Rabbit Liver Microsomal System: Study of Interaction with Two Model N-Nitrosamines and Their Metabolism

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Abstract. Rabbit liver microsomes of control (non-treated) or animals induced either by ethanol (EtOH) or phenobarbital (PB) were incubated with N-nitrosodimethylamine (NDMA) or N-nitrosomethylaniline (NMA). Difference spectroscopy showed that NMA is bound to the substrate-binding site of cytochrome P-450 (CYP) isoforms as heme ligand in control and EtOH pre-treated microsomes. On the other hand, PB-induced microsomes exhibit with NMA substrate type of spectra. NDMA does not provide any type of binding spectra with used microsomal systems. Oxidative bio-activation of N-nitrosamines by the microsomal CYP isoforms was measured as formaldehyde formation. Analysis of reaction kinetics in control microsomes revealed, for both substrates, two values of Michaelis–Menten constant (K_m) for, K_m values of 0.03 and 0.13 mmol/l for NDMA, and 0.30 and 0.82 mmol/l for NMA. Induction of animals with EtOH resulted in a decrease in the K_m value for both substrates. In contrast, PB treatment caused an elevation of K_m value for NDMA. Based on these data, we conclude that EtOH-inducible microsomal CYP isoforms (mainly CYP2E1) are responsible for binding and N-demethylation metabolism of both studied N-nitrosamines in rabbit liver microsomal system. The role of the other CYP isoforms involved in the metabolism of mentioned N-nitrosamines is discussed.

Key words: Rabbit — Microsomes — N-nitrosamine — Cytochrome P-450 — CYP2E1

Abbreviations: CYP, cytochrome P-450; ΔA_{SAT}, maximal difference between the actual minimum and maximum of absorbance observed in difference spectrum; DLPC, dilauroyl phosphatidyl choline; DNA, deoxyribonucleic acid; 7ER, 7-ethyl-7-hydroxy-3H-phenoxazin-3-one; EtOH, ethanol; HCHO, formaldehyde; K_m, Michaelis–Menten constant; LD_{50}, amount of a material, given all at once, which

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causes the death of 50 % of a group of test animals; NADPH, nicotinamid adenine dinucleotide phosphate reduced form; NDMA, N-nitrosodimethylamine; NMA, N-nitrosomethylaniline; 7PR, 7-pentyl-7-hydroxy-3H-phenoxazin-3-one; PB, phenobarbital; V_{max}, maximal reaction velocity.

Introduction

N-nitroso compounds represent a group of unique chemicals exerting wide organ- and species-specificities in their carcinogenic effects. N-nitrosamines, depending on their structure and route of their administration, cause tumours in nearly all organs. Both studied compounds, N-nitrosodimethylamine (NDMA) or N-nitrosomethylaniline (NMA), cause tumours in rats, hamsters and mice (Lijinsky 1987). The prototype carcinogenic N-nitrosamine, NDMA, is a potent hepatotoxin, with a LD_{50} in rats of 30–40 mg/kg as a high single dose, and develops kidney but no liver tumours (Hard and Butler 1970). On the other hand, moderate-to-low doses (continuous treatment in food or drinking water) induce liver tumours, but no kidney tumours (Terracini et al. 1967; Lijinsky and Reuber 1984). NDMA gives rise to a similar extent of DNA methylation in liver and kidneys of rats and hamsters (Lijinsky 1985), yet single or a few pulsed doses of this N-nitrosamine, close to the LD_{50}, induce liver tumours but no kidney tumours in hamster (Tomatis and Cefis 1967). Although NDMA causes alkylation of nucleic acids in a rat esophagus, it fails to induce tumours there. The NMA is a carcinogen of considerable potency in rats, giving rise only to tumours of esophagus (Goodall et al. 1970). Like many other carcinogens, most N-nitrosamines are believed to require metabolic activation to exert their carcinogenic effects. Some of the target organs for N-nitrosamines-induced tumorigenesis are rich in specific cytochrome P-450 (CYP) isoforms (e.g., liver, nasal cavity, esophagus), some are rich in peroxidases (e.g., kidney, lung, urinary bladder) which are supposed to be responsible for the metabolic initiation of N-nitrosamines-induced carcinogenesis in these organs (Schuller et al. 1990). The specificity of particular CYPs in reactions converting carcinogenic N-nitrosamines in rat or rabbit crude liver microsomes (Lorr et al. 1982; Gold et al. 1987) or in the reconstituted NADPH-dependent system (Tu and Yang 1985; Yang et al. 1985) has, therefore, been extensively investigated. However, the *in vitro* obtained values of Michaelis–Menten constant (K_m) of the NDMA conversion in rat crude liver microsomes do not correspond to results of *in vivo* experiments with NDMA in perfused liver (8.3 μmol/l) (Skipper et al. 1983). In the latest works, the K_m values were corrected (the lowest K_m values, 15–20 μmol/l, were obtained for NDMA) for rat liver microsomal systems with respect to inhibitory effects of high concentration of glycerol, thiol-compound, or protein (Yoo et al. 1987, 1990). Since these corrections of reaction conditions have not been performed with crude rabbit microsomal systems, we decided to investigate them in this paper.

