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The binding affinity of carcinogenic N-nitrosodimethylamine and N-nitrosomethylaniline to cytochromes P450 2B4, 2E1 and 3A6 does not dictate the rate of their enzymatic N-demethylation

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Abstract. The interaction of carcinogenic N-nitrosodimethylamine (NDMA) and N-nitrosomethylaniline (NMA) with cytochromes P450 (CYP), CYP2B4, CYP2E1 and CYP3A6, and their metabolism by these enzymes reconstituted with NADPH-CYP reductase in liposomes were studied. Using the difference spectroscopy, the lowest values of spectral dissociation constants (K_S) of the binary complex of NDMA and NMA with the enzyme were found for CYP2E1. Both N-nitrosamines bind to the heme iron atom as ligands. On the contrary, the binding of NDMA and NMA to CYP2B4 gives the type I spectra. NDMA is bound to CYP3A6 analogously as to CYP2B4, whereas no difference spectra were acquired with NMA and CYP3A6. All tested CYPs oxidize NDMA and NMA. CYP2E1 exhibits the lowest K_m values, 7.5 and 5.0 μmol/l for NDMA and NMA, respectively, and for CYP3A6 we found 30.0 and 10.0 μmol/l for NDMA and NMA, respectively, while CYP2B4 exhibits the lowest affinity for both carcinogens.. In spite of different binding affinities of NDMA and NMA, the values of V_{max} for their oxidation were, however, similar for all tested CYPs. The results demonstrate that investigations utilizing several enzymatic approaches are necessary to properly evaluate the mechanism and efficiency of NDMA and NMA oxidation by CYPs *in vitro*.

Key words: Cytochrome P450 — CYP2B4 — CYP2E1 — CYP3A6 — N-nitrosamines

Abbreviations: CYP, cytochrome P450; DLPC, dilauroylphosphatidylcholine; MALDI-TOF, matrix assisted laser desorption-ionization time of flight; NDMA, N-nitrosodimethylamine; NMA, N-nitrosomethylaniline; K_m, Michaelis-Menten constant; K_S, spectral dissociation constants; V_{max}, maximal reaction velocity; 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.

Introduction

N-Nitrosamines are a unique group of chemicals exerting wide organ and species specificity in their carcinogenic effects. Tumors can be obtained in nearly all organs, depending on the route of administration and the structure of the nitrosamine (Preussmann and Stewards 1984a,b; Dipple et al. 1987; Preussmann and Weissler 1987). Two members of this class of carcinogens, N-nitrosodimethylamine (NDMA) and N-nitrosomethylaniline (NMA) are present in cigarette smoke, as well as many other sources (Bartsch and Montesano 1984; Adams et al. 1987; Sen 1991), and cause development of tumors in rats, hamsters and mice (Lijinski 1987). Both compounds are also supposed to play a role in the etiology of human cancer. Even though most of the present research investigating carcinogenic potencies of N-nitrosamines is targeted to other tobacco-specific N-nitrosamines (Hecht 2007; Cheng et al. 2008; Liu and Glatt 2008), examination of these prototype members of this group of chemicals is still challenging (Hecht 2008).

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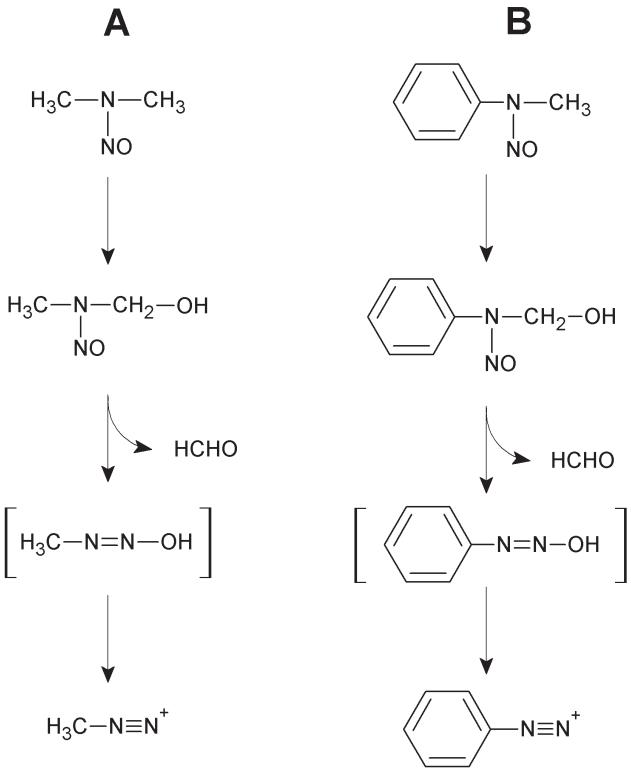

Figure 1. Proposed metabolic scheme of reaction of NDMA (A) and NMA (B) with CYP enzymes. The metabolites in parentheses are unstable and therefore not detectable.

