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MICROSOMAL AND CYTOSOLIC EPOXIDE HYDROLASES IN RHESUS MONKEY LIVER, AND IN NORMAL AND NEOPLASTIC HUMAN LIVER

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Summary

The cytosolic epoxide hydrolase (EH-LC) was observed in rhesus monkey liver cytosol, and in both normal and neoplastic human liver. Microsomal epoxide hydrolase (EH-LM) was detected not only in the microsomes of normal and neoplastic human liver and normal rhesus monkey liver, but also in the cytosol of these tissues. No apparent differences were observed between the EH-LM in liver cytosol and that in microsomes. No major differences were observed between the levels of EH-LM in the cytosol of normal and that in neoplastic human liver.

Epoxides are metabolized to 1,2-diols or undergo conjugation with glutathione, reactions catalyzed by epoxide hydrolases (EH, EC 3.3.2.3) and glutathione transferases, respectively (1). Liver EH's are either largely membrane bound, as in the endoplasmic reticulum, and nuclear and plasma membranes, or occur largely in the soluble form in cell cytosol and mitochondrial matrix/ intermembrane space fraction (1-4). In this study these enzyme activities will be referred to as the liver microsomal (EH-LM) and cytosolic EH's (EH-LC), respectively. In laboratory animals studied EH-LM and EH-LC are distinct proteins as indicated by their differential inhibition, induction, pI, molecular weight, antigenic determinants, and their species, strain, age, sex, tissue and subcellular distribution (3,5-7). However, preliminary studies indicate that the situation may be more complex in humans (8).

EH-LM may also be of significance in hepatocarcinogenesis and liver cancer detection as demonstrated by Farber and associates (9-10), who showed the presence of an antigen in the cytosol of preneoplastic and neoplastic tissues of rats. Subsequent work by various workers has demonstrated that this antigen is or is antigenically related to EH-LM as evidenced by their similar catalytic and immunogenic reactions (11-14). Thus in normal rats EH-LM is largely membrane bound, while in preneoplastic and neoplastic rat liver EH-LM is present not only in the microsomes but also in the cytosol (13). It is not known whether the same phenomenon of release of EH-LM is observed in humans. In addition, while there are a number of reports on EH-LM activity in humans and other primates, EH-LC activity has only recently been observed in humans (15). This report thus summarizes some observations on the catalytic activity of EH's in human and rhesus liver.

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Materials and Methods

Chemicals: ^{3}H-Trans- and cis-stilbene oxides (TSO and CSO, respectively) >99% radiochemical and >97% geometrical purity; ^{3}H-trans- and cis-β-ethylstyrene oxides (TESO and CESO, respectively), >98% radiochemical and >99% geometrical purity were synthesized as previously reported (16,17). ^{3}H-Benzo(a)pyrene 4,5-oxide (BPO) was kindly provided by F. Oesch (University of Mainz, W. Germany). 4-Phenyl-, 4-isopropyl-and 4-bromochalcone oxides were synthesized by C. Mullin (18). All other chemicals used were the best grade commercially available.

Enzyme Preparation: Monkey liver obtained from the University of California Primate Research Center rhesus colony (male, 4 kg) was homogenized in 4 vol of 0.25M sucrose-10mM Tris-HCl, pH 7.4. The homogenate was centrifuged sequentially at 600 g for 10 min, the 600 g supernatant at 10,000 g for 10 min and the 10,000 g supernatant at 100,000 g for 60 min to give the cell nuclei and debris, mitochondrial, and microsomal pellets, respectively, and the 100,000 g supernatant as the cytosol. Cell nuclei debris, mitochondrial, and microsomal pellets were resuspended and recentrifuged before use. Liver samples from human patients with liver cancer were obtained by surgical biopsies. Both normal and tumor tissues from these patients were utilized. The liver samples were homogenized and centrifuged as described for monkey liver. Normal liver samples, obtained from human subjects who died accidentally, were supplied by M. Arnott (University of Texas, Houston) and were prepared as follows. The liver tissues were homogenized in 2.2 vol of 0.25M sucrose buffered with 10mM Tris-HCl, pH 7.4 and 3mM 2-mercaptoethanol. The homogenate was centrifuged at 9,000 g for 15 min and the supernatant subsequently centrifuged at 140,000 g for 90 min to give the microsomal fraction and the cytosolic supernatant. Microsomes were washed once in the same buffer at 140,000 g for 60 min. The fractions were diluted in appropriate buffers prior to use. Protein concentrations were determined following Bradford (19) using bovine serum albumin fraction V as standard.

