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TOXICOKINETICS AND METABOLISM

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Metabolism of benzene in human liver microsomes: individual variations in relation to CYP2E1 expression

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Abstract In human liver microsomes the oxidations of benzene, chlorzoxazone, aniline, dimethylformamide, and 4-nitrophenol were significantly correlated with each other and with the level of cytochrome P450 (CYP) 2E1 estimated by immunoblotting. Moreover, benzene oxidation to water-soluble metabolites was suppressed by 0.1 mM diethyldithiocarbamate, supposedly a specific inhibitor of CYP2E1 at this level. None of these metabolic rates correlated with immunochemically determined levels of CYP1A2, 2C9, and 3A4 nor oxidation of 7-ethoxyresorufin, tolbutamide, and nifedipine. Benzene oxidation to water-soluble metabolites was characterized by typical Michaelis-Menten kinetics. The different benzene K_m values seen in individual human microsomal samples were not correlated with the level or activity of CYP1A2, 2C9, 2E1, and 3A4 but could be due to CYP2E1 microheterogeneity. The lowest K_m for benzene oxidation could be related to C/D and/or c1/c2 polymorphism of CYP2E1 gene. Covalent binding of benzene reactive metabolites to microsomal proteins was also correlated with the CYP2E1 metabolic rates and immunochemical levels. At high concentrations of benzene covalent binding was inversely related to benzene concentrations (as well as to formation of water-soluble metabolites) in agreement with the view that secondary metabolites, mainly benzoquinone, are responsible for the covalent binding.

Key words Benzene · Chlorzoxazone · Covalent binding · Cytochrome P450 · Enzyme kinetics

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Introduction

Benzene is a human carcinogen (IARC 1987): continuing occupational exposures to benzene in some countries still cause leukaemia (Yin 1995). Benzene toxicity is postulated to be due to its oxidation to benzene oxide, 1,4-benzoquinone, 1,2,4-trihydroxybenzene and *trans*,-*trans*-muconaldehyde, which can alkylate proteins and DNA (Irons 1985; Souček et al. 1994). The putative rearrangement of benzene oxide to phenol and cleavage to *trans*,*trans*-muconaldehyde are considered to be very rapid, while benzene and phenol are oxidized by cytochrome P450 (CYP; EC 1.14.14.1) enzymes (for nomenclature see Nelson et al. 1996). Hydroquinone oxidation to benzoquinone is either non-enzymatic in the presence of dioxygen or catalysed by peroxidases in bone marrow (Greenlee et al. 1981; Smith et al. 1989).

Rat and rabbit CYP2E1, major catalysts in the oxidation of *c*. 75 carcinogenic and toxic chemicals, most efficiently oxidize benzene to soluble products, mainly phenol and hydroquinone (Johansson and Ingelman-Sundberg 1983; Guengerich et al. 1991; Gut et al. 1993) and to covalently binding metabolites (Gut et al. 1996). In contrast, other rat and rabbit CYP enzymes except 2B1 apparently do not effectively oxidize benzene (Koop et al. 1989, Gut et al. 1993). CYP2E1 was also the most efficient CYP enzyme in benzene oxidation in human liver microsomes (Guengerich et al. 1991; Schlosser et al. 1993; Seaton et al. 1994), but was measured only at one low benzene level. The evidence that human CYP2E1 is the principal enzyme producing reactive products covalently bound to biomacromolecules is limited. CYP1A1 which oxidizes phenol to hydroquinone in rabbits, is absent in human liver. Rat but not human

CYP1A2 was shown to metabolize benzene (Nakajima et al. 1992; Guengerich et al. 1991).

Individual differences in CYP2E1 expression could contribute to susceptibility to cancer and toxicity (Nedelcheva and Gut 1994). CYP2E1 expression and activity in human biopsy samples from alcoholics was *c*. two-fold (Perrot et al. 1989) or three-fold (Ekström and Ingelman-Sundberg 1989) higher than in abstainers, but did not correlate with the level of alcohol intake in other subjects (Lucas et al. 1993). Moreover, the CYP2E1 content in human liver samples from transplantation donors varied more than sevenfold (Peter et al. 1990), and even higher (Ekström et al. 1989). Alcoholics had twofold higher CYP2E1 activity measured by chlorzoxazone metabolism (Girre et al. 1994), but the level was similar to controls in some of them. Although alcohol intake increased benzene metabolism and toxicity in rats (Nakajima et al. 1985), it is not clear whether alcohol-induced CYP2E1 expression would cause this effect in humans. Moreover, CYP2E1 degradation is decreased by its substrates but increased by adrenalin and glucagon (Eliasson et al. 1990).