Materials and Methods

Chemicals

Chlorzoxazone, dithiothreitol, dilauroyl phosphatidylcholine (DLPC), erythromycin, and NADPH were purchased from Sigma Chemical Co. (St. Louis, USA). 7-pentyl (7PR) and 7-ethyl (7ER) derivatives of 7-hydroxy-3-H-phenoxazin-3-one (resorufin) were obtained from Fluka Chemie AG (Switzerland). Phenobarbital (PB) was from Kulich Co. (Hradec Králové, Czech Republic), and D-glucose-6-phosphate dehydrogenase from Serva (Heidelberg, Germany). Bicinchoninic acid was from Pierce (Rockford, USA), and D-glucose-6-phosphate was from Reanal (Budapest, Hungary). Other chemicals were obtained from Lachema (Praha, Czech Republic). All chemicals were of reagent grade or higher quality. NMA and NDMA were gracious gifts of Dr. M. Stiborová (Department of Biochemistry, Faculty of Science, Charles University, Prague), and were synthesized as described by Stiborová et al. (1992).

Animals and pretreatment

Adult male rabbits (2.5–3.0 kg, VELAZ, Czech Republic) had free access to pellet chow and water for one week before treatment. Six days prior to the forced ethanol (EtOH) administration regimen, tolerance to EtOH was initiated by addition of EtOH to the drinking water, 5 % (v/v) solution. EtOH was then administered by gavage as 20 % (v/v) solution given in three doses at 8 a.m., 2 p.m. and 8 p.m. for three consecutive days. The total EtOH daily dose was increased from 3.2 g/kg to 4.7 g/kg body weight in fasted animals. In addition, animals given EtOH were maintained on the 5 % (v/v) EtOH drinking fluid. PB-treated rabbits were drinking 0.1 % (w/v) PB solution in water for six days. Control microsomes were prepared from non-treated rabbits (Hu et al. 1995).

Isolation and characterization of microsomes

Microsomes were prepared according to Haugen and Coon (1976), and stored in aliquots in liquid nitrogen until use. The total CYP content was measured based on complex of reduced CYP with CO (Omura and Sato 1964), and the total NADPH : CYP reductase activity was determined as NADPH : cytochrome C reductase activity based on increasing absorbance of reduced cytochrome C at 550 nm (Williams and Kamin 1962). The protein concentration was measured using bicinchoninic acid as described by Wiechelman et al. (1988) with bovine serum albumin as a standard.

Spectral measurements

Binding of NMA and NDMA to CYP in microsomes prepared from livers of rabbits treated with different inducers was monitored by difference spectroscopy (Specord M-42, Carl-Zeiss, Jena, Germany) using cuvettes of 1 cm optical path at ambient temperature (Schenkman et al. 1967). The concentration of microsomal CYP was adjusted to 0.1 μmol/l by 0.1 mol/l potassium phosphate buffer, pH 7.4. To