Both carcinogenic N-nitrosamines require metabolic activation to form DNA adducts that are critical for their carcinogenic effects (Preussmann and Weissler 1987). The well-established major activation pathway is cytochrome P450 (CYP)-mediated α-hydroxylation (adjacent to the N-nitroso group), leading to the formation of alkyl- or aryldiazonium ions (Fig. 1), which are responsible for the covalent modification of DNA (in other words for the initiation of chemical carcinogenesis) (Druckrey et al. 1967; Dipple et al. 1987; Preussmann and Weissler 1987). Furthermore, parallel reaction of this methyl hydroxylation is production of formaldehyde, which is also known to modify DNA, generating several products including hydroxymethyl adducts and cross-links (Spratt et al. 1990; U. S. Department of Health and Human Services 2004). Formaldehyde is mutagenic in a variety of different test systems and carcinogenic in laboratory animals (IARC 2006). Formaldehyde has been evaluated 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 (U. S. Department of Health and Human Services 2004; IARC 2006). Therefore, it is plausible that though a major activation pathway is CYP-mediated α-hydroxylation, the formaldehyde-DNA adducts could also play a role in carcinogenesis caused by N-nitrosamines.

The target organs for NDMA- and/or NMA-induced tumorigenesis such as liver and esophagus are rich in specific CYPs, while such as kidney and lung express also considerable amounts of peroxidases. Both two classes of enzymes have been found to activate these carcinogens, which might lead to their carcinogenic effects in the target organs (Schuller et al. 1990; Stiborova et al. 1992, 1996a,b, 1997; Sulc et al. 2004). The specificity of individual CYP enzymes involved in metabolism of both NDMA and NMA has been extensively investigated, utilizing microsomes isolated from target organs (livers and esophagus) of several species including humans, isolated CYPs reconstituted with NADPH: CYP reductase and/or human recombinant CYP enzymes (Yang et al. 1985; Stiborova et al. 1996b, 1999; Bernauer et al. 2003). The CYP2E1 is believed to be the most important enzyme activating NDMA in liver and esophagus (Tu and Yang 1985; Smith et al. 1998; Stiborova et al. 1999a; Sulc et al. 2004), whereas CYPs of 2B and 2A subfamilies are supposed to be efficient in oxidative activation of NMA (Tu and Yang 1985; Stiborova et al. 1996a,b, 1999; Kushida et al. 2000). In addition, whereas CYPs of a 3A subfamily are also included in oxidation of some tobacco-specific N-nitrosamines (Smith et al. 1998; Kushida et al. 2000), their role in oxidation of NDMA and NMA have not been properly investigated as yet.

It is noteworthy that the *in vitro* kinetic data determined for oxidation of NDMA and NMA in microsomal preparations or using isolated enzymes are not consistent with results found *in vivo*. For example, the value of Michaelis-Menten constant (K_{m1}) of 0.07 mmol/l was found for NDMA oxidation with rabbit hepatic microsomes, the value of 2.9 mmol/l when NDMA was oxidized with isolated CYP2E1 reconstituted with NADPH-CYP reductase, while 8.3 μmol/l in perfused liver (Skipper et al. 1983). This discrepancy is likely caused by various artificial effects such as high concentrations of glycerol, thiol-compounds or residual detergents from purification procedures, which are present in *in-vitro* experiments, whereas other compounds such as proteins and nucleic acids, influencing the kinetics of N-nitrosamine oxidation, are present in tissue cells *in vivo* (Yoo et al. 1987, 1990). Furthermore, when microsomes were used to study a demethylation of NDMA and NMA, the multiplicity of CYP enzymes in these subcellular systems resulted in determination of more than one K_m (Tu and Yang 1985; Yoo et al. 1990; Stiborova et al. 1996a; Sulc et al. 2004).