CYP2E1 expression in human diabetics was due to mRNA stabilization, but in rats after birth and after starvation is increased by transcriptional activation (Ingelman-Sundberg et al. 1993). It is therefore of interest whether genetic polymorphism of CYP2E1 could influence its catalysis. Two kinds of CYP2E1 gene polymorphisms have been reported [using restriction fragment length polymorphism (RFLP)]: the c1/c2 polymorphism in the 5′-flanking region and a C/D polymorphism in intron 6. A higher frequency of the mutated alleles c2 and C, respectively was reported in Japanese lung cancer patients than in a control population (Uematsu et al. 1992); however, in the Caucasian lung cancer patients the frequency of mutated alleles did not differ from healthy subjects (Persson et al. 1993; Hirvonen et al. 1993). However, it has been suggested that the c2 allele is related to lower inducibility of CYP2E1 by alcohol (Lucas et al. 1995). Thus, although CYP2E1 expression could influence benzene toxicity, the main control of CYP2E1 activity is still unclear. The aim of our study was to evaluate the role of human CYP 1A2, 2C, 2E1, and 3A4 enzymes in benzene oxidation, activation to reactive products, and inter-individual differences in human oxidation of benzene and their possible relation to polymorphism of CYP2E1 gene.

Materials and methods

Chemicals

Chemicals of highest purity were purchased from Sigma Chemical Co., St. Louis, Mo., USA (CYP substrates) and from Bio-Rad Laboratories, Hercules, Calif., USA (electrophoresis and immunoblotting). [^{14}C]Benzene (121 MBq/mmol), was obtained from Isocommerz, Dresden, Germany or Sigma Chemical Co. and purified by distillation before use.

Human liver samples

Human liver samples were obtained from donors who died accidentally as a result of brain injury from Transplantcentrum IKEM, Czech Republic. Liver samples were obtained up to 30 min after death and were stored in liquid nitrogen until microsomes were isolated. Some donors underwent therapy including antibiotics (chloramphenicol, penicillin, ampicillin, gentamycin), mannitol, and hormones (hydrocortisol, dexamethasone) for 24–48 h before death. Small pieces of frozen liver samples were added to KCl-TRIS buffer, 150 mM KCl/50 mM TRIS, pH 7.4, and homogenized using the OMNI 1000 (OMNI International, Waterburg, CT, USA) for 20 s on ice. The homogenate was centrifuged at 9000 *g*, for 20 min at 4 °C; the resulting supernatant was used to prepare microsomes by centrifugation as described in Gut et al. (1993). Addition of 1 mM phenylmethyl sulphonyl fluoride (PMSF; often used during CYP isolation) to homogenization buffer did not improve P450/P420 levels in microsomes or enzyme activities. The resulting CYP content was 0.13–0.31 nmol CYP/mg of microsomal protein. Protein content was measured according to Lowry et al. (1951) with bovine serum albumin as standard. Total CYP content was estimated by the method of Omura and Sato (1964).

Electrophoresis and immunoblotting

Electrophoresis of microsomal proteins was performed according to Laemmli (1970) using a MiniProtean II apparatus (Bio-Rad). For immunoblotting 20 μg of microsomal protein per lane was used and 1 pmol of purified CYP standard was applied corresponding to the antibody used (parent antigen). Immunoblotting was done as described by Towbin et al. (1979) using a Miniblotter device (Bio-Rad). Blots were incubated with primary antibody for 1 h at 37 °C in PBST buffer (20 mM potassium phosphate/150 mM NaCl/0.05% Tween 20) using rabbit anti-human CYP1A2, 2C9, 2E1, and 3A4 polyclonal antibodies. The next incubation was with goat anti-rabbit immunoglobulin G (IgG) coupled to horseradish peroxidase as a secondary antibody (for 1 h at room temperature; Johansson et al. 1988; Tindberg and Ingelman-Sundberg 1989). For visualization of the bands, either 3-amino-9-ethyl- carbazole or 1-chloro-4-naphthol was used. The bands of 2–3 separate samples (per liver sample) were quantitated using a Bio-Rad model 620 densitometer.