eliminate interaction of solvent with CYP2E1 (Guengerich et al. 1991), the tested N-nitrosamines were dissolved in chloroform solution of DLPC evaporated to dryness and, after addition of used potassium phosphate buffer, sonicated for 5 min. Resulted suspension of 0.75 mmol/l DLPC and tested N-nitrosamines were directly added to the sample cuvette containing microsomes. The same volume of the DLPC suspension without substrate was added to the referent cuvette to compensate volume change in blank. Absorption spectra were recorded between 350 and 500 nm for the final concentrations of studied N-nitrosamines from 0.5 μmol/l till 25 mmol/l. When shift of minimum and maximum of absorbance was observed, ΔA between the actual minimum and maximum was determined. Spectral dissociation constants (K_S) and the maximal differences between the actual minimum and maximum of absorbance (ΔA_{SAT}) were calculated using double reciprocal Lineweaver-Burk plots of the absorbance difference ΔA *versus* substrate concentrations.

Selective CYP isoform assays

Selective CYP activities were measured using selective substrates in microsomes from rabbits pretreated with different inducers. 7ER *O*-deethylase (CYP1A1/2 selective activity) with 5 μmol/l 7ER and 7PR *O*-depentylase (CYP2B4 selective activity) with 2 μmol/l 7PR were assayed with NADPH-generating system (10 mmol/l $MgCl_2$, 4 mmol/l D-glucose-6-phosphate, 0.04 U D-glucose-6-phosphate dehydrogenase, 0.5 mmol/l NADPH) and 0.08 mmol/l CYP in 0.1 mol/l potassium phosphate buffer, pH 7.4, for 10 min in total volume of 0.5 ml at 37 ℃ in a shaking incubator. The reaction was terminated by addition of 1.0 ml of methanol and, after centrifugation, the fluorescence of supernatant was measured by spectrofluorimeter ($\lambda_{excitation}$ 530 nm/slot 15, $\lambda_{emission}$ 588 nm/slot 10) (Burke et al. 1985). Erythromycin N-demethylation (CYP3A6 selective activity) was carried out with 10 μmol/l substrate, NADPH-generating system and 0.08 mmol/l CYP in 0.1 mol/l potassium phosphate buffer, pH 7.4, for 10 min in total volume of 0.4 ml at 37 ℃ in a shaking incubator. The reaction was terminated by addition of 100 μl 70 % (w/v) trichloroacetic acid. The amount of formed HCHO in supernatant was determined after centrifugation as described by Nash (1953). Chlorzoxazone 6-hydroxylation (CYP2E1 activity) was assayed with 0.1 mmol/l substrate dissolved in 0.15 mmol/l DLPC, NADPH-generating system and 0.08 mmol/l CYP in 0.1 mol/l potassium phosphate buffer, pH 7.4, for 10 min in total volume of 0.4 ml at 37 ℃ in a shaking incubator. The reaction was terminated by addition of 20 μl 43 % (w/v) phosphoric acid. Then, phenacetin (10 μmol/l in final concentration) was added as an internal standard, and each mixture was extracted with 1.5 ml of dichlormethane and 1.0 ml of extract was evaporated to dryness. The residues were dissolved in 50 μl of methanol and the 5 μl was analysed on Nukleosil (C18) HPLC column (4.6 × 25 mm, 5 μm, Macherey-Nagel, Germany) under isocratic conditions (65 % (v/v) methanol) using 290 nm wavelength for metabolites detection (Peter et al. 1990).

N-demethylation metabolism of N-nitrosamines

N-demethylations of NMA and NDMA were measured as HCHO formation. The reaction mixture contained within total volume 0.4 ml of 0.1 mol/l potassium phosphate buffer, pH 7.4, 0.075 mmol/l CYP, NADPH-generating system and 0.01–0.7 mmol/l NDMA or 0.01–1.0 mmol/l NMA dissolved in 0.15 mmol/l DLPC suspension. The reaction was started after 3 min of preincubation by addition of NADPH-generating system. The mixture was incubated for 10 min at 37 °C in a shaking incubator. The reaction was terminated by addition of 100 μl 70 % (w/v) trichloroacetic acid. The amount of formed HCHO was determined as described by Nash (1953). The control experiment was performed without substrate.