Enzyme Assays: EH-LM was monitored with ^{3}H-CSO following published procedures (16,17) which were briefly as follows. CSO (final concentration 5 x 10^{-5}M, in 1 µl ethanol), added to a microsomal suspension (100 µl) in 0.1M Tris-HCl buffer, pH 9.0, was incubated for 10 min at 37°C after which the mixture was extracted with n-dodecane (200 µl). The aqueous phase (50 µl) containing the product diol was removed after centrifugation and quantified by liquid scintillation counting (lsc). To detect the formation of glutathione conjugate, incubations were alternatively extracted with 1-hexanol. Under these conditions, the epoxide and diol partitions into the organic phase while the glutathione conjugate remains in the aqueous phase, and quantified by lsc. BPO hydrolysis was monitored following the thin-layer chromatographic procedure of Jerina et al. (20). EH-LC was monitored with TSO and TESO in assays similar to that employed for CSO but determined in 0.08M sodium phosphate buffer, pH 7.4 (16,17). K_m and V_{max} of EH-LM was determined with the microsomal and cytosolic fractions at various substrate concentrations (1.8×10^{-6}M to 1×10^{-4}M) and time (2-20 min). Inhibition assays were performed by preincubating the inhibitor with enzyme suspension for 1 min after which CSO as substrate was added except for 4-bromobenzyl bromide and 2-bromo-4'-nitroacetophenone where a 30 min preincubation was utilized. The stability of EH-LM at 5°C and 37°C in 0.1M Tris-HCl, pH 9.0 was assessed by incubating the microsomal and cytosolic fractions of normal and tumor tissue for varying times after which an aliquot was removed and activity monitored by CSO. Isoelectric focusing was performed in 5% polyacrylamide gels (2% LKB Ampholines, pH 4-6) (21).

Results and Discussion

This study clearly demonstrates the presence of an EH in the cytosol of rhesus and human liver which is analogous to EH-LC activity described in other common laboratory species (3,6). On the other hand EH activity also occurs in the cytosol which appears catalytically identical to EH-LM. CSO, which is hydrolyzed primarily by EH-LM in laboratory animals (17), is similarly hydrolyzed largely by EH(s) in the endoplasmic reticulum and nuclear fractions (Table I). The rate of CSO hydration by monkey liver microsomes, is 13 and 6 times faster than that observed with mouse or rat liver microsomes, respectively. Similar higher rates of hydration by monkey liver microsomes have been reported for styrene oxide (22). Of the total postmitochondrial CSO hydrolase (pmol min^{-1} mg tissue $equiv^{-1}$) ca. 14% is in the cytosolic fraction. Throughout this study, the hydration of CSO at pH 9.0 is taken as an indication of EH-LM, whether the assay is performed using microsomes or cytosol because of the similarity in CSO hydrolase in these two fractions. GSH transferase activity was negligible in both monkey or human liver cytosol if no exogenous GSH was added during the partition assay when using CSO as substrate, and when the assay was performed at pH 9.0. Thus it was not necessary to dialyze the cytosol before using the partition assay to monitor EH activity.

TSO and TESO, selective substrates for mouse EH-LC (16,17), are hydrolyzed by monkey liver cytosol (Table I). While TSO is selectively hydrolyzed by monkey liver cytosol, such selectivity is not observed with TESO as substrate. Nevertheless, the highest levels of TESO and TSO hydrolase(s) (pmol min^{-1} mg tissue $equiv^{-1}$) are observed in the cytosol indicating the overall importance of the cytosol in the liver as a whole. EH activity in the microsomes may also be due to a membrane bound form of EH-LC as previously observed with other substrates. Substrates known to be hydrated preferentially by the EH-LC are sometimes hydrated by exhaustively washed microsomes with a pH optimum of 7.4 (23,24). Unlike mice (4) only low levels of EH activity are observed in the mitochondria of monkey liver. Some mitochondria, however, could have appeared in the 600 g pellet.

EH-LM and EH-LC activity in tumor tissue is lower than in adjacent normal tissue of human liver (Table II-IV). This lower activity is not due to necrosis because detailed histological examination of all tissues revealed negative necrosis. Similar differences in EH-LM activity between tumor and normal tissue have been reported previously in man (25,26). In rat liver, in

TABLE I

Distribution of Epoxide Hydrolase Activity in Various Subcellular Fractions of Rhesus Monkey Liver

Subcellular Fraction	Specific Activity[a]					
	pmol min^{-1} mg tissue $equiv^{-1}$			nmol min^{-1} mg $protein^{-1}$		
	CSO[b]	TSO[c]	TESO[c]	CSO[b]	TSO[c]	TESO[c]
Cell Nuclei and Debri	370	13	110	8.1	0.29	2.5
Mitochondria	92	10	80	3.5	0.37	3.0
Microsomes	1200	3.3	150	71	0.19	8.7
Cytosol	190	78	640	1.1	0.45	3.7

[a]Values reported are means of experiments performed in triplicate from a single monkey liver. [b]Assayed in 0.1M Tris-HCl, pH 9.0 buffer. [c]Assayed in 0.08M sodium phosphate, pH 7.4 buffer.