Our previous studies have been focused on oxidative activation of NDMA and NMA with rat and rabbit hepatic microsomal systems or partially purified CYPs. In these experiments either formaldehyde production (Stiborova et al. 1996a,b; Sulc et al. 2004) or the formation of DNA adducts were analyzed (Stiborova et al. 1992, 1999). Kinetics

of the reactions catalyzed by microsomes of control (uninduced) animals as well as those of rats and rabbits treated with inducers of CYP2B and CYP2E1, phenobarbital and ethanol, respectively, were extensively examined in some of these studies (Stiborova et al. 1996a; Sulc et al. 2004). However, because of CYP multiplicity and their different levels expressed in various microsomal samples, these studies did not bring unambiguous results. For example, in the control rabbit hepatic microsomes, two values of K_m constants for NDMA and NMA were detected; K_{m1} of 0.03 and K_{m2} of 0.13 mmol/l for NDMA, and K_{m1} of 0.30 and K_{m2} of 0.82 mmol/l for NMA (Sulc et al., 2004). In addition, the mechanism by which the individual CYPs demethylate both N-nitrosamines has not been investigated as yet.

The present study was undertaken to investigate the efficiency of rabbit CYP2B4, CYP2E1 and CYP3A6 to oxidize (demethylate) NDMA and NMA. Two experimental approaches were employed: i) investigation of interaction of NDMA and NMA with purified rabbit CYPs by difference spectra measurements and ii) determination of kinetic characteristics (parameters) of NDMA and NMA oxidation by isolated CYPs reconstituted with NADPH-CYP reductase. In addition, because various solvents utilized in former studies investigating oxidation of NMA and/or other N-nitrosamines might influence kinetics of their oxidation, the effect of some of such solvents was also examined.

Material and Methods

Chemicals

Dithiothreitol, dilauroylphosphatidylcholine (DLPC), α-cyano-4-hydroxy-cinnamic acid, rifampicin, trichloroacetic acid and NADPH were purchased from Sigma Chemical Co. (St. Louis, USA). Phenobarbital 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). The other chemicals were obtained from Pliva-Lachema (Brno, Czech Republic). All these and other chemicals were of analytical purity or better. N-nitrosomethylaniline and N-nitrosodimethylamine were synthesized as described elsewhere (Stiborova et al., 1996a). The diamantane was gracious gift of Pliva-Lachema (Brno, Czech Republic).

Animals pre-treatment

The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic) and EU (86/609/EEC), which complies with Declaration of Helsinki (1975). Adult male rabbits (2.5–3.0 kg, VELAZ, Czech Republic) had free access to pellet chow and water for one week before treatment. The ethanol administration regimen by gavage and additional fluid drinking was described previously (Hu et al. 1995). Phenobarbital treated rabbits were drinking 0.1% (w/v) phenobarbital solution in water for ten days (Sulc et al. 2004). Rifampicin administration was performed for three consecutive days (*i.p.* dose of 50 mg/kg body weight in 40 mmol/l NaOH) (Borek-Dohalska et al. 2001).

Preparation 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 carbon monoxide (Omura and Sato 1964). The NADPH:cytochrome *c* reductase activity was acquired as described previously (Williams and Kamin 1962). Protein concentrations were assessed by bicinchoninic acid using bovine serum albumin as a standard (Wiechelman et al. 1988).

Purification of CYP2B4, CYP2E1 and CYP3A6 and NADPH-CYP reductase

Rabbit liver CYPs and NADPH-CYP reductase were purified from microsomes isolated from livers of rabbits after different animal pre-treatment: CYP2B4 and NADPH-CYP reductase after pre-treatment with phenobarbital, CYP2E1 after that with ethanol, and CYP3A6 after that with rifampicin. The procedures used for purification of CYPs and NADPH-CYP reductase were analogous to those described previously (CYP2B4 (Haugen and Coon 1976), CYP2E1 (Larson et al. 1991), CYP3A6 (Johnson et al. 1988), NADPH-CYP reductase (Digman and Strobel 1977)), generally starting with microsome solubilisation by different types of detergents, followed by a combination of cation and/or anion-exchange chromatographies and finalized with adsorption or affinity chromatography. The SDS-PAGE (Smith 1995) was used to prove electrophoretic homogeneity of pure proteins and prepare enzyme samples for mass spectrometry analysis. The total NADPH-CYP reductase content was determined based on assessed absorbance of purified protein at 455 nm (Vermilion and Coon 1978).

MALDI-TOF mass spectrometry analysis

Stained protein spot was excised from SDS-PAGE gel and processed for MALDI-TOF mass spectrometry by digestion with trypsin as described previously (Osicka et al. 2004). MALDI-TOF mass spectra of the peptide mixtures in α-cyano-4-hydroxy-cinnamic acid were compared with theoretical tryptic peptides and also used for Peptide Mass Fingerprint analysis.