Results

We used two inducers, PB or EtOH, to enrich the content of several CYP isoforms in microsomal fractions. Rabbit liver microsomes of control (non-induced) or induced animals either by EtOH or PB were isolated by differential centrifugation and the concentrations of protein, CYP and activity of NADPH : cytochrome C reductase (reductase) were determined. The results are shown in Table 1. Although the EtOH pre-treatment decreased the total content of CYP and reductase two-fold in whole liver tissue, the protein concentration reduction (three-fold decrease) resulted in 1.5-fold increase in specific CYP content and specific reductase activity. On the other hand, PB administration caused three-fold increase in protein concentration and total CYP content, and reductase activity was elevated as well. This inducer doubled specific CYP content but specific reductase activity was slightly reduced. The changes in CYP isoform patterns after animal pre-treatment are very important to explain metabolism of studied N-nitrosamines in crude microsomal systems (Figure 1). The activity of CYP2B4 was more then seven-fold induced by PB while weak CYP1A1/2 and CYP2E1 activities were slightly sup-

Table 1. Characterisation of the isolated rabbit liver microsomal systems

Parameters	Microsomes induced by		
	no addition (control)	EtOH	PB
Protein concentration (mg/ml)	34.9 ± 1.0	10.0 ± 0.5	96.9 ± 2.0
Specific content of CYP (nmol/mg)	1.1 ± 0.2	1.5 ± 0.3	2.0 ± 0.3
Specific reductase activity (nmol cytochrome C/min *per* mg)	84.0 ± 5.3	50.0 ± 4.5	139.0 ± 5.9

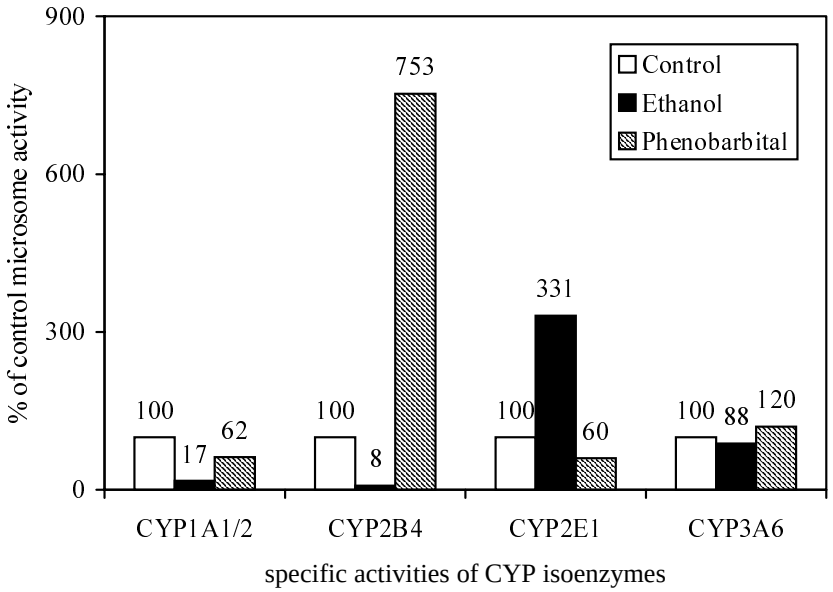

Figure 1. Relative changes of selective CYP isoforms activities in EtOH-induced (black) and PB-induced (hatched) microsomes as compared to non-treated microsomal fraction (white). 7ER *O*-deethylase (CYP1A1/2 activity), 7PR *O*-depentylase (CYP 2B4 activity), chlorzoxazone 6-hydroxylation (CYP2E1 activity) and erythromycin N-demethylation (CYP3A6 activity). Control microsomal activities were 108 ± 9 pmol resorufin/min·nmol CYP for CYP1A1/2 activity, 10.4 ± 1.2 pmol resorufin/min·nmol CYP for CYP 2B4 activity, 0.0096 ± 0.0009 amount of 6-hydroxychlorzoxazone/amount of phenacetin/min·nmol CYP for CYP2E1 activity and 4.0 ± 0.5 nmol HCHO/min·nmol CYP for CYP3A6 activity.