TABLE II

Hydration of Cis- and Trans-Stilbene Oxide, Benzo(a)pyrene 4,5-Oxide and Trans-β-Ethylstyrene Oxide by Mitochondria, Microsomes, Cytosol and Microsomal Wash of Pathologically Normal and Tumor Tissue from an 11-Month-Old Male Hispanic with Hepatoblastoma

Subcellular Fraction	Specific Activity[a] pmol min^{-1} mg tissue $equiv^{-1}$				Specific Activity[a] nmol min^{-1} mg $protein^{-1}$			
	BPO	CSO	TSO	TESO	BPO	CSO	TSO	TESO
Normal tissue:								
Mitochondria	230	390	20	10	2.0	3.4	0.17	0.10
Unwashed microsomes	1500	4200	160	24	13	35	1.4	0.20
Cytosol	615	1800	540	140	1.1	3.3	1.0	0.26
Washed microsomes	1400	4000	24	10	27	76	0.47	0.20
Microsomal wash	130	220	13	0.8	5.0	8.4	0.51	0.03
Tumor tissue:								
Mitochondria	90	180	9	44	1.8	3.6	0.2	0.04
Unwashed microsomes	590	1000	58	3.4	10	17	1.0	0.05
Cytosol	190	350	83	36	0.4	0.8	0.2	0.17
Washed Microsomes	510	840	9	1.1	17	28	0.3	0.04
Microsomal wash	38	60	95	0.2	2.0	3.2	5.0	0.01

[a]Values are means of experiments performed in triplicate. BPO was assayed in 0.1M Tris-HCl, pH 9.0. Buffers for other substrates are in Table I.

contrast, EH-LM levels are increased in hyperplastic nodules and benign tumors, but not in malignant tissue (27). Whether similar changes in EH-LM levels occur with liver tumor progression in man is not known.

While the distribution pattern of EH activity is similar in both man and monkey there is, however, a greater proportion of the postmitochondrial CSO hydrolase or EH-LM activity in the cytosolic fraction of apparently normal human liver than that observed in monkey liver. Approximately 29% of the postmitochondrial CSO hydrolase (pmol min^{-1} mg tissue $equiv^{-1}$) is in the cytosol of normal liver (Table II). In human tumor tissue, a similar proportion of the postmitochondrial CSO hydrolase activity, ca. 28% is in the cytosol. When BPO is used as substrate, similar proportions of BPO hydrolase or EH-LM are observed in the cytosolic fractions of normal and tumor tissues. There is variability, however, in the distribution of postmitochondrial CSO activity in liver cytosol. With patient in Table III, ca. 20% and 22% of the postmitochondrial CSO hydrolase are in the cytosol of normal and tumor tissues, respectively, while with patient in Table IV, ca. 24% and 25% of such activity are in the cytosol of normal and tumor tissues, respectively. The corresponding percentages of postmitochondrial CSO hydrolase in the cytosol of three human subjects with no liver tumor are 33, 60 and 57 (Table V). These values indicate that there is a great variability between individuals in the levels of EH-LM in the cytosol in human subjects who died accidentally. It is possible that the higher levels of EH-LM in the cytosol in human subjects who died accidentally, are due to changes in liver membrane structure post-mortem before the liver was removed, or due to storage in liquid nitrogen before separation of microsomes and cytosol. However, even when short post-mortem times are involved, viz 3 hr, and the tissue processed immediately, significant EH-LM activity is observed in liver cytosol (Table V). Both EH-LM and EH-LC can be stored for long periods in the freezer without appreciable loss of activity.