Antibody specificity tests with purified CYP 1A1, 1A2, 2C9, 2E1, and 3A4 showed that anti-human CYP antibodies reacted only with parent antigen under these conditions. In human liver microsomes anti-CYP1A2 antibodies showed only one band, comigrating with the standard. Anti-2C9 antibodies showed three to four well-resolved bands; only the intensity of the band comigrating with CYP2C9 standard was taken into consideration. Anti-CYP2E1 antibody showed two bands, the unspecific band had an M_r approximately 2 kDa lower than CYP2E1. Anti-3A4 antibodies showed one major and one very weak band of slightly higher M_r than standard CYP3A4 (most probably representing CYP3A5).

Enzyme assays

Catalytic activities of CYP2E1 were assayed by oxidation rates of CYP2E1 related substrates 4-nitrophenol, chlorzoxazone, dimethylformamide and aniline. 4-Nitrophenol hydroxylation (Reinke and Moyer 1985) was assayed for 20 min with 0.2 mM 4-nitrophenol, 0.5 mM nicotinamide adenine dinucleotide phosphate reduced form; NADPH), and 200 μg microsomal protein. Chlorzoxazone oxidation (Peter et al. 1990) was determined with 0.5 mM substrate, 0.5 mM NADPH, and 200 μg microsomal protein for 30 min. Dimethylformamide, 2 mM, was incubated in KCl-TRIS buffer with 0.5 mM NADPH, 1 mg protein and 10 mM glutathione for 30 min to form *S*-(*N*-methylcarbamoyl) glutathione, and assayed according to Hyland et al. (1991). Oxidation of aniline (Gram et al. 1967) was estimated with 4 mM aniline, 1 mM NADPH, and 1 mg microsomal protein/ml for 30 min. Other CYP activities were assayed using 3 μM 7-ethoxyresorufin, 10 μM

7-benzyloxyresorufin, 10-μM 7-pentoxyresorufin (Lubet et al. 1985) with 0.5 mM NADPH, 200 μg microsomal protein/ml for 30 min. Tolbutamide hydroxylation (Knodell et al. 1987) was assayed with 2.5 mM tolbutamide. Nifedipine oxidation (Guengerich et al. 1986) was made with 0.2 mM nifedipine and 50 pmol CYP. All incubations were carried out in 1.0 ml volume (0.5 ml in the case of nifedipine), at 37 °C in a shaking water bath.

Metabolism of [^{14}C]benzene

Incubation mixtures (2 ml total volume, final levels 150 mM KCl/50 mM TRIS pH 7.4, 1 mg of microsomal protein/ml, 6 mM $MgSO_4$, 4 mM glucose 6-phosphate, 1 mM $NADP^+$, and 0.5 IU glucose 6-phosphate dehydrogenase/ml) were incubated at 37 °C in 25 ml glass-stoppered Erlenmeyer flasks for 30 min, in a shaking water bath. Benzene was added as saturated solution in KCl/TRIS buffer. In the enzyme kinetic assays, larger (105 ml) screw-cap bottles with Teflon-lined septa were used in order to achieve lower concentrations of [^{14}C]benzene. Water-soluble metabolites were assayed by measuring radioactivity of the alkaline water phase and metabolites covalently bound to microsomal proteins were assayed in aliquots of the incubation mixture, as described (Gut et al. 1996). Radioactivity was measured by liquid scintillation with SLD31 cocktail (Spolana, Czech Republic) in Betaszint BF5000 scintillation counter (Bethold-7547 wildbad Germany). Benzene concentration was measured at the beginning and end of the incubation.

Inhibition with diethyldithiocarbamate

Diethyldithiocarbamate (DEDTC), a selective inhibitor of CYP2E1 at 0.1 or 0.3 mM (Guengerich et al. 1991) was preincubated with microsomes and NADPH generating system for 5 min. The oxidation of [^{14}C]benzene was started with addition of the substrate and carried out as described above.

