : 1, v/v) were continuously infused through a capillary held at 1.8 kV into the dynamic Finnigan nano-electrospray ion source via a linear syringe pump (Harvard Apparatus Model 22) at a rate of 1 µl/min. The ionizer and ion transfer optics parameters of the ion trap were as follows: spray voltage 1800 V, capillary temperature 150 °C, capillary voltage 14 V, tube lens offset – 22 V, octapole 1 offset – 7.4 V, lens voltage -16 V, octapole 2 offset – 11.3 V, octapole r.f. amplitude 450 V peak-to-peak (pp), and entrance lens voltage – 66.9 V. Helium was introduced at a pressure of 0.1 Pa to improve the trapping efficiency of the sample ions. The spectra were scanned in the range *m*/*z* 50–800 and the gating time was set to accumulate and trap $1 \times 10^7$ ions. The mass isolation window for precursor ion selection was set to 2 amu and centered on the $^{12}C$ isotope of the pertinent ion. The background helium gas served as the collision gas for the collision-induced dissociation (CID) experiment. The relative activation amplitude was 35 % and the activation time was 30 ms. No broadband excitations were applied.

*Animal experiments*

Six male Wistar rats (125–150 g) were treated once a day for 5 consecutive days with *o*-nitroanisole or *o*-anisidine dissolved in sunflower oil (0.15 mg/kg body wt i.p. per day). Two control animals received an equal volume of solvent only. Rats were placed in cages in temperature and humidity controlled rooms. Standardized diet and water were provided *ad libitum*. Animals were killed 24 h after the last treatment by cervical dislocation[4, 7]. Liver and kidney were removed immediately after death and used for isolation of microsomal and cytosolic fractions[20, 21].

*Estimation of CYP, NADPH:CYP reductase and NAD(P)H: quinone oxidoreductase 1 content in microsomes and cytosols of rat liver and kidney*

Immunoquantitation of rat liver microsomal CYPs (CYP1A1, 1A2, 2B, 2E1 and 3A), NADPH:CYP reductase and of cytosolic NAD(P)H:quinone oxidoreductase 1 (NQO1) was estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Samples containing 75 µg microsomal or cytosolic proteins were subjected to electrophoresis on SDS/10% polyacrylamide gels[22]. After migration, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. CYP, NADPH:CYP reductase and NQO1 proteins were probed with a chicken polyclonal antibody raised against various CYPs (CYP1A, 2B, 2E1 and 3A), NADPH:CYP reductase and NQO1, as reported elsewhere[22]. The complex antigen-antibody was visualized with an alkaline phosphatase-conjugated anti-chicken IgG rabbit antibody and 5-bromo-4-chloro-3-indolylphosphate/nitrobluetetrazolium as dye. The microsomal samples were also characterized for CYP1A activity using EROD activity[23] and for the oxidation of Sudan I (a marker substrate for CYP1A1)[22, 24]. The cytosolic samples were characterized for NQO1 activity, using menadione as a substrate[25].

## RESULTS

*o-Anisidine oxidation by rat and rabbit CYP enzymes*

When *o*-anisidine was incubated with rat and rabbit hepatic microsomes in the presence of NADPH, one major and two minor spots were separated by TLC on silica gel. On the basis of mass spectroscopy, the structure of the major *o*-anisidine metabolite was identified. In the positive-ion electrospray mass-spectrum, the metabolite showed the protonated molecule at *m*/*z* 140.1 (Fig. 1), indicating the molecular mass of a hydroxylated derivative of methoxyaniline. The CID of this ion afforded a fragment at *m*/*z* 125.2 showing the mass difference equaled to 15, representing a methyl group. Other fragments at *m*/*z* 108.1 and 109.1 show the molecular masses of pro-

