METABOLISM OF CARCINOGENIC 2-NITROANISOLE IN RAT, RABBIT, PORCINE AND HUMAN HEPATIC CYTOSOL

Markéta MIKŠANOVÁ[a1], Petr NOVÁK[b], Eva FREI[c] and Marie STIBOROVÁ[a2,*]

[a] *Department of Biochemistry, Charles University in Prague, Albertov 2030, 128 40 Prague 2, Czech Republic; e-mail: [1] mikmar@natur.cuni.cz, [2] stiborov@natur.cuni.cz*

[b] *Institute of Microbiology, Academy of Sciences of the Czech Republic, 142 20 Prague 4, Czech Republic; e-mail: pnovak@ca.sandia.gov*

[c] *Department of Molecular Toxicology, German Cancer Research Center, 69120 Heidelberg, Germany; e-mail: e.frei@dkfz-heidelberg.de*

Received June 9, 2003
Accepted September 1, 2003

Dedicated to the 50th anniversary of the foundation of the Department of Biochemistry, the first biochemical department in Czechoslovakia.

We investigated the ability of hepatic cytosolic samples from human, rat, rabbit and pig to metabolize an important industrial pollutant and a potent carcinogen for rodents, 2-nitroanisole (1-methoxy-2-nitrobenzene). A comparison between experimental animals and the human enzymatic system is essential for the extrapolation of animal carcinogenicity data to humans to assess a health risk to humans. Two major metabolites produced from 2-nitroanisole by cytosols of all species were *N*-(2-methoxyphenyl)hydroxylamine and 2-methoxyaniline. An additional minor product of 2-nitroanisole metabolism has not yet been characterized. Both the identified metabolites are generated from 2-nitroanisole by reduction of the nitro group. To define the role of cytosolic reductases in the reduction of 2-nitroanisole, we investigated the modulation of 2-nitroanisole reduction by cofactors of the cytosolic reductases, DT-diaphorase and xanthine oxidase. The role of the human enzymes in 2-nitroanisole reduction was also investigated by correlating the xanthine oxidase-linked catalytic activities in each human cytosolic sample with the concentration of the 2-nitroanisole reduction product, 2-methoxyaniline, formed by the action of the same cytosol. On the basis of these analyses, most of hepatic cytosolic reduction of 2-nitroanisole was attributed to xanthine oxidase, but participation of DT-diaphorase in the reduction of this carcinogen in hepatic cytosols of rabbit and pigs cannot be excluded. Using the purified xanthine oxidase, its participation in 2-nitroanisole reduction was confirmed. The data clearly demonstrate the predominant role of xanthine oxidase in 2-nitroanisole reduction in human and rat hepatic cytosols and suggest a carcinogenic potency of this rodent carcinogen for humans.

Keywords: Nitro compounds; Environmental pollutants; Carcinogens; Risk assessment; Metabolism; Cytosolic reductases; Xanthine oxidase; DT-diaphorase; Enzymatic reductions.

Aromatic nitro compounds rank among potent toxic or carcinogenic compounds, presenting a considerable danger for human population[1,2]. They are widely distributed environmental pollutants found at workplaces (e.g. in chemical industry), in emissions from diesel and gasoline engines and on the surface of ambient air particulate matter[2], where they add to local and regional pollution (car exhausts, technological spills). The toxicity and carcinogenicity of these compounds, their metabolic pathways and the persistence of their residues and/or their metabolites in organisms have been examined[2–9]. However, the knowledge of the fate of most aromatic nitro compounds and their physiological significance in humans is still scarce[3].

This is also the case for 2-nitroanisole (1-methoxy-2-nitrobenzene, 2-NA)[3 +]. 2-NA is used primarily as a precursor in the synthesis of 2-methoxyaniline, an intermediate in the manufacture of many azo dyes[10]. This chemical exhibits carcinogenic activity, causing neoplastic transformation in the urinary bladder and, to a lesser extent, in spleen, liver and kidneys in rodents[10,11]. 2-NA is also a toxic compound, causing anemia. The anemia is characterized by increased levels of methemoglobin and accelerated destruction of erythrocytes[10]. The compound became interesting for numerous institutions mainly in 1993. At that time, an industrial accident in the Hoechst company in Germany caused a large-scale leakage of 2-NA and subsequent local and regional contamination. Various dermatological changes were found among children living in the area affected by the above-mentioned accident[12]. Furthermore, single- and double-strand breaks were induced in DNA of the firemen working in the place of the accident[13].