Genetic polymorphism of human CYP2E1 gene

The c1/c2 and C/D genotypes were analysed as described before (Nedelcheva et al. 1996).

Statistics

All results are given as mean ± SD or were subject to correlation analysis. K_m and V_{max} calculations were done by computer program based on Fortran.

Results

Optimal conditions of [^{14}C]benzene oxidation and DEDTC inhibition

PMSF, a protease inhibitor often used in CYP purification procedures, added to homogenization buffer, did not specifically protect CYP2E1 since it did not influence P450/P420 levels and [^{14}C]benzene oxidation (data not shown). The NADPH concentration, time of incubation, and microsomal protein concentration were varied to select optimal incubation conditions (up to 1 mg microsomal protein/ml, 1 mM NADPH, and 30 min incubation time). For kinetic assays, the incubation time was 15 min. The water-soluble metabolites represented the sum of all benzene metabolites and therefore optimally reflected the first step of benzene oxidation to benzene oxide. In microsomes of three different human liver samples, the markedly different formation of soluble metabolites of [^{14}C]benzene (0.32 ± 0.03, 0.51 ± 0.04 and 1.45 ± 0.11 nmol/nmol CYP per min, mean ± SD, $n = 3$, respectively) was inhibited by 0.1 mM DEDTC by 100, 100 and 89%, respectively, and 0.3 mM inhibitor completely suppressed the activity in all three microsome preparations.

Immunoblotting analysis and oxidation rates of CYP2E1 substrates

Metabolism of several CYP2E1 substrates was investigated in a set of 12 human liver samples. All correlations (Table 1) among the oxidation rates of the five different metabolites of four specific CYP2E1 substrates and their correlations with the immunochemically detected CYP2E1 levels were significant ($P < 0.01$). The high correlation between oxidation of chlorzoxazone and dimethylformamide ($r = 0.94$) is comparable to that between chlorzoxazone and 4-nitrophenol ($r = 0.90$)

Table 1 Correlation of benzene metabolism with series of cytochrome P450 (CYP) specific activities and immunochemical levels. (*BROD* 7-benzyloxyresorufin O-debenzylation, *B-CB* benzene oxidation – covalently binding metabolites, *B-SM* benzene oxidation – soluble metabolites, *CLZ* chlorzoxazone 6-hydroxylation, *DMF* dimethylformamide oxidation, *EROD* 7-ethoxyresorufin O-deethylation, *NF* nifedipine oxidation, *PNP* *p*-nitrophenol oxidation, *PROD* 7-pentoxyresorufin O-dependentylation, *TB* tolbutamide methylhydroxylation, *2C9*; *2E1*; *3A4* immunochemically detected level of CYP isoenzymes)

	EROD	BROD	PROD	TB	PNB	Aniline	CLZ	DMF	NF	B-SM	B-CB	2C9	2E1	3A4
EROD	1													
BROD	−0.19	1												
PROD	0	0.11	1											
TB	−0.1	0.08	0.75	1										
PNP	0.17	0.02	−0.19	−0.74	1									
Aniline	−0.26	0.28	−0.09	−0.49	0.79	1								
CLZ	−0.14	0.06	−0.2	−0.64	0.9	0.89	1							
DMF	−0.29	0.22	−0.32	−0.68	0.83	0.95	0.94	1						
NF	−0.24	0.05	0.36	0.37	−0.19	−0.31	−0.24	−0.27	1					
B-SM	−0.32	0.29	−0.11	−0.37	0.64	0.66	0.78	0.86	−0.23	1				
B-CB	−0.26	0.46	−0.18	−0.52	0.58	0.81	0.67	0.77	−0.59	0.82	1			
2C9	0.04	−0.55	0.44	0.51	−0.43	−0.51	−0.33	−0.55	0	−0.06	−0.41	1		
2E1	−0.48	0.45	−0.08	−0.41	0.63	0.78	0.82	0.82	−0.03	0.92	0.72	−0.32	1	
3A4	0.35	0.13	0.25	−0.26	0.66	0.28	0.4	0.28	0.67	0.08	0	−0.35	0.27	1

and confirms that these substrates are primarily oxidized by CYP2E1. Benzene oxidation to soluble products was also correlated well with that of dimethylformamide ($r = 0.86$), in agreement with the view that benzene is a CYP2E1 substrate. Differences of up to fivefold were observed in the individual immunochemically determined CYP2E1 levels and the substrate oxidation rates. None of the rates of oxidation of other CYP (1A2, 2C9 and 3A4) substrates or their immunochemically determined levels were correlated with rates of oxidation of benzene.