Spectral measurements with purified CYP2B4, CYP2E1 and CYP3A6

Binding of NMA and NDMA to purified CYPs 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 oxidized CYP was adjusted to 2.0 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 added into chloroform solution of DLPC, evaporated to dryness and sonicated in potassium phosphate buffer for 5 min. Resulted suspension of 0.75 mmol/l DLPC and N-nitrosamines were directly added to the sample cuvette containing solution of CYP. The same volume of the DLPC suspension without substrate was added to the reference cuvette to compensate optical density and volume changes in blank. Absorption spectra were recorded 30 second after mixing between 350 nm and 500 nm for the gradually increasing concentration of studied N-nitrosamines from 0.5 μmol/l to 25 mmol/l. The spectral dissociation constants (K_S) and the maximal difference between the actual minimum and maximum of absorbance (ΔA_{SAT}) were calculated using double reciprocal plots of the absorbance difference ΔA (between the actual minimum and maximum) *versus* substrate concentrations.

To investigate the effect of DLPC suspension on binding properties of CYP2B4 the following systems were employed: i) diamantane (dissolved in methanol or DLPC suspension), ii) NDMA (dissolved in H_2O or DLPC suspension), and iii) NMA (dissolved in methanol or DLPC suspension).

N-demethylation of N-nitrosamines by CYP2B4, CYP2E1 and CYP3A6 reconstituted with NADPH-CYP reductase

N-demethylations of NMA and NDMA were measured as formaldehyde formation. Incubation mixtures contained 0.1 mol/l potassium phosphate buffer, pH 7.4, 0.1 μmol/l CYP, 0.1 μmol/l NADPH-CYP reductase, 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 0.01–0.7 mmol/l NDMA or 0.01–1.0 mmol/l NMA in 0.15 mmol/l DLPC suspension in a final volume of 0.4 ml (Stiborova et al. 1996). The reaction was initiated after 3 min of preincubation by adding the NADPH-generating system. After incubation in open tubes (37°C, 10 min, shaking) the reaction was stopped by adding 100 μl 70% (w/v) trichloroacetic acid. The amount of formed formaldehyde was determined as described by Nash (1953). The blank experiment was performed without substrates.

To estimate the optimal conditions in the reconstitution experiments (e.g. ratio of CYPs and NADPH-CYP reductase, DLPC vesicles preparation, and time of demethylation metabolism linearity), the N-demethylation of dimethylaminopyrine by CYP2B4 was used. The optimal molar ratio of CYPs to reductase were close to a value of 1.0, linearity of metabolism was validated till 20 minutes, and kinetics parameters were also estimated (K_m = 0.6 mmol/l and V_{max} = 6.75 nmol formaldehyde/min *per* nmol CYP). Kinetic analyses were carried out by using the non linear least-square method described previously (Cleland 1979).

Results

Characterization of CYP and NADPH-CYP reductase enzymes

The purity of CYP2B4, CYP2E1, CYP3A6 and NADPH-CYP reductase isolated to electrophoretic homogeneity from rabbit hepatic microsomes were proved by the SDS-PAGE and the specific content of these enzymes in the purified preparations was determined (Table 1). Because in some cases the lower specific content was determined than would be theoretically expected for pure enzymes, the MALDI-TOF mass spectrometry after in-gel trypsin digestion was performed to verify identity and purity of all proteins (Appendix, Table S1). The results of all analyses indicate high purity of all enzymes and, therefore, their suitability for further metabolic studies. The lower specific content was probably caused by presence of traces of apoenzyme and not by any protein contaminations in CYP samples.

Binding of NDMA and NMA to CYP2B4, CYP2E1 and CYP3A6

Although NDMA is water-soluble compound, the solubility of NMA in water is negligible. NMA is soluble only in organic solvents such as methanol that was frequently used for its dissolving in former studies (Tu and Yang 1985; Yang et al. 1985; Stiborova et al. 1992, 1996a,b, 1997; Sulc et al. 2004). However, solvents such as methanol, ethanol, acetone, dimethylsulfoxide, acetonitrile, hexane or benzene are known to influence enzymatic activities of several CYPs

Table 1. Characterization of purified rabbit liver microsomal CYPs and NADPH-CYP reductase

	Protein concentration	Specific content of enzymes
Purified preparation	(mg/ml)	(nmol/mg)
CYP2B4	3.8 ± 0.3	11.0
CYP3A6	1.5 ± 0.2	7.1
CYP2E1	1.5 ± 0.2	5.2
NADPH-CYP reductase	1.4 ± 0.2	10.1

Means ± S.D. (n = 3). The determined specific reductase activity was 9.6 nmol cytochrome *c*/min *per* mg.