pressed. Conversely, EtOH increased three-fold 6-hydroxylation activity of chlorzoxazone (CYP2E1 selective activity), and almost eliminated O-dealkylation of 7PR and 7ER (CYP2B4 and CYP1A1/2 activity). Both inducers had no influence on N-demethylation of erythromycin (CYP3A6 selective activity) in microsome fractions (Table 1, Fig. 1) After characterization, all microsomal systems were incubated with two model substrates (NDMA or NMA) to determine the interaction of the substrate with each of microsomes by binding spectra and N-demethylation metabolism. Data of difference spectroscopy experiments (Table 2) showed that NMA is bound to the substrate-binding site of CYP isoforms as a heme ligand in control and EtOH pre-treated microsomes. On the other hand, PB-induced microsomes provided the substrate type of spectra (no heme ligand) with NMA, and the spectral dissociation constants K_S (2.1 mmol/l) and ΔA_{SAT} (0.0625) were determined by double reciprocal plot. NDMA did not induce any detectable difference spectra with used microsomal systems (Table 2).

Oxidative bio-activation of N-nitrosamines was measured as HCHO formation

Table 2. Spectral parameters of CYP isoforms interaction with NMA in isolated rabbit liver microsomal systems. The NDMA does not provide any type of spectra with used microsomal systems

Parameters	Microsomes induced by		
	no addition (control)	EtOH	PB
K_S (mmol/l)	[a]	[a]	2.1 ± 0.3
ΔA_{SAT}			0.0625 ± 0.003
λ_{max} (nm)	423	425	390
λ_{min} (nm)	395	395	416
Type of spectra	II	II	I

EtOH, ethanol; PB, phenobarbital; [a] not detectable; K_S, spectral dissociation constant; ΔA_{SAT}, maximal difference between the actual minimum and maximum of absorbance; λ_{max}, wavelength of maximum observed in difference spectrum; λ_{min}, wavelength of minimum observed in difference spectrum.

Table 3. Kinetic parameters of NMA and NDMA N-demethylation in isolated rabbit liver microsomal systems

Substrate	Parameters	Microsomes induced by		
		no addition (control)	EtOH	PB
NMA	K_{m1} (mmol/l)	0.30 ± 0.03	0.10 ± 0.02	0.48 ± 0.03
	V_{max1}*	0.9 ± 0.1	1.5 ± 0.05	4.2 ± 0.1
	K_{m2} (mmol/l)	0.82 ± 0.1	[a]	4.0 ± 0.3
	V_{max2}*	1.4 ± 0.1	[a]	8.3 ± 0.1
NDMA	K_{m1} (mmol/l)	0.03 ± 0.01	0.01± 0.004	0.83 ± 0.1
	V_{max1} *	0.5 ± 0.05	1.4 ± 0.1	0.4 ± 0.05
	K_{m2} (mmol/l)	0.13 ± 0.01	0.14 ± 0.01	2.5 ± 0.3
	V_{max2}*	0.6 ± 0.04	6.2 ± 0.15	0.6 ± 0.05

[a] not detected; K_m, Michaelis–Menten constant; V_{max}, maximal reaction velocity; * nmol HCHO/min·nmol CYP.

by the microsomal CYP isoforms (Table 3). In non-treated microsomes both substrates provided two values of K_m for, K_{m1} value of 0.03 mmol/l and K_{m2} value of 0.13 mmol/l for NDMA, and K_{m1} value of 0.30 mmol/l and K_{m2} value of 0.82 mmol/l for NMA. EtOH treatment of the animals resulted in three-fold decrease of the K_{m1} value for both substrates. The K_{m2} value of NMA was not detectable and K_{m2} value of NDMA was unchanged. The corresponding maximal reaction velocity values (V_{max}) increased for both substrates. In contrast, PB treatment caused ele-

vations of both K_m values for NDMA (27 times for the K_{m1} value and 19 times for the K_{m2} value) and both K_m values for NMA, too (1.6 times for the K_{m1} value and 4.9 times for the K_{m2} value). Their corresponding maximal reaction velocity values were unchanged for NDMA and four-times higher for NMA (Table 3). Our results show that only microsomal fraction of the EtOH induced animals followed classical Michaelis–Menten kinetics with NMA as a substrate. The other used microsomal systems yielded two values of K_m and V_{max} for both substrates.