Metabolism of [^{14}C]benzene to water soluble and to covalently bound products

Oxidation of benzene was studied at different concentrations of [^{14}C]benzene; 0.2 mM, 0.89 mM, and 2.8 mM (saturation activity; Fig. 1). The [^{14}C]benzene concentrations were analysed at the start in some and at the end of incubations in all samples. The highest and lowest rates of formation of water soluble products in individual human liver samples were 4.5-fold different at the benzene saturation level, and the rates correlated well with the CYP2E1 immunochemical levels and the other CYP2E1 activities (Table 1). Maximum rates in individual human liver samples were reached at different substrate levels and suggested different K_m values (see subsequently). The oxidation rates of benzene to water soluble products in all human liver microsomal samples were more than threefold higher than in microsomes prepared from untreated rats (Gut et al. 1996).

Covalent binding of reactive metabolites of [^{14}C]benzene to microsomal proteins was correlated with the immunochemical levels of CYP2E1 as well as with oxidation rates for marker CYP2E1 substrates. However, with increasing concentration of [^{14}C]benzene (approx. 0.1, 0.4 mM and up to 1.0 mM), formation of water soluble benzene products increased, whereas that of covalently bound products decreased. The same trend was seen with different human liver microsomes (Fig. 2). At the saturation level of benzene, 1 mM ascorbate did not influence the formation of water soluble metabolites (no addition, 1.84 ± 0.15 nmol/nmol CYP per min; ascorbate, 1.93 ± 0.10), but significantly decreased the formation of covalently bound products (no addition, 0.08 ± 0.02; ascorbate, 0.02 ± 0.03). Glutathione, 4 mM, suppressed the covalent binding almost completely (Fig. 3), while formation of soluble metabolites was not influenced. Addition of widely used solvent, methanol (2%), to the incubation mixture did not show significant inhibitory effect on benzene metabolism to soluble and covalently bound products (Fig. 3).

The different rates of benzene oxidation to water soluble products in various liver samples at 0.2, 0.89 and 2.8 mM (Fig. 1) indicated possible inter-individual difference in K_m values. Therefore a further detailed analysis of steady-state kinetics was done at 14 benzene concentrations (5–400 μM). Six different human liver samples were selected based on oxidation rates (both high and low) and the c1/c2 and C/D polymorphisms of the CYP2E1 gene. The Michaelis-Menten kinetic analysis (Table 2) showed markedly different V_{max} and K_m values for the microsomes from different livers. The lowest K_m value was in the c1/c1 plus C/D polymorphic liver and the next lowest was in the c1/c2 plus C/D liver, whereas all other livers with the wild-type alleles (c1/c1 plus D/D) had higher K_m values.

Discussion

Our multiple significant correlations between the rates of oxidation of chlorzoxazone, benzene, 4-nitrophenol and dimethylformamide comply with reports that chlorzoxazone (Peter et al. 1990), benzene (Guengerich et al. 1991; Schlosser et al. 1993; Seaton et al. 1994), dimethyl-