It has not been determined exactly if 2-NA is a genotoxic or epigenetic carcinogen. Recently, we found that 2-NA is reductively activated to species forming DNA adducts detected by ^{32}P-postlabeling[14], which might suggest a genotoxic mechanism of 2-NA carcinogenicity. However, the in vivo genotoxicity of 2-NA has not yet been confirmed. In spite of potent rodent carcinogenicity of 2-NA, this chemical is weakly mutagenic in the Ames test with the *Salmonella typhimurium* TA100 strains[10]. This carcinogen also exhibits a low activity in cytogenetic tests. It induces a slight increase in chromosomal aberrations and in sister chromatid exchanges, but only at high concentrations[10]. An explanation for these discrepancies could be different

+ *Abbreviations used*: CID, collision-induced dissociation; CYP, cytochrome P450; dGp, deoxyguanosine 3′-monophosphate; NAT, *N*,*O*-acetyltransferase; 2-NA, 2-nitroanisole; PAPS, phosphoadenosylphosphosulfate; r.t., retention time; SULT, sulfotransferase; XO, xanthine oxidase.

enzyme patterns, which are responsible for 2-NA activation or detoxication in different cells, organs and species.

Most nitroaromatic hydrocarbons require metabolizing to reactive species in order to exert their genotoxic activity. The activation of nitroaromatic hydrocarbons to reactive *N*-hydroxyarylamine intermediates is through nitro reduction catalyzed primarily by several cytosolic reductases (i.e. xanthine oxidase (XO), DT-diaphorase, aldehyde oxidase) and/or microsomal enzymes such as NADPH:cytochrome P450 reductase, whereas cytochrome P450 enzymes are primarily responsible for the oxidative metabolism of these compounds[3,4,7,9,15]. *N*-Hydroxyarylamine intermediates can further be metabolized by phase II enzymes, such as *N*,*O*-acetyltransferases (NATs) or sulfotransferases (SULTs), leading to the formation of reactive esters, e.g. *N*-acetoxy- or *N*-sulfooxyarylamines which undergo heterolysis of the N–O or S–O bond to produce electrophilic nitrenium ions capable of reacting with DNA to form DNA adducts[16,17]. The major route of metabolism of 2-NA in vivo is oxidative demethylation to 2-nitrophenol, which appears in urine predominantly as the sulfate conjugate[18]. A second pathway involves reduction to 2-methoxyaniline; at blood concentrations at which the metabolism and elimination of 2-NA are linear, 2-methoxyaniline is a minor metabolite formed in liver[18]. However, at higher doses the 2-nitrophenyl sulfate pathway may reach saturation, leading to the formation of proportionally more 2-methoxyaniline[10]. Hence, nitro reduction of 2-NA, which is considered an activation pathway for aromatic nitro compounds, was clearly detected in the in vivo study[18]. Nevertheless, the enzymes participating in such reactions remain to be resolved.

The present study was undertaken to identify the cytosolic reductases responsible for 2-NA metabolism and to characterize the structure of 2-NA metabolites generated in reactions catalyzed by these enzymes. Furthermore, to assess the human health hazard of 2-NA, we have compared the capacity to reduce 2-NA of hepatic cytosolic samples from humans, and species, which succumb to tumors in exposition studies with this carcinogen.

EXPERIMENTAL

Chemicals

Chemicals were obtained from the following sources: 2-nitroanisole (>99% based on HPLC) from Fluka Chemical Co. (Switzerland), NADH, NADPH, hypoxanthine and butter milk XO from Sigma Chemical Co. (St. Louis, MO, U.S.A.), bicinchoninic acid from Pierce (Rockford, IL, U.S.A.), menadione from Merck (Darmstadt, Germany), Affi-Gel Blue (Cibacron Blue Agarose, Porcine Blue HB, C.T. 61211 Agarose) from Bio-Rad (Richmond, CA, U.S.A), and *N*-

methylnicotinamide from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). All chemicals were of analytical purity or better. *N*-(2-Methoxyphenyl)hydroxylamine was synthesized by a procedure similar to that described earlier[19]. To a solution of ammonium chloride (2 g) and 2-NA (90 mmol) in 60% ethanol/water, zinc powder (180 mmol) 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. Now a cooling bath (ice/sodium chloride mixture) is applied to the flask and temperature inside the reaction mixture was kept at 10–15 °C by slowly adding additional doses of zinc powder. After 1 h, the excess of zinc was removed by filtration and ethanol was removed under reduced pressure. The residue is taken up with ethyl acetate (100 ml) and separated from water. The *N*-(2-methoxyphenyl)hydroxylamine then can be crystallized by addition of hexane to give white needles in 60% yield.