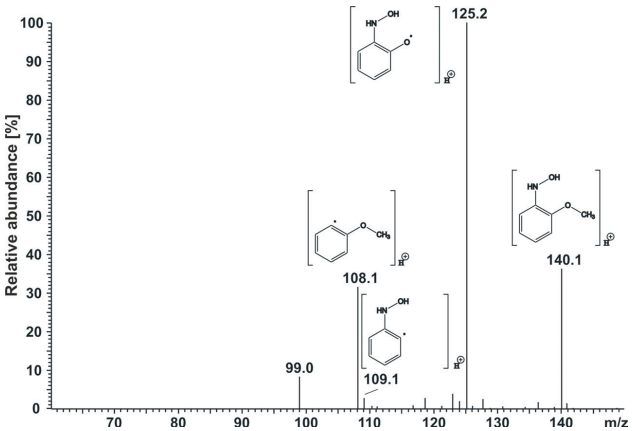


**Fig. 1.** MS/MS spectrum of *N*-(2-hydroxyphenyl)hydroxyl amine.

tonated methoxybenzene and *N*-phenylhydroxylamine, respectively. Collectively, these results indicate that the analyzed compound is a *N*-(2-methoxyphenyl)hydroxylamine metabolite. Indeed, the analyzed metabolite is identical with authentic *N*-(2-methoxyphenyl)hydroxylamine (by chromatography on thin layers of silica gel).

When incubation mixtures were analyzed by HPLC, two product peaks with retention times of 6.4 (M1) and 10.0 min (M2) were observable (Fig. 2). The co-chromatography of these metabolites with *N*-(2-methoxyphenyl)-hydroxylamine showed that chromatographic properties of either of them did not correspond to those of this synthetic standard. This finding suggests that *N*-(2-methoxyphenyl)hydroxylamine might be further oxidized by microsomal enzymes. Characterization of the metabolites remains to be performed. However, the co-chromatography of these metabolites with *o*-aminophenol (r.t. of 8.4 min), *o*-nitrosoanisole (2-methoxynitrosobenzene) (r.t. of 8.8 min) and *o*-nitroanisole (2-methoxynitrobenzene) (r.t. of 57.5 min) showed that they were none of these compounds. The results of additional experiments demonstrate that both two metabolites are products formed from *N*-(2-methoxyphenyl)hydroxylamine. In addition, the time-dependent inter-conversion between metabolites M1 and M2 occurs in the incubation mixture even after the termination of the reaction. An increase in formation of