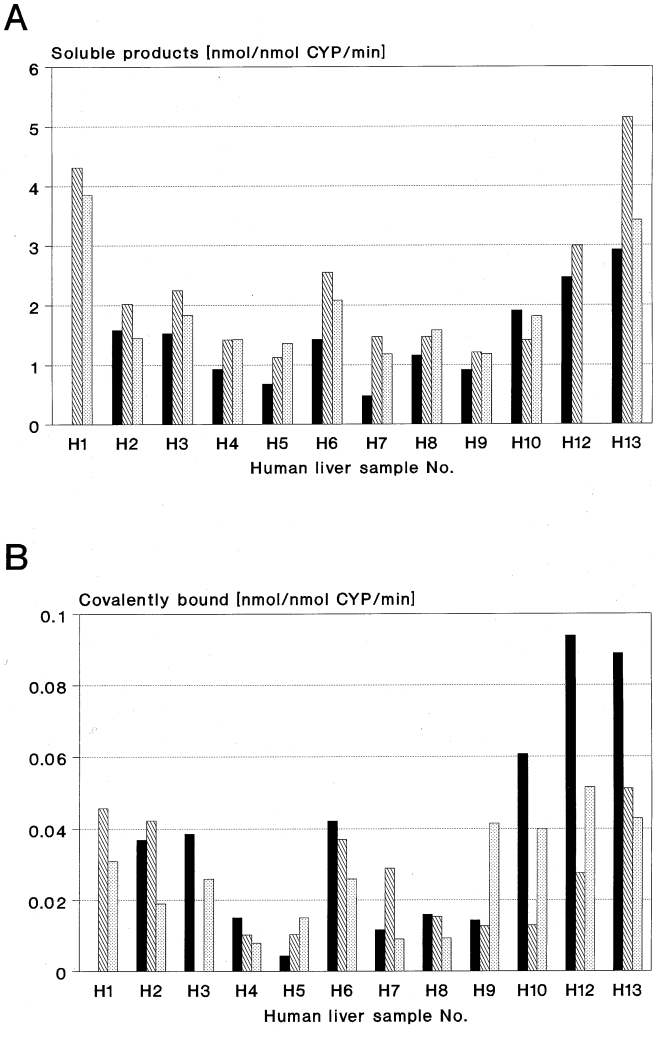

Fig. 1 Metabolism of [^{14}C]benzene to water soluble products (**A**) and to products covalently bound to microsomal proteins (**B**) at 0.2 mM ■, 0.89 mM ▨, and 2.8 mM ⬚ benzene concentrations. Incubations were carried out as described in the Materials and methods. Missing bars represent missing samples. (*CYP* Cytochrome P450)

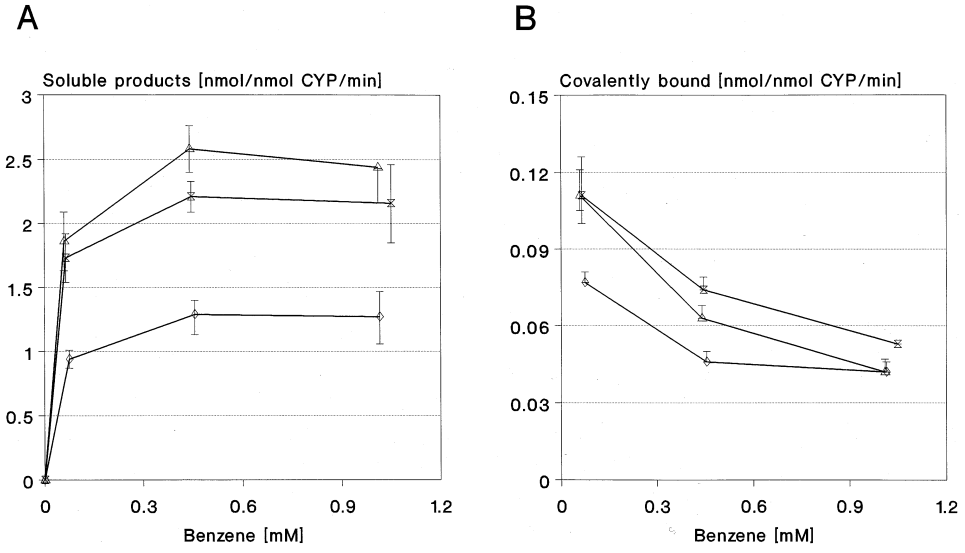

Fig. 2 Effect of benzene concentration on oxidation of benzene to water-soluble (**A**) and covalently bound (**B**) products in different human liver microsomes: H6 (×), H12 (◇), and H13 (△).

formamide (Mraz et al. 1993) and 4-nitrophenol (Tassaneeyakul et al. 1993) are oxidized principally by CYP2E1 in human liver. Higher correlations between the oxidation rates than to immunochemically determined levels of CYP2E1 in microsomes may reflect the fact that substrate rates correspond to the active enzyme, while the immunoblots may also detect some inactive CYP2E1 protein. In this regard, Johansson et al. (1988) found that 48 h after CYP2E1 induction, expression of the protein was still increased five fold, while benzene oxidation in vitro returned from a five fold to *c*. 2.5-fold increase above control values.