Hepatic Cytosolic Samples

Ten human hepatic cytosolic fractions were from Gentest Corp. (Woburn, MA, U.S.A.) – samples H803, H806, H823, H842, H843, H856, H866, H870, H889, H8112 – and stored at –80 °C. The donors ranged in age from 2 to 71 years and included 2 men and 8 women. A drug and/or alcohol abuse history of the samples was described in Gentest protocols. Cytosolic fractions from livers of male Wistar rats, rabbits and pigs (always from three animals) were prepared by differential centrifugation as described previously[20]. The 105 000 *g* supernatant was taken as cytosol[21] and used for studies presented in the paper. Each cytosolic preparation was analyzed for specific enzyme activities. The assays used were as follows.

DT-Diaphorase, XO and Aldehyde Oxidase Assays

DT-diaphorase activity was measured essentially as described by Ernster[22]. The standard assay system was 25 mM in Tris-HCl (pH 7.4), 400 μM in NADH (or NADPH), 100 μM in menadione (2-methyl-1,4-naphthoquinone, the solvent for this compound was methanol) and contained 0.2% Tween 20, 0.07% bovine serum albumin and 1 mg of human, rat, rabbit or porcine hepatic cytosolic protein. The enzyme activity was determined by following the oxidation of NADH (NADPH) spectrophotometrically at 340 nm on a Hewlett–Packard 8453 diode array spectrophotometer. The activity unit is defined as the amount of enzyme catalyzing the oxidation of 1 nmol of NADH (molar absorption coefficient is 6.27 l $mmol^{-1}$ cm^{-1}) per minute. The activities of XO in cytosolic fractions were measured as described by Ichikawa et al.[23] using hypoxanthine as a substrate. Briefly, the standard assay system was 100 mM in sodium phosphate (pH 10), 6 mM in hypoxanthine, 3.75 μM in cytochrome c, and contained 1 mg of human, rat, rabbit or porcine hepatic cytosolic protein. XO activity was determined by following the reduction of cytochrome c spectrophotometrically at 550 nm on a Hewlett–Packard 8453 diode array spectrophotometer. The activity unit is defined as the amount of enzyme catalyzing the reduction of 1 nmol of cytochrome c (molar absorption coefficient is 21.0 l $mmol^{-1}$ cm^{-1}) per minute. The aldehyde oxidase activity was assayed as described by Felsted and coworkers[24] using *N*-methylnicotinamide as a substrate. Protein concentration was assessed using the bicinchoninic acid protein assay with serum albumin as a standard[25].

Isolation of DT-Diaphorase

DT-diaphorase was isolated as described earlier[21]; liver cytosol from Sudan I-treated rats was used. Briefly, proteins of cytosol (20 ml, 24 mg ml^{-1}) were fractionated with ammonium sulfate and the fraction of 30–90% saturation containing most of the DT-diaphorase activity was dialyzed against 2000 ml of 150 mM KCl in 50 mM Tris (pH 7.4). The dialyzed enzyme preparation was chromatographed on a Sephadex G-150 column and DT-diaphorase was eluted with the same buffer. Pooled fractions containing the DT-diaphorase activity were applied onto a column of Affi-Gel Blue and non-DT-diaphorase proteins were eluted with the same buffer and subsequently with a NaCl gradient (0–3.5 mol l^{-1}) in this buffer. DT-diaphorase was eluted from Affi-Gel Blue with 20 mM Tris-buffer (pH 10.0) containing 1 mM NADH. In order to remove residual protein impurities, the DT-diaphorase sample was applied onto a Sephadex G-150 column and rechromatographed. The eluate was concentrated by ultrafiltration and stored at –80 °C.

Incubations

The deaerated and argon-purged incubation mixtures were in a final volume of 100 µl: 100 mM in sodium phosphate (pH 7.4), 0.1–1.0 mM in 2-NA (the solvent for this compound was methanol; 1 µl/100 µl incubation), 1 mM in cofactors of cytosolic reductases (hypoxanthine for XO, NADH or NADPH for DT-diaphorase), and contained human, rat, rabbit or porcine cytosolic fractions (1 mg of cytosolic protein). The reaction was initiated by adding 2-NA. Control incubations were carried out either without cytosolic samples or with cytosolic samples, which were boiled for 10 min before use, or with cytosol but without 2-NA. Incubations with butter milk XO were in a final volume of 100 µl: 100 mM in sodium phosphate (pH 7.4), 1.0 mM in 2-NA (the solvent for this compound was methanol; 1 µl/100 µl incubation), 1 mM in hypoxanthine, and contained 2 mg of butter milk XO (150 units) instead of cytosolic fractions. The reaction was initiated by adding 2-NA. Control incubations were performed without hypoxanthine or without XO. After incubation of reaction mixtures (37 °C, 30–240 min), proteins were precipitated by addition of 12 µmol of $HClO_4$ and the mixture centrifuged at 7500 *g* for 5 min. A 5 µl aliquot of the mixtures was injected directly onto the HPLC. The incubation time of reactions, whose results are shown in Table I, was 180 min. The HPLC was performed on a C-18 reversed-phase column (250 × 4.6 mm, 5 µm, Nucleosil 100-5, Macherey–Nagel, Duren, Germany). Metabolites were eluted with 40% methanol in 50 mM sodium phosphate pH 7.4 (v/v) at a flow rate of 0.5 ml min^{-1} and monitored at 254 nm. To characterize 2-NA metabolites (*N*-(2-methoxyphenyl)hydroxylamine and 2-methoxyaniline), fractions containing the metabolites were collected from multiple HPLC runs, concentrated on a speed-vac evaporator and analyzed by mass spectrometry. The metabolites were also identified by comparison of their chromatographic properties on HPLC with those of synthetic standards.