Table 2. Influence of DLPC suspension on spectral parameters of purified CYP2B4 with different substrates

CYP/binding substrate	solvent	K_S (μmol/l)	ΔA_{SAT}	λ_{max} (nm)	λ_{min} (nm)	Type of spectra
CYP2B4/diamantane	methanol	1.1 ± 0.3	0.093 ± 0.003	386	420	I
	DLPC	15.0 ± 1.6	0.138 ± 0.006	387	420	I
CYP2B4/NDMA	H_2O	9.1 ± 0.6	0.006 ± 0.001	383	420	I
	DLPC	10.0 ± 1.2	0.011 ± 0.004	383	420	I
CYP2B4/NMA	methanol	730.0 ± 21.5	0.079 ± 0.008	388	416	I
	DLPC	770.0 ± 14.5	0.091 ± 0.012	388	416	I

K_S, spectral dissociation constant; ΔA_{SAT}, the maximal difference between the actual minimum and maximum of absorbance; λ_{max}, wavelength of maximum; λ_{min}, wavelength of minimum observed in difference spectrum. Means ± S.D. (n = 3).

and their interactions with substrates (Guengerich et al. 1991; Chauret et al. 1998). Therefore, to find a suitable system dissolving NMA without effects on CYPs, DLPC compound, used for preparation of liposomes necessary for reconstitution of CYP enzymes with NADPH-CYP reductase, was evaluated. First of all, the effect of DLPC on interaction of one of the CYPs used in the study, CYP2B4, with NDMA and NMA as well as with a prominent substrate of CYP2B, diamantane (Hodek et al. 1988, 1995, 2005), was examined in comparison with introducing of substrates in methanol or water.

Diamantane, NDMA and NMA were dissolved in either methanol or water or liposomes (prepared from DLPC in phosphate buffer) and their binding to CYP2B4 were analyzed by a spectral titration. As shown in Table 2, DLPC liposomes used to dissolve the tested substrates essentially does not influence the binding of NDMA and NMA to CYP2B4. The NMA binding indicates similar values of K_S and the maximal difference, ΔA_{SAT}, for both solvents (methanol or DLPC suspension). Also spectral binding constant K_S as well as ΔA_{SAT} for NDMA dissolved in DLPC suspension were only slightly elevated when comparing with spectral titrations performed with NDMA dissolved in water. In the case of diamantane, however, the DLPC suspension increased the value of the dissociation constant (K_S) of the binary complex of this substrate with CYP2B4 when compared with its methanol solution. Another important finding of these spectroscopic experiments is that for all tested substrates, utilization of DLPC suspension as a substrate solvent does not change a type of difference binding spectra or their local maxima or minima. These results

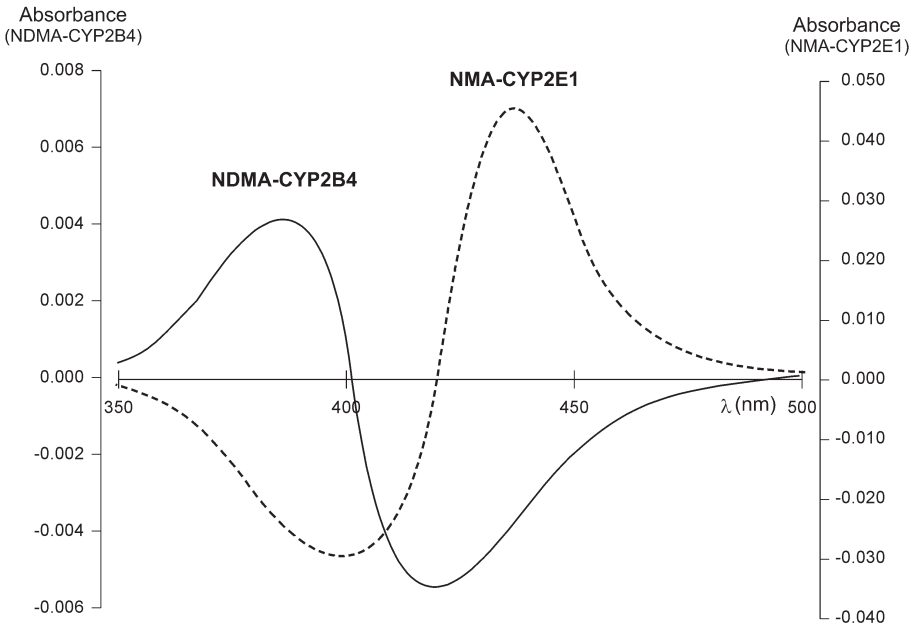

Figure 2. Spectral changes caused by addition of NDMA to CYP2B4 in DLPC liposomes (filled line) and NMA to CYP2E1 in DLPC liposomes (dashed line). For detailed conditions, see Materials and Methods.

proved application of DLPC suspension of NMA and NDMA to be useful to eliminate the effect of organic solvents on at least the CYP2B4 enzyme.