Discussion

In this study, we concentrated on the interaction and metabolism of NMA and NDMA in the rabbit liver microsomes in the respect of correction of the reaction conditions as have been shown in earlier publications (Yoo et al. 1987, 1990). Several liver microsomal CYPs are known to interact with N-nitrosamines (Lorr et al. 1982; Yang et al. 1985; Scheper et al. 1991). Yang et al. (1990) postulated that the most probable CYP isoform involved in the substrate metabolism is the isoform with the lowest K_m and the highest V_{max}. Consequently, the second goal of this paper was to clarify which isoforms of CYP can play the major role in studied N-nitrosamines metabolism.

Using only a technique of spectral measurements, we answered this question only partially. The CYP isoforms of non-induced and EtOH induced microsomes behave similarlly in respect to binding NMA (type II of binding spectra). On the contrary, PB-induced microsomes differ significantly, since type I of binding spectra with NMA is detected. This result partially indicates importance of CYP isoformes induced by EtOH for interaction with this substrate. Because no spectral response was detected with NDMA, we directly continued with N-demethylation metabolism of both N-nitrosamines.

This metabolic activation of the NMA and NDMA clarifies our answer more precisely. Since a different CYP isoform pattern present in the studied microsomal systems, different affinity for NMA and NDMA and thus multiple K_m values are determined. Obtained K_{m1} value (30 μmol/l) for NDMA in non-induced microsomal fraction is well corresponding with that described in literature for rat liver microsomal system (15–20 μmol/l) in respect to inhibition effect of high glycerol, thiol compound or protein concentration (Yoo et al. 1987, 1990). The similar value of K_m (8.3 μmol/l) was determined in *in vivo* experiment with NDMA in perfused liver (Skipper et al. 1983).

The slight increase in the K_{m1} value for NMA after PB induction partially agrees with high accumulation of CYP2B4 and partial suppression of CYP2E1 or CYP1A1/2. On the other hand, three-fold reduction of the K_{m1} value after EtOH pre-treatment corresponds with three-fold increase in CYP2E1 activity, moreover, only one value of K_m was observed at the same time with a deprivation of CYP2B4 and CYP1A1/2 selective activity. On the bases of spectral and metabolism data we provide the evidence that the CYP2E1 plays major role in interaction with NMA and its metabolism. This result is consistent with observations described

in literature for PB-induced mouse (Scheper et al. 1991) and rat (Tu and Yang 1985; Stiborová et al. 1996) liver CYP-dependent microsomal systems and their major CYP isoforms in reconstitution systems. To exclude role of other rabbit CYP isoforms in NMA activation, the reconstitued systems of purified CYP isoforms will be used in future.

Although no spectral data for NDMA were obtained in any microsomal systems, it is clear from the results of NDMA N-demethylation metabolism that PB increases both K_{m1} and K_{m2}. This is in accordance with a suppression of CYP2E1 and high accumulation of CYP2B4. On the contrary, the three-fold decrease in K_{m1} constant by EtOH induction agrees very well with three-fold accumulation of CYP2E1 activity in this microsomal system. This is the same result as we described for NMA. These results indicate the major and crucial role of CYP2E1 in N-demethylation metabolism of small, aliphatic and symmetric molecule as NDMA. This conclusion is consistent with the results of the experiments performed with rat microsomal systems (Yoo et al. 1990), or with an observation of inhibition effect of anti-CYP2E1 antibodies affected the first value of K_m described by Amelizad et al. (1988) in rat microsomal fraction. Similarly, the inhibition of DNA methylation observed in rat hepatocytes by pyrazol that is known as CYP2E1 inhibitor support our conclusions, too (Encell et al. 1996).

The fact that the value of the second K_m for NDMA is not changed by EtOH pre-treatment of the animals indicates the participation of one (or several) more isoform(s) of CYP induced by EtOH and not by PB in metabolism of NMA and NDMA. Based on the CYP2B4 and CYP1A1/2 activities elimination after EtOH induction we conclude that CYP2B4 and CYP1A1/2 should not play important role in metabolism of these N-nitrosamines.

Taking all the data together, there is an evidence that CYP isoform(s) induced by EtOH (mainly CYP2E1) participate(s) in activation metabolism of NMA and NDMA in rabbit liver. To elucidate their roles, the experiments with purified CYP isoforms and in the reconstituted systems will be performed.

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