Mass Spectroscopy

The metabolites collected from HPLC were analyzed by mass spectrometry. Positive-ion ESI mass spectra were recorded on a Finnigan LCQ-DECA quadrupole ion trap mass spectrometer (FinniganMAT, San Jose, CA, U.S.A.). 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^{-1}. 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 1×10^7 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.

Statistical Analyses

Statistical association between DT-diaphorase- or XO-catalytic activities in human hepatic cytosolic samples and levels of 2-methoxyaniline formed by the same cytosolic samples were determined by linear regression correlation coefficients using Statistical Analysis System software version 6.12. Correlation coefficients were based on a sample size of ten. All *Ps* are two-tailed and considered significant at the 0.05 level.

TABLE I
XO- and DT-diaphorase-dependent catalytic activities and rates of formation of 2-methoxyaniline from 2-NA in human, rat, rabbit and porcine hepatic cytosolic samples

Hepatic cytosol	XO	DT-diaphorase units mg^{-1} [a]	Rate of 2-methoxyaniline formation in the presence of 1 mM		
			none	NADPH pmol min^{-1} mg^{-1}	hypoxanthine
Human[b]	29.3	12.7	1.6	1.9	15.2
	(8.9)	(20.9)	(0.9)	(0.1)	(5.1)
Rat[c]	142.8	790.0	9.1	5.8	21.3
	(11.3)	(80.0)	(0.1)	(1.3)	(1.2)
Rabbit[d]	5.7	92.4	4.9	15.8	6.5
	(0.3)	(10.1)	(0.8)	(2.0)	(0.7)
Porcine[e]	62.8	123.0	43.8	106.7	67.9
	(4.4)	(15.3)	(3.1)	(15.8)	(7.1)

[a] The activity units, see Experimental. The values are arithmetic means for cytosolic samples from livers of ten different human donors (H803-H8112)[b], and from livers of three different rats[c], rabbits[d] and pigs[e]. Values in parentheses are standard deviations, showing the inter-individual variability.

RESULTS

Comparison of Metabolism of 2-Nitroanisole (2-NA) by Human, Rat, Rabbit and Porcine Hepatic Cytosolic Samples

When 2-NA was incubated with human, rat, rabbit or porcine hepatic cytosolic fractions, three product peaks were observed by HPLC analysis (Fig. 1). On the basis of mass spectrometry and chromatography with synthetic standards, the structures of two 2-NA metabolites (peaks 2 and 3) were identified. In the positive-ion electrospray mass spectrum, the metabolite