Our data show that titration of CYP2B4 with NDMA or NMA dissolved in DLPC liposomes typically gave type I spectra with the pure CYP2B4 enzyme (see Fig. 2 for NDMA:CYP2B4 binary complex, filled curve). For this type of binding spectrum, an increase in absorbance at about 390 nm and a decrease in absorbance at about 420 nm are typical (Table 3). The magnitude of the difference in the absorbance maximum and minimum for both compounds is assumed to represent the extent of their binding to the catalytic site. The values of the spectral dissociation constants for the complexes of NDMA and NMA with this CYP are shown in Table 3. The K_S values indicate that NDMA binds to CYP2B4 with higher affinity than NMA.

The binding titration of NDMA with CYP3A6 gives an analogous spectrum of type I, whereas NMA does not provide any detectable minimum or maximum of absorbance in the binding titration with this enzyme (Table 3).

In the case of CYP2E1, the binding titration of NDMA and NMA gives type II spectra, indicating that both compounds bind to this enzyme as heme ligands (see Fig. 2 for NMA:CYP2E1 binary complex, dashed curve). For this type of binding spectrum, an increase in absorbance at 437 nm and a decrease in absorbance at 396 nm are typical (Table 3). The lowest values of the spectral dissociation constants for the complexes of NDMA and NMA with CYP2E1 among the studied CYPs, as well as the highest value of ΔA_{SAT}, demonstrate high affinities of these N-nitrosamines to bind to this enzyme.

CYP2B4, CYP2E1 and CYP3A6 reconstituted with NADPH-CYP reductase N-demethylate NDMA and NMA

Using CYP2B4, CYP2E1 and CYP3A6 reconstituted with NADPH-CYP reductase in DLPC liposomes (vesicles), kinetics of a demethylation of NDMA and NMA yielding a formaldehyde product were investigated. This reaction is the major CYP-mediated activation pathway for NMA and NDMA, leading beside generation of formaldehyde to the formation of methyl- or benzene-diazonium ions, the species responsible for the covalent modification of DNA.

Enzymes, CYP and NADPH-CYP reductase in liposomes are necessary for the reactions, which were expected for the CYP-mediated monooxygenase reactions. The classical

Table 3. Spectral parameters of interactions of pure CYPs with NMA and NDMA dissolved in DLPC suspension

CYP	Substrate	K_S (μmol/l)	ΔA_{SAT}	λ_{max} (nm)	λ_{min} (nm)	Type of spectra
CYP2B4	NMA	770.0	0.091	388	416	I
	NDMA	10.0	0.011	383	420	I
CYP3A6	NMA	n.d.	n.d.	n.d.	n.d.	n.d.
	NDMA	33.0	0.011	388	418	I
CYP2E1	NMA	40.0	0.074	436	396	II
	NDMA	5.0	0.017	437	396	II

n.d., not detectable; K_S, spectral dissociation constant; ΔA_{SAT}, the maximal difference between the actual minimum and maximum of absorbance; λ_{max}, wavelength of maximum; λ_{min}, wavelength of minimum observed in difference spectrum. Means ± S.D. (n = 2 for CYP3A6 and CYP2E1; n = 3 for CYP2B4).

Table 4. Kinetic parameters of NMA and NDMA N-demethylation by CYP2B4, CYP2E1 and CYP3A6 reconstituted with NADPH-CYP reductase

CYP reconstituted with NADPH-CYP reductase	Substrate	K_m (μmol/l)	V_{max} (nmol formaldehyde/min *per* nmol CYP)
CYP2B4	NMA	700.0 ± 33.2	3.0 ± 0.08
	NDMA	180.0 ± 25.5	1.8 ± 0.05
CYP3A6	NMA	10.0 ± 3.2	2.5 ± 0.06
	NDMA	30.0 ± 5.5	1.3 ± 0.04
CYP2E1	NMA	5.2 ± 1.6	3.5 ± 0.08
	NDMA	7.5 ± 1.2	3.8 ± 0.05

K_m, Michaelis-Menten constant; V_{max}, maximal reaction velocity. Means ± S.D. (n = 3).

Michaelis-Menten kinetics was found for a demethylation of both N-nitrosamines by all used CYPs (data not shown). The values of K_m and V_{max} determined from the kinetic data are shown in Table 4. CYP2E1 reconstituted with reductase revealed the lowest values of K_m and the highest values of V_{max} for both substrates. In contrast to these results, a demethylation of both N-nitrosamines by CYP2B4 exhibits the highest values of K_m. Although CYP3A6 did not provide any type of difference spectra with NMA and the highest value of K_S was determined for NDMA in spectroscopic experiments, both N-nitrosamines are readily N-demethylated by this enzyme. The K_m values of NDMA and NMA for CYP3A6 are similar to those found for CYP2E1 (Table 4).