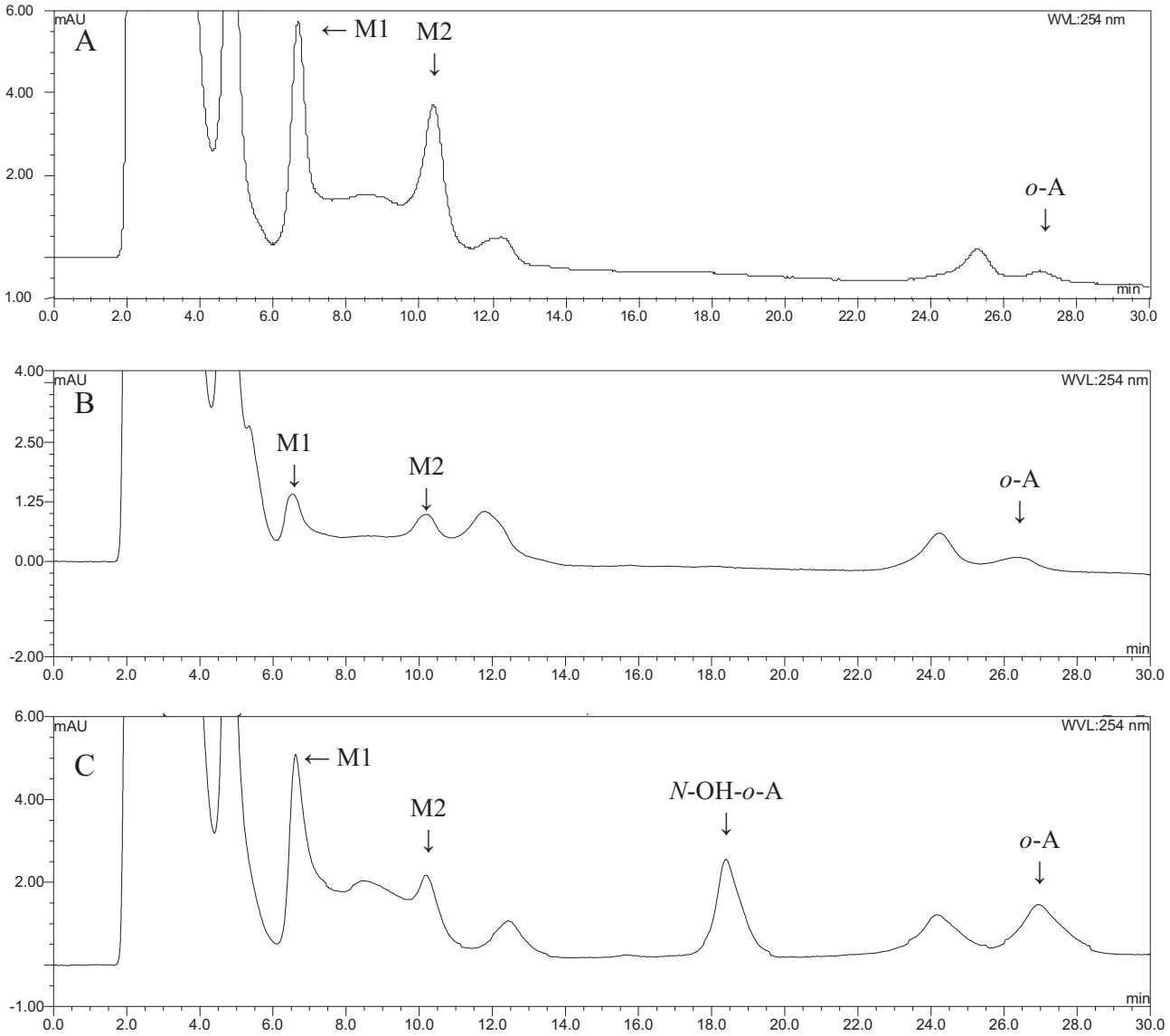


**Fig. 2.** HPLC elution profiles of metabolites of 0.1 mM *o*-anisidine (*o*-A) (A), 0.1 mM *N*-(2-methoxyphenyl)hydroxylamine (*N*-OH-*o*-A) (B) and 0.5 mM *N*-OH-*o*-A (C) formed by rabbit hepatic microsomes. Incubations (1 mM $NADP^+$, 10 mM D-glucose 6-phosphate 1 U/ml D-glucose 6-phosphate dehydrogenase, 1 mg rabbit hepatic microsomal protein and 0.1 mM *o*-anisidine or *N*-(2-methoxyphenyl)hydroxylamine or 0.5 mM *N*-(2-methoxyphenyl)hydroxylamine dissolved in 1 µl of methanol in 100 mM sodium phosphate buffer pH 7.4, final volume of 100 µl) were stopped after 30 min by addition of 100 µl of methanol and analyzed by HPLC.