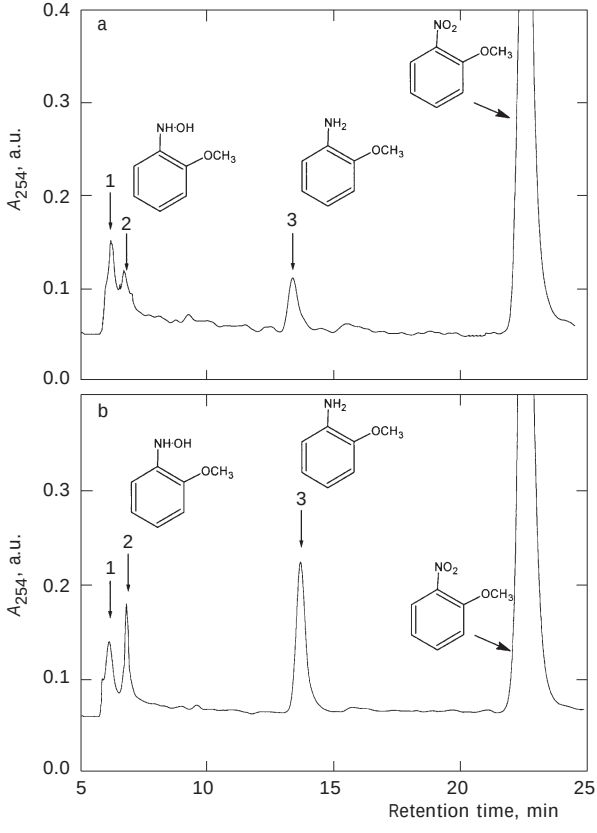

FIG. 1

HPLC chromatograms of 2-nitroanisole metabolites formed by human hepatic cytosol (a) and purified XO (b). Incubations (100 mM sodium phosphate (pH 7.4), 0.1 mM 2-NA, 1 mM hypoxanthine, and 1 mg of human hepatic cytosolic protein; or 0.25 units butter milk XO in a final volume of 100 μl) were stopped after 4 h by addition of 12 μl of $HClO_4$ and analyzed by HPLC (see Experimental)

eluting at retention time (r.t.) of 7.1 min (peak 2) showed the protonated molecule at *m*/*z* 140.1 (Fig. 2), 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 equal to 15, representing a methyl group. Other fragments at *m*/*z* 108.1 and 109.1 show the molecular masses of protonated 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). An additional product of 2-NA metabolism eluting as peak 3 at 13.9 min is identical with a 2-methoxyaniline standard (by chromatography). The mass spectra of this metabolite and 2-methoxyaniline were identical (not shown). The structure of a metabolite eluted as peak 1 has not yet been elucidated. Reduction of 2-NA with cytosolic systems was time-dependent and linear until 180 min (Fig. 3).

The porcine cytosolic samples were more efficient in 2-NA reduction to 2-methoxyaniline than those of human, rat and rabbit (Table I). The species difference in catalytic activities of cytosolic enzymes responsible for 2-NA metabolism in cytosol might be the cause of these metabolic differences. To confirm this suggestion, it is necessary to identify the most efficient enzymes metabolizing 2-NA in cytosol of all the used animal species.

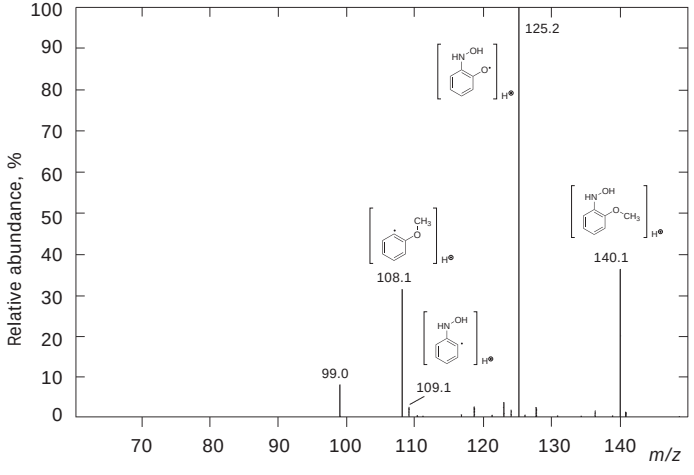

FIG. 2
MS/MS spectrum of metabolite eluting at retention time of 7.1 min (peak 2 in Fig. 1). Structure of the assigned fragments is shown

It is evident that the two metabolites found in hepatic cytosolic samples are generated from 2-NA by nitro group reduction. Therefore, the cytosolic fractions tested in this study contain the enzymes, which are capable of catalyzing the reductive metabolism of 2-NA leading to formation of the metabolites. XO, DT-diaphorase and aldehyde oxidase are major candidates for the reduction of 2-NA.

Catalytic activities known to be associated with XO and DT-diaphorase enzymes were analyzed in all hepatic cytosolic preparations. The assays used were with hypoxanthine[17] and menadione[16] as known substrates of both enzymes. Wide variations in catalytic activities were evident among different species (Table I). Moreover, activities of XO and DT-diaphorase in ten human samples differ considerably among individuals, but less among individual experimental animals, rats, rabbits and pigs (three animals always) (Table I). The activity of another reductase, aldehyde oxidase, measured with *N*-methylnicotinamide as a substrate[26] was not detectable in cytosols under the conditions used.