In spite of large differences among the K_m values of NDMA and NMA (the values differ from 5 to 700 μmol/l for individual CYPs) the values of V_{max} for their oxidation were, however, similar for all tested CYPs (Table 4). The lowest and highest values of V_{max} were 1.3 and 3.8 nmol formaldehyde/min *per* nmol CYP, respectively.

Discussion

Since N-nitrosamines are carcinogens requiring the CYP-mediated activation to exert their carcinogenic effects, the CYP enzymes responsible for such activation to species modifying DNA have already been extensively studied (for a summary, see: Skipper et al. 1983; Hecht 2007, 2008). Even though the CYP2E1 and CYP2B enzymes were shown to oxidize two members of this group of carcinogens, NDMA (Tu and Yang 1985; Amelizad et al. 1988; Yang et al. 1990, 1994; Yoo et al. 1990; Encell et al. 1996; Stiborova et al. 1996; Smith et al. 1998; Sulc et al, 2004) and NMA (Tu et al. 1985; Stiborova et al. 1996a,b, 1999; Kushida et al. 2000; Sulc et al. 2004), respectively, there are still some discrepancies found in earlier studies about their efficiencies in the reactions. Likewise, the knowledge on the detailed kinetics of reactions catalyzed by these enzymes is still limited. Therefore, here we increased our knowledge on the kinetics of NDMA and NMA oxidation by CYP enzymes.

To avoid any potential interference found in former studies utilizing perfused livers, hepatocytes, microsomes and/or partially purified enzymes (Skipper et al. 1983; Tu and Yang 1985; Yang et al. 1985, 1990; Yoo et al. 1990; Encell et al. 1996; Stiborova et al. 1996a,b; Smith et al. 1998; Sulc et al. 2004), the CYP2E1 and CYP2B4 enzymes, isolated from rabbit livers to homogeneity, were used. The enzymatic experiments were performed under the reaction conditions minimizing any inhibition effects that might be caused by some compounds present in incubation mixtures of former studies. In addition, utilizing another rabbit enzyme, CYP3A6, whose activity to oxidize both N-nitrosamines has not been studied in detail as yet we demonstrate clearly its participation in NDMA and NMA oxidation. The results found in this study, utilizing two different enzymatic approaches, shed some light to the real impact of these CYPs (rabbit CYP2B4, CYP2E1 and CYP3A6) in a demethylation of NDMA and NMA and mechanisms of such reactions.

Here, we found that NDMA and NMA are N-demethylated by all used CYPs, by the reactions following a classical Michaelis-Menten kinetics. The CYP2E1 is the most efficient enzyme to oxidize NDMA, as was found for this enzyme also previously (Tu and Yang 1985; Yang et al. 1985, 1990; Amelizad et al. 1988; Yoo et al. 1990; Smith et al. 1998; Sulc et al. 2004). The lowest K_m and the highest V_{max} were found for NDMA oxidation by CYP2E1. The K_m value of 7.5 μmol/l corresponds well to that of 8.3 μmol/l found for NDMA oxidation in perfused liver (Skipper et al. 1983). Only one K_m was detectable by us using this purified enzyme and NDMA. This finding, which is in accordance with that described previously (Tu and Yang 1985; Yang et al. 1985, 1990; Stiborova et al. 1996a), clearly confirmed the hypothesis that determination of more than one K_m for NDMA in the microsomal system is caused by multiplicity of CYPs present in this subcellular system.

Of the CYP enzymes tested in this work, CYP2E1 was the most efficient enzyme N-demethylating NMA under the conditions used, and exhibiting even higher affinity to bind to this CYP than NDMA. This result is a novel finding that has not been described till the present time.