TABLE III

Hydration of Cis- and Trans-Stilbene Oxides and Cis- and Trans-β-Ethylstyrene Oxide by the Microsomal and Cytosolic Fractions of Pathologically Normal and Tumor Tissue from a 4-Month-Old White Female with Hemangioendothelioma

Subcellular Fraction	Specific Activity[a]									
	pmol min^{-1} mg tissue equiv^{-1}					nmol min^{-1} mg protein^{-1}				
	CSO	TSO	TESO	CESO at pH 9.0	CESO at pH 7.4	CSO	TSO	TESO	CESO at pH 9.0	CESO at pH 7.4
Normal tissue:										
Microsomes	1000	6.2	52	150	88	15	0.1	0.8	2.2	1.3
Cytosol	250	30	340	39	77	0.6	0.1	0.8	0.1	0.2
Microsome wash	32	0.6	9.1	11	7.1	1.1	ND	0.3	0.4	0.3
Tumor tissue:										
Microsomes	44	4.4	14	25	12	1.2	0.1	0.4	0.7	0.3
Cytosol	13	9.4	31	9.4	19	ND	ND	0.1	ND	0.1
Microsome wash	1.5	0.9	3.8	0.9	ND	0.1	0.1	0.2	0.1	ND

[a]Values reported are means of experiments performed in triplicate. CESO was assayed either at pH 9.0 or 7.4. Buffers for other substrates are as in Table I. ND: values are <0.1 nmol min^{-1} mg protein^{-1} or 0.1 pmol min^{-1} mg tissue equiv^{-1}.

There is significant CSO hydrolase (pmol min^{-1} mg tissue equiv^{-1}) activity in microsomal washes (Tables II-IV), ca. 2.5-6.1% and 2.6-5.1% of the postmitochondrial activity, in normal and tumor tissues. These results indicate that unlike rat EH-LM (13), human EH-LM is loosely bound and significant amounts become detached with washing of the microsomal pellet. There is apparently no difference in the amounts of EH-LM that become detached from normal and tumor tissue microsomes. Although further work needs to be done to confirm these findings, the high levels of CSO hydrolase found in the cytosol of normal patients (Table V) suggests that the EH-LM in humans is easily detached. Thus, it appears that unlike the rat where EH-LM does not appear in the cytosol of normal rat but does appear in the cytosol of preneoplastic and neoplastic liver tissue (9-13), EH-LM in humans is present in the cytosol of apparently both normal and neoplastic human liver tissue.

CSO hydrolase(s) found in the microsomal and the cytosolic fractions are similar; they show similar substrate selectivity (Tables II-V), and are inhibited similarly by a variety of compounds (Table VI). Trichloropropene and cyclohexene oxides, both good inhibitors of EH-LM, readily inhibit CSO hydrolase in both the microsomal and cytosolic fractions. Metyrapone, a known stimulator of rat EH-LM (28), also stimulates human CSO hydrolase in both microsomes and cytosol (Table VI). However, the CSO hydrolase in microsomes and that in cytosol behave differently when exposed to a number of chalcone oxides. The K_m of CSO hydrolase in the microsomes and cytosol are also very similar, 2.40 x 10^{-6}M and 2.54 x 10^{-6}M, respectively. The corresponding V_{max} values are 63.8 and 2.64 nmol min^{-1} mg protein^{-1}. The pH optima of CSO hydrolase are similar, 9.0-9.2, in both microsomal and cytosolic fractions. CSO hydrolase in the microsomal and cytosolic fractions of both normal and tumor tissue show similar stability when incubated for varying lengths of time at 5°C and 37°C.

TABLE IV

Hydration of Cis- and Trans-Stilbene Oxides and Trans-β-Ethylstyrene Oxide by Microsomes, Microsomal Wash, and Cytosol of Apparently Normal and Tumor Tissue from a 52-Year-Old Oriental Female with Hepatocellular Carcinoma

	Specific Activity[a]					
	pmol min^{-1} mg tissue equiv^{-1}			nmol min^{-1} mg protein^{-1}		
Subcellular Fraction	CSO	TSO	TESO	CSO	TSO	TESO
Normal tissue:						
Microsomes	1500	8.4	190	29	0.2	3.7
Cytosol	500	160	1400	1.3	0.4	3.6
Microsome wash	91	7.3	130	4.4	0.4	6.2
Tumor tissue:						
Microsomes	920	8.7	190	22	0.2	4.7
Cytosol	530	42	430	1.6	0.1	1.3
Microsome wash	72	4.9	100	2.6	0.2	3.7

[a]Values reported are means of experiments performed in triplicate. Assay conditions are as in Table 1.