Chlorzoxazone is considered as a marker substrate of CYP2E1 (Peter et al. 1990). The drug is also oxidized by the expression product of human CYP1A1 cDNA in vitro (Carriere et al. 1993), but this form is absent in human liver and the V_{max}/K_m is very low (Yamazaki et al. 1995). In our assays, chlorzoxazone oxidation did not correlate with the oxidation of 7-ethoxyresorufin ($r = 0.14$), 7-benzyloxyresorufin ($r = 0.06$), tolbutamide ($r = -0.64$), and nifedipine ($r = -0.24$) indicating that

Fig. 3 Effect of glutathione (4 mM) and methanol (2%) on oxidation of [^{14}C]benzene to water soluble products (**A**) and to products covalently bound to microsomal proteins (**B**) at different benzene concentrations in human H13 liver. (*GSH* Glutathione, *MeOH* methanol)

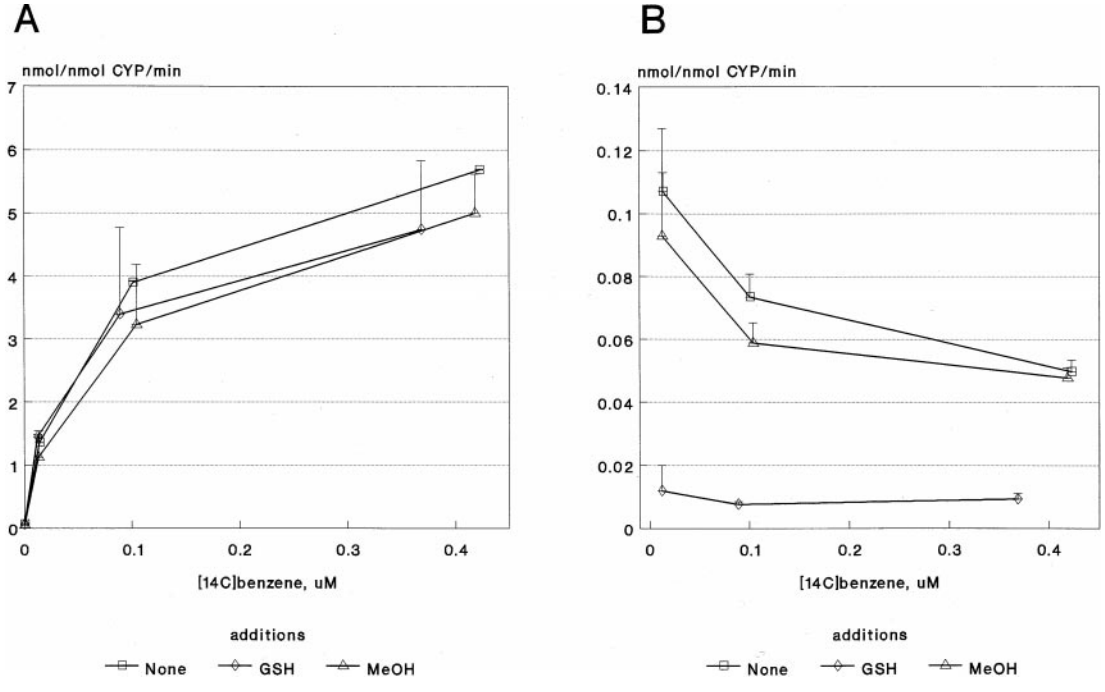

Table 2 Benzene oxidation and CYP2E1 genotype

Human liver no.	K_m (μM)	V_{max} (nmol/nmol CYP per min)	CYP2E1 genotype
H2	17.4 ± 6.8	3.0 ± 0.8	c1/c2 and C/D
H4	20.1 ± 5.3	3.5 ± 0.7	c1/c1 and D/D
H6	26.4 ± 11.6	3.6 ± 1.3	c1/c1 and D/D
H9	11.2 ± 1.9	0.7 ± 0.1	c1/c1 and C/D
H12	21.2 ± 7.2	2.3 ± 0.6	c1/c1 and D/D
H13	54.7 ± 9.5	6.1 ± 2.0	c1/c1 and D/D

Note: Restriction fragment length polymorphism (RFLP) analysis of CYP2E1 gene polymorphism revealed that the other livers in the H1–H13 series contained wild type alleles (c1/c1 and D/D genotype)

CYP1A2, 2C9 and 3A4 do not significantly contribute to the oxidation of chlorzoxazone in human liver.