To investigate which cytosolic reductases are mainly responsible for 2-NA reduction, the influences of various structurally diverse compounds, serving as cofactors (electron donors) for these enzymes, upon 2-NA metabolism catalyzed with cytosol were examined. As shown in Table I the formation of the reductive metabolite of 2-NA, 2-methoxyaniline, was stimulated

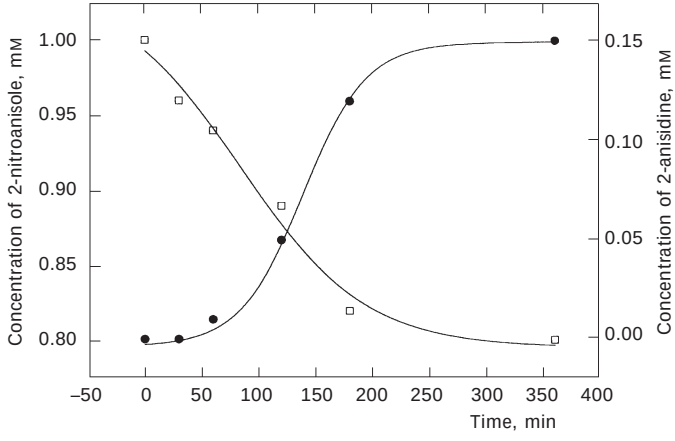

FIG. 3

Time dependence of 2-nitroanisole reduction (measured as decrease in 2-nitroanisole) (□) and 2-methoxyaniline formation (●) by the action of rat cytosolic system. Incubations were carried out using the procedure described in the text, except that incubation times were 0–360 min (see Experimental)

by addition of hypoxanthine, a known cofactor of XO [23], in cytosolic samples of all tested species, but to different degrees. NADPH, a cofactor of DT-diaphorase[22], was ineffective in rat and human cytosols, while it stimulated the 2-NA reduction in rabbit and porcine cytosols (Table I). 2-Hydroxypyrimidine, an electron donor (cofactor) of the cytosolic aldehyde oxidase[24], was not analyzed in the samples, because its activity was negligible in the cytosols (see above). These results suggest that XO would participate in 2-NA reductive metabolism in hepatic cytosol of all species, mainly in rats and humans, but in rabbit and porcine cytosols, DT-diaphorase seems to be also efficient.

Correlations between XO catalytic activities and the rate of formation of the 2-NA metabolite, 2-methoxyaniline, in the same set of human hepatic cytosolic samples were used to confirm its role in metabolism of 2-NA by human cytosol. Significant correlations were found between the levels of 2-methoxyaniline and XO activities (r = 0.735, P = 0.012), but not with catalytic activities of DT-diaphorase (r = 0.095, P = 0.793). These results strongly suggest that XO catalyzes reduction of 2-NA in human hepatic cytosol.

Reduction of 2-NA by Purified XO

To further confirm the role of XO in 2-NA reduction, comercial XO purified from butter milk was used in additional experiments. Incubations of 2-NA, XO and its cofactor, hypoxanthine, resulted in the formation of the same pattern of metabolites as that determined in cytosol (Fig. 1). This enzyme efficiently reduced 2-NA to 2-methoxyaniline (the rate of its formation was 300 pmol min^{-1} mg^{-1} XO protein). Control incubations carried out in parallel, without the cofactor, hypoxanthine, or without XO, were free of 2-NA metabolites.

DT-diaphorase does not seem to be the enzyme reducing 2-NA in human and rat hepatic cytosol, while its participation in such a reaction catalyzed with hepatic rabbit and porcine cytosols cannot be excluded. Therefore, we investigated if the purified rat hepatic DT-diaphorase might catalyze reduction of this carcinogen, but no 2-NA metabolites were detected using purified rat hepatic DT-diaphorase.

DISCUSSION

We present for the first time the finding that hepatic cytosols of different species including humans can metabolize carcinogenic 2-NA. The results of

this study clearly demonstrate that human, rat, rabbit and porcine hepatic cytosols catalyze the reduction of nitro group, which is considered to be an activation pathway for nitroaromatics in rodents. Hepatic cytosols of all species tested in the study produced two reduction metabolites, *N*-(2-methoxyphenyl)hydroxylamine and 2-methoxyaniline. In addition, the present study documents the role of specific cytosolic enzymes in reduction pathways of 2-NA. XO seems to be the principal enzyme responsible for the reductive metabolism of 2-NA in hepatic cytosol of humans and rats, while DT-diaphorase might be additional enzyme participating in 2-NA reduction in rabbits and pigs. However, no 2-NA metabolites were detected with purified rat DT-diaphorase. The activities of XO and DT-diaphorase differ considerably among human individuals. This follows from modulation of expression and from activities of both the enzymes in humans by several factors; a number cytokines, including interferon γ, interleukins, some drugs such as dexamethasone for XO [27,28], and by smoking, environmental chemicals, drugs and genetic polymorphism for DT-diaphorase[29–31].