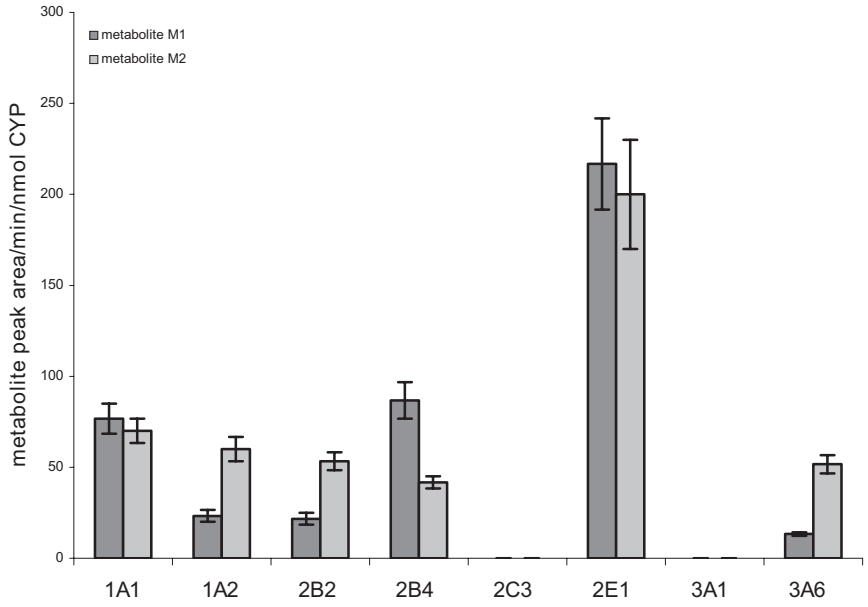


**Fig. 3.** Oxidation of *o*-anisidine by purified rat and rabbit CYPs reconstituted with rabbit NADPH:CYP reductase. Experimental conditions are described in the Material and Methods section.

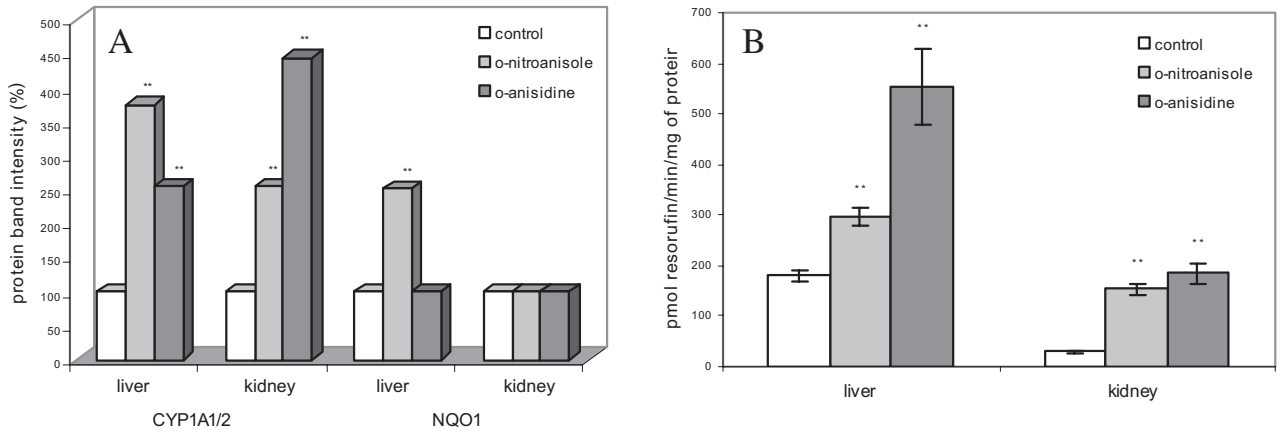


**Fig. 4.** Expression of CYP1A1/2 and NQO1 (A) and EROD activity, a marker for CYP1A1/2, (B) in hepatic and renal microsomes and cytosols of rats treated with *o*-nitroanisole or *o*-anisidine, and those of control rats. **, $P < 0.01$ [n = 3 (A), n = 6 (B)] for Student's t-test.

the metabolite M2 is followed by decrease in levels of the metabolite M1 (results not shown). After the incubation of *N*-(2-methoxyphenyl)hydroxylamine with rabbit hepatic microsomes and NADPH, a parental compound of the *N*-(2-methoxyphenyl)hydroxylamine metabolite, *o*-anisidine (r.t. of 26 min), was detected by HPLC. All these results indicate that *o*-anisidine is subject of complex redox cycling reactions. It is primarily oxidized to *N*-(2-methoxyphenyl)hydroxylamine, which is additionally both converted either spontaneously or enzymatically to two additional metabolites with yet unknown structures and reduced to the parental compound, *o*-anisidine.

In order to identify the rat and rabbit CYPs capable of oxidizing *o*-anisidine, the purified CYPs reconstituted with NADPH:CYP reductase were employed. *o*-Anisidine seems to be a promiscuity substrate of both rat and rabbit CYP enzymes as CYPs of 1A, 2B, 2E and 3A subfamilies oxidize *o*-anisidine. In the reconstituted CYP system, rabbit CYP2E1 was the most efficient enzyme oxidizing *o*-anisidine (Fig. 3).