TABLE V

Hydration of Cis- and Trans-Stilbene Oxides and Trans-β-Ethylstyrene Oxide by the Microsomal and Cytosolic Fractions of Apparently Normal Human Liver

		Specific Activity[a]					
Patient Description	Subcellular Fraction	pmol min^{-1} mg tissue equiv^{-1}			nmol min^{-1} mg protein^{-1}		
		CSO	TSO	TESO	CSO	TSO	TESO
Black, ♂ 22 yrs.[b]	Microsomes	3100	10	240	54	0.2	4.1
	Cytosol	1500	310	2300	5.5	1.1	8.5
White, ♂ 25 yrs.[c]	Microsomes	420	14	170	7.3	0.2	2.9
	Cytosol	620	220	1900	1.8	0.7	5.7
White, ♀ 22 yrs.[d]	Microsomes	1600	15	200	28	0.3	3.5
	Cytosol	2100	490	3600	4.0	0.9	6.7

[a]Values reported are means of experiments done in triplicate. Assay conditions are as in Table I. [b,c,d]Livers were obtained 7.5[b], 3[c] and 10[d] hr after post mortem, and stored for 8[b], 0[c] and 2[d] days in liquid nitrogen before being worked up. The cytosolic and microsomal fractions were stored at -68°C for 8 month before transport to U.C. Davis in dry ice.

Human liver fractions are also capable of hydrolyzing TSO and TESO, selective substrates for rodent EH-LC, indicating the presence of the EH-LC in humans. The relative rates of hydration by EH-LC when TSO and TESO are used as substrates are lower than that of EH-LM hydration of CSO. It demonstrates, however, the importance of EH-LC in human liver in metabolizing epoxides which are poorly hydrolyzed by EH-LM, both in the microsomal and cytosolic fractions of human liver. The EH-LC activity from apparently normal human liver demonstrated a single, sharp, acidic pI similar to that observed with mouse EH-LC (3). Under the same conditions the EH-LM in the cytosol failed to

TABLE VI

Inhibition of Cis-Stilbene Oxide Hydrolase in the Microsomal and Cytosolic Fractions of Patient with Hepatoblastoma

Compound	Inhibitor Concentration (M)	Percent Inhibition	
		Microsomes	Cytosol
4-Bromobenzyl bromide[a]	1×10^{-3}	66.5[b]	67.5[b]
2-Bromo-4'-nitroacetophenone[a]	3×10^{-4}	22.4[b]	30.0[b]
Cyclohexene oxide	1×10^{-4}	90.2[b]	91.1[b]
Trichloropropene oxide	1×10^{-4}	100.3[b]	98.3[b]
Metyrapone	1×10^{-4}	-20.3[b]	-13.8[b]
Chalcone	1×10^{-5}	-2.5	3.6
Chalcone oxide	1×10^{-5}	-1.8	16.7[b]
4-Phenylchalcone oxide	1×10^{-5}	-10.9	13.6[b]
4-Isopropylchalcone oxide	1×10^{-5}	-21.2[b]	7.8
4-Bromochalcone oxide	1×10^{-5}	-3.2	18.1[b]

[a]Preincubated for 30 min. [b]Values are significantly different from control values at the 95% level.

focus. CESO, a substrate previously shown to be poorly hydrolyzed by both EH-LC and EH-LM of mouse (16) is hydrolyzed apparently by both human EH-LC and EH-LM, with hydrolysis occurring at a faster rate at pH 9.0, optima for the EH-LM, than at pH 7.4, as was previously observed for guinea pig. This parallel in substrate selectivity may correlate with the antigenic similarity of guinea pig and human EH's (29).

This study demonstrates that the subcellular distribution of human EH-LM is rather more complex than those of commonly used laboratory animals (9-14), because unlike them there is significant EH-LM activity in the cytosol of human liver. The levels of EH-LM in the cytosol of normal and neoplastic tissues of liver are similar. Although only one individual was studied in detail, there are apparently few differences in the EH-LM found in microsomes and cytosol of both normal and neoplastic tissues as evidenced by their similar substrate selectivity, inhibition, pH optima, and K_m. It is possible that differences in the EH-LM in microsomes and cytosol occur, as their stimulation to chalcone oxides, and these could conceivably be in the structure of the enzyme itself, in membrane structure or both. In addition, it is possible that multiple forms of the EH-LM occur in man (30). The use of EH-LM as a preneoplastic antigen, as suggested for the rat (9-13), therefore will require further study, because unlike the rat significant activity is found in pathologically normal liver cytosol.

This report confirms the presence of EH-LC in man as shown recently (15), and thus the occurrence of EH-LC is apparently ubiquitous in mammalian systems. The role of EH-LC, like that of the EH-LM is not established. However, it is probably of major importance in the metabolism of epoxidized xenobiotics and endogenous compounds not metabolized primarily by the membrane bound EH's.

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