Interestingly, both N-nitrosamines bind to CYP2E1 as heme ligands. It is well known that these type II ligands should cause inhibition of initiation of the CYP reaction cycle, by direct coordination to the heme iron atom, preventing oxygen binding to the heme. Thus, this finding is contradictory to NDMA and NMA oxidation by these enzymes. However, similarly to NDMA and NMA, some other type II ligands are also substrates of CYPs (Stiborova et al. 2004; Aimova and Stiborova 2005; Pearson et al. 2006; Locuson et al. 2007; Ahlstrom and Zamora 2008). In addition, even more surprising results were found when we investigated the binding of the studied N-nitrosamines to and their oxidation by CYP3A6. While NDMA gives a type I spectrum with this enzyme, any spectral changes were acquired for CYP3A6 by spectroscopic titration with NMA. This finding strongly suggests that this compound is not the CYP3A6 substrate. But NMA is easily N-demethylated by CYP3A6. Now, we can only speculate on mechanisms how such compounds might be hydroxylated. One can suppose that moving of NDMA and/or NMA molecules in the active centers of CYP2E1 and CYP3A6 might cause such orientations that the compounds are bound, at least in second periods, to the protein molecule as substrates (type I ligands), thus initiating the CYP reaction cycle. Because the time period

is, however, very short, this situation is not measurable by the difference spectroscopy, resulting in no detection of either type I spectra (NDMA or NMA with CYP2E1) or no binding spectra at all (NMA with CYP3A6). Because of the data found previously, this suggestion seems to be reasonable (Narasimhulu 2007). Namely, it is widely suggested that many compounds bind with multiple orientations within CYP active sites, due to multiplicity of products generated from a single substrate (Korzekwa et al. 1989; Iyer et al. 1997; Pearson et al. 2006). Moreover, in several cases, substrates appear to rapidly re-oriented near the heme iron, without dissociation from the active site, on the basis of intermolecular kinetic deuterium isotope effects (Korzekwa et al. 1989; Iyer et al. 1997). These results reveal substrate reorientation on a dramatically different, but functionally relevant, time scale. The *in silico* docking of NDMA and NMA to the active sites of CYP2E1 and CYP3A6 employing soft-soft (flexible) docking procedure (Reynisson et al. 2008) is planned to be utilized for further studies to support or exclude the supposed molecular mechanism of NDMA and NMA oxidation by these CYPs.

In contrast to CYP2E1 and CYP3A6, NDMA and NMA behave as classical substrates of CYP2B4, exhibiting type I binding spectra. However, the K_m values for N-demethylation of both N-nitrosamines by this CYP are much higher than those found for other CYPs. These results, together with those found for NDMA and NMA binding to CYP2E1 and CYP3A6 indicate that the binding affinities of the individual N-nitrosamine substrates to CYPs tested in the study do not dictate the velocities of their oxidation. Indeed, the differences among the values of V_{max} for NDMA and NMA oxidation by individual CYPs are minimal. Other steps of the CYP reaction cycle such as electron transfers and/or velocities of product dissociation from the active site seem to be the factors limiting the product formation. Nevertheless, this suggestion awaits further investigation.

We have reported for the first time that the rabbit enzyme CYP3A6 (orthologue to human CYP3A4) is also capable of catalyzing the NMA and NDMA N-demethylation, an activation reaction for these carcinogens, and exhibits kinetical parameters similar to those for CYP2E1. Therefore, due to high content of CYP3A4 in human liver and the results found for the CYP3A4 and CYP2E1 efficiencies to activate both N-nitrosamines underline their physiological significance as a risk factor for human population. In addition, in humans consumed alcohol beverages, producing an increase in CYP2E1 levels in livers, together with simultaneous N-nitrosamine administration from foodstuff with any content of N-nitrosamines (e.g. beer, smoked meats) or from cigarette smoking, an increase in a risk for the cancer development is enormous.

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Appendix

Table S1. Proteins characterization by Peptide Mass Fingerprint identification using MASCOT or ProFound searching software

Protein	Protein number	Protein name	m.w. (Da)	pI	Mowse score	ProFound Est'd Z	Sequence coverage (%)	matched/ searched peptides
CYP2B4	O4RBPC	cytochrome P450 2B4 – rabbit	55678	9.01	288	2.35	46	26/28
CYP3A6	A34236	cytochrome P450 3A6 (version 2) – rabbit	57413	9.08	370	2.4	63	54/60
CYP2E1	A26579	cytochrome P450 2E1, hepatic – rabbit	56909	8.42	300	2.42	47	31/34
NADPH-CYP reductase	A25505	NADPH-ferrihe moprotein reductase – rabbit	76540	5.38	337	2.33	45	33/34

Mowse Score (Probability Based Mowse score is –10*log (P), where P is the probability that the observed match is a random event. Protein scores greater than 59 are significant ($p < 0.05$).), ProFound Est'd Z (The estimated Z score 2.326 corresponds to 99.0 percentile in an estimated random match population). Peak lists were searched against a UniProtKB protein database subset of the other mammalian taxonomy group using MASCOT™ or ProFound™ software with the following settings: enzyme chemistry – trypsin, missed cleavages 2, carbamidomethyl modification of cysteine, variable single oxidation of methionine and peptide mass tolerance ±0.2 Da.

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