Benzene oxidation to water soluble products was correlated with oxidation of other CYP2E1 substrates and immunochemical level, but not with the activities for marker substrates or immunochemically determined levels of CYP 1A2, 2C9, and 3A4. This findings indicated their negligible roles in benzene oxidation in humans. The hypothesis suggesting a significant role of CYP2E1 in benzene activation to haematotoxic metabolites has been recently supported by a dramatic decrease of benzene toxicity in transgenic Cyp2e1 knock-out mice (Valentine et al. 1996).

The formation of covalently bound products during oxidation of benzene was also correlated with immunochemically determined CYP2E1 level and CYP2E1 catalytic activities, but not with activities or immunochemically determined levels of CYP1A2, 2C9, and 3A4. Moreover, benzene oxidation to both soluble and covalently bound products was almost completely inhibited by 0.1 mM DEDTC, which selectively inhibits CYP2E1 at this concentration (Guengerich et al. 1991). Ascorbate, which reduces benzoquinone to hydroquinone, almost abolished covalent binding. Guengerich et al. (1991) indicated that human CYP2E1 is the main enzyme oxidizing benzene to phenol and hydroquinone at 300 μM benzene. Schlosser et al. (1993) measured benzene oxidation to various water soluble products at 4 μM and Seaton et al. (1994) at 3.4 μM benzene only.

Our study on the effect of benzene concentration (200–2800 μM, 100–1000 μM and 20–400 μM in different experiments) revealed for the first time that the formation of water soluble products increased with benzene concentration, whereas formation of covalently bound products was inversely related to benzene concentration (in 9 of 11 samples). Moreover, different human livers had different K_m values ranging for benzene from 11 μM to 55 μM. Similar data were earlier reported for chlorzoxazone (Peter et al. 1990). K_m values observed with [^{14}C]benzene do not necessarily give the same values that would have been obtained with unlabelled benzene. For instance, deuteration of substrate raises the K_m without affecting V_{max} . Thus K_m cannot be a direct measure of substrate affinity. The K_m is a complex ratio of sums of microscopic rate constants, which is heavily influenced by the rate of the isotopically sensitive step (Yang et al. 1991; Guengerich et al. 1995). However, individual differences in K_m values apparently reflect true differences, since ^{14}C-labelled benzene was used in all measurements.

Increasing benzene concentration resulted in increasing formation of water soluble products and decreasing formation of covalently bound products. Moreover, ascorbate and glutathione inhibited the formation of covalently bound products, but not of soluble products. The results support the view that benzoquinone, the oxidation product of hydroquinone, is mainly responsible for the covalent binding and comply with data in rat liver microsomes (Gut et al. 1996): benzoquinone destroyed various proteins and CYP heme, while hydroquinone did not (Souček et al. 1994). Human and rabbit CYP2E1 very actively oxidize phenol to hydroquinone (Koop et al. 1989). However, the fact that glutathione abolished covalent binding does not exclude the possibility that benzene oxide may participate in covalent binding.

In our experiments the rate of formation of water soluble products was highest in the first 15 min of incubation, but covalently bound products were formed at significantly higher rates in 30–60 min incubation and a duration of 30 min was therefore selected. This is in accordance with the results of Seaton et al. (1994), who found that at 4 μM benzene, phenol is formed mainly in the first 15–30 min and is further oxidized to hydroquinone at levels exceeding phenol concentration after 45 min.

RFLP analysis of CYP2E1 gene for c1/c2 and C/D polymorphisms indicated that the lowest K_m value belonged to c1/c1 plus C/D and the next lowest to c1/c2 plus C/D genotype. Although this result is interesting, further analysis of higher numbers of human liver samples will be needed to address the hypothesis of an actual link between CYP2E1 genotype and phenotype. Such a relationship might be helpful in cancer risk assessment, since CYP2E1 is a very efficient enzyme involved in catalysis of many carcinogenic and mutagenic substrates including benzene.

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