The role of XO in 2-NA reduction was fully confirmed by experiments using pure XO. The reduction of 2-NA with purified XO mediated the formation of the same two 2-NA reduction metabolites as hepatic cytosol. Moreover, recently we demonstrated that this enzyme is capable of activating 2-NA to form DNA adducts detected by ^{32}P-postlabeling[14]. These results suggest that one major pathway of bioactivation of 2-NA is simple nitro reduction by XO. The DNA adduct formation may be due to the reactivity of the *N*-(2-methoxyphenyl)hydroxylamine intermediate, which can form, to some extent, DNA-binding arylnitrenium ions spontaneously. We confirmed the structure of this metabolite and showed that human cytosol and XO reduce 2-NA to *N*-(2-methoxyphenyl)hydroxylamine. Furthermore, preliminary results of our laboratory indicate that the same 2-NA–DNA adducts detected by ^{32}P-postlabeling are formed by 2-NA activated with XO and by direct reaction of DNA with *N*-(2-methoxyphenyl)hydroxylamine[32]. A detailed study of this reaction is under way in our laboratory.

In mammals, the liver and intestine have the highest XO activity of all tissues. The XO activities of mammalian species, however, can differ substantially, with human organs showing relatively low XO activity[28,33]. What appears to be clear is that human liver and intestine have the highest XO activity of all tissues and this is largely due to their XO-rich parenchyme cells. The data on lung and kidney tissues are inconclusive as some samples appeared to have some XO activity while other samples tested had none[28,34]. However, several recent immunohistochemical studies have shown the presence of XO in most human tissues and provided answers to

some of the discrepancies between the studies. These studies identified microvascular endothelial cells from several human tissues as being rich in XO activity[28,34–37]. These XO-rich but relatively small subpopulations of cells could account for the extremely low tissue activity found when relatively large pieces of tissue are homogenized for enzymatic activity or blotting[28]. Collectively, because the XO enzyme is present in many human tissues, it might reduce 2-NA to its proximate carcinogenic metabolite, *N*-(2-methoxyphenyl)hydroxylamine, which induces DNA adduct formation[32].

One of the most important results found in the present study is the finding that reductive activation of 2-NA by the human enzymatic system is analogous to that observed in rats, the species for which 2-NA is carcinogenic. Human hepatic cytosolic samples produce not only the same metabolites as the cytosolic subcellular fraction of rats, but the same enzyme, XO, is responsible for such metabolism in both species. In contrast to these results, the role of DT-diaphorase in 2-NA reduction catalyzed with rabbit and porcine hepatic cytosols cannot be excluded, because NADPH, a cofactor of this enzyme, stimulated the formation of 2-methoxyaniline in cytosols of rabbits and pigs. Therefore, rats are more suitable animal models than rabbits and pigs to evaluate metabolism and carcinogenicity of 2-NA for humans.

CONCLUSIONS

The results presented in this work suggest that rats may predict human susceptibility to carcinogenic 2-NA. This is important in view of the estimation of 2-NA carcinogenicity for humans. While 2-NA is carcinogenic for rats[10], its carcinogenicity for humans has not yet been proven. The results of our study, showing for the first time a similarity in the 2-NA reduction to a proximate metabolite, *N*-(2-methoxyphenyl)hydroxylamine, catalyzed by human and rat hepatic cytosolic XO, suggest a carcinogenic potential of this rat carcinogen for humans. An increased cancer risk should be taken into account mainly for individuals working in a chemical industry and exposed to 2-NA in production of this chemical for the manufacture of azo dyes. The analysis of 2-NA reduction metabolites in urine of such groups of individuals as well as that of 2-NA-mediated adducts in DNA of their blood should confirm or exclude this suggestion.

Supported by the Grant Agency of the Czech Republic (grant No. 203/03/0283) and the Ministry of Education, Youth and Sports of the Czech Republic (grant MSM 113100001).