*The effect of o-nitroanisole and o-anisidine on expression of biotransformation enzymes*

We evaluated the effect *o*-nitroanisole and *o*-anisidine on the expression of enzymes metabolizing xenobiotics, including these pollutants, in rats. Rats were treated intraperitoneally with *o*-nitroanisole or *o*-anisidine (0.15 mg/kg of body weight daily for five consecutive days) using the procedure as described[4,7]. Utilizing Western blotting with immunoquantification employing chicken polyclonal antibodies[22] raised against various CYPs (CYP1A, 2B, 2E1 and 3A), NADPH:CYP reductase and NAD(P)H:qui-

none oxidoreductase 1 (NQO1), the expression of hepatic and renal CYP1A1/2 was found to be strongly induced in rats treated with either studied chemicals (Fig. 4A). In addition, the level of hepatic NQO1 was enhanced 2.5-fold in rats treated with *o*-nitroanisole, while no induction was seen in rats exposed to *o*-anisidine (Fig. 4A).

To confirm the results obtained from Western blot analysis, specific catalytic activities of all studied enzymes in hepatic and renal microsomal fractions were examined. 7-Ethoxyresorufin *O*-deethylation (EROD) activity, a marker for CYP1A1 and 1A2 (ref.[23]), was significantly increased in livers and kidneys of rats treated with either carcinogens (Fig. 4B). Moreover, the 1.7-fold increase in oxidation of Sudan I, a marker substrate of CYP1A1 (ref.[22,24]), was observed in hepatic microsomes of rats treated with *o*-anisidine, and 9- and 8.1-fold increase in renal microsomes of rats treated with o-nitroanisole and o-anisidine, respectively. The pattern of NQO1 activities (measured with menadione as a substrate)[25] in liver and kidney cytosols was very similar to that of expression of NQO1 protein obtained by Western blot analysis. The 2- and 1.4-fold increase in the NQO1 activity was detected in livers of rats treated with o-nitroanisole and *o*-anisidine, respectively. However, there were no significant changes in NQO1 activity in kidneys of either tested rats.

## DISCUSSION

The results of this study show that rat and rabbit hepatic microsomes can oxidize carcinogenic *o*-anisidine. The hepatic microsomes of both species catalyze *N*-hydroxylation of *o*-anisidine to form a reactive metabolite *N*-(2-methoxyphenyl)hydroxylamine, which was found previously to be responsible for formation of deoxyguanosine adducts in DNA *in vitro* after *o*-anisidine oxidation by human hepatic microsomes and *in vivo* in rats treated with this carcinogen[7] or with its oxidative counterpart, *o*-nitroanisole[4]. Therefore, the enzymes of liver microsomes of both species participate in the activation pathway of *o*-anisidine. Moreover, the CYP enzymes, analogous to those catalyzing the *o*-anisidine oxidation in humans[7], were found to be responsible for this oxidation. Our results, showing an analogy in the formation of *N*-(2-methoxyphenyl)hydroxylamine as the *o*-anisidine metabolite leading to its activation to species forming DNA adducts catalyzed by human, rat and rabbit enzymes and in rats *in vivo*, strongly suggest a carcinogenic potential of this rodent carcinogen for humans.

As shown here and in the previous work[6,7], both *o*-anisidine and o-nitroanisole are metabolized besides CYP2E1 also by CYP1A and CYP2B; the reactions lead to the *o*-nitroanisole detoxication[6] and o-anisidine bioactivation (ref.[7] and present paper). Moreover, since both compounds induce the expression of enzymatically functional CYP1A in rats, it could enhance considerably the participation of these enzymes in *o*-anisidine and *o*-nitroanisole metabolism and influence the genotoxic potential of these chemicals. In the case of *o*-nitroanisole, higher activity of CYP1A allows its easier oxidative detoxication and excretion from the organism[6], decreasing its carcinogenic potential mediated mainly by its reduction with xanthine oxidase[4,5]. On the contrary, the induction of the same enzymes leads to increased cancer risk of *o*-anisidine that is oxidatively activated by CYPs to species capable of binding to DNA (ref.[7]).

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