REFERENCES

1. Garner R. C., Martin C. N., Clayson D. B. in: *Chemical Carcinogens*, 2nd ed., ACS Monograph 182 (C. Searle, Ed.), Vol. 1, p. 175. American Chemical Society, Washington D.C. 1984.
2. *IARC, Diesel and Gasoline Engine Exhausts and Some Nitroarenes*. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, No. 46, Lyon 1989.
3. Purohit V., Basu A. K.: *Chem. Res. Toxicol.* **2000**, *13*, 673.
4. Sabbioni G., Jones C. R.: *Biomarkers* **2002**, *7*, 347.
5. El-Bayoumy K., Desai D., Boyiri T., Rosa J., Krzeminski J., Sharma A. K., Pittman B., Amin S.: *Chem. Res. Toxicol.* **2002**, *15*, 972.
6. Cui X. S., Bergman J., Moller L.: *Mutat. Res.* **1996**, *369*, 147.
7. Ritter C. L., Culp S. J., Freeman J. P., Marques M. M., Beland F. A., Malejka-Giganti D.: *Chem. Res. Toxicol.* **2002**, *15*, 536.
8. Cui X. S., Eriksson L. C., Moller L.: *Mutat. Res.* **1999**, *442*, 9.
9. Ritter C. L., Decker R. W., Malejka-Giganti D.: *Chem. Res. Toxicol.* **2000**, *13*, 793.
10. *Toxicology and Carcinogenesis. Studies of o-Nitroanisole*, No. 416, NTP Technical Report. National Institute of Health, U.S. Department of Health and Human Services, Research Triangle Park (NC) 1991.
11. *Bioassay of o-Anisidine Hydrochloride for Possible Carcinogenicity*. NTP Technical Report 89, Research Tringle Park (NC) 1978.
12. Traupe H., Menge G., Kandt I., Karmaus W.: *Dermatology* **1997**, *195*, 112.
13. Hengstler J. G., Fuchs J., Bolmaudorff U., Meyer S., Oesch F.: *Scand. J. Work Environ. Health* **1995**, *21*, 36.
14. Stiborová M., Schmeiser H. H., Frei E.: *Collect. Czech. Chem. Commun.* **1998**, *63*, 857.
15. Fu P. P.: *Drug Metab. Rev.* **1990**, *22*, 209.
16. Hein D. W., Doll M. A., Fretland A. J., Gray K., Deitz A. C., Feng Y., Jiang W., Rustan T. D., Satran S., Wilkie T. R.: *Mutat. Res.* **1997**, *376*, 101.
17. Glatt H.: *Chem.-Biol. Interact.* **2000**, *129*, 141.
18. Miller M. J., Sipes I. G., Perry D. F., Carter D. E.: *Drug Metab. Dispos.* **1985**, *13*, 527.
19. Balaban A. T., Garfield R. E., Lesko M. J., Seitz W. A.: *Org. Prep. Proced. Int.* **1998**, *30*, 439.
20. Stiborová M., Asfaw B., Frei E., Schmeiser H. H., Wiessler M.: *Chem. Res. Toxicol.* **1995**, *8*, 489.
21. Stiborová M., Hájek M., Vošmiková H., Frei E., Schmeiser H. H.: *Collect. Czech. Chem. Commun.* **2001**, *66*, 959.
22. Ernster L.: *Methods Enzymol.* **1967**, *10*, 309.
23. Ichikawa M., Nishino T., Nishino T., Ichikawa A.: *J. Histochem. Cytochem.* **1992**, *40*, 1097.
24. Felsted R. L., Chu A. E.-Y., Chaykin S.: *J. Biol. Chem.* **1973**, *248*, 2580.
25. Wiechelman K. J., Braun R. D., Fitzpatrick J. D.: *Anal. Biochem.* **1988**, *175*, 2317.
26. Pius J., Xu Y., Jaiswal A. K.: *Int. J. Cancer* **1996**, *65*, 263.
27. Pfeffer K. D., Huecksteadt T. P., Hoidal J. R.: *J. Immunol.* **1994**, *153*, 1789.
28. Pritsos C. A.: *Chem.-Biol. Interact.* **2000**, *129*, 195.
29. Ross D., Kepa J. K., Winski S. L., Beall H. D., Anwar A., Siegel D.: *Chem.-Biol. Interact.* **2000**, *129*, 77.

30. Schulz W. A., Krummeck A., Rosinger I., Eickelmann P., Neuhaus C., Ebert T., Schmitz-Drager B. J., Sies H.: *Pharmacogenetics* **1997**, *7*, 235.
31. Chen H. W., Lum A., Seifried A., Wilkens L. R., Le Marchand L.: *Cancer Res.* **1999**, *59*, 3045.
32. Stiborová M., Mikšanová M., Rýdlová H., Schmeiser H. H., Frei E.: *Chem. Listy* **2002**, *96*, 946.
33. Sarnesto A., Linder N., Raivio K. O.: *Lab. Invest.* **1996**, *74*, 48.
34. Saksela M., Lapatto R., Raivio K. O.: *Biol. Neonate* **1998**, *74*, 274.
35. Jarasch E.-D., Bruder G., Heid H. W.: *Acta Physiol. Scand.* **1986**, *548*, 39.
36. Moriwaki Y., Yamamoto T., Suda M., Nasako Y., Takahashi S., Agbedana O. E., Hada T., Higashino K.: *Biochim. Biophys. Acta* **1993**, *1164*, 327.
37. Vickers S., Schiller H. J., Hildreth J. E. K., Bulkley G. B.: *Surgery* **1998**, *124*, 551.