Rat and Human Liver Cytosolic Epoxide Hydrolases: Evidence for Multiple Forms at the Level of Protein and mRNA

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Two forms of human liver cytosolic epoxide hydrolase (cEH) with diagnostic substrate specificity for trans-stilbene oxide (cEH_{TSO}) and cis-stilbene oxide (cEH_{CSO}) have been identified, and cEH_{CSO} was purified to apparent homogeneity. The enzyme had a monomer molecular weight of 49 kDa and an isoelectric point of 9.2. Pure cEH_{CSO} hydrolyzed CSO at a rate of 145 n mole/min/mg. TSO was not metabolized at a detectable level, and like cEH_{TSO}, the enzyme was about three times more active at pH 7.4 than at pH 9.0. Unlike cEH_{TSO}, cEH_{CSO} was efficiently inhibited by 1 mM 1-chloropropane oxide (90.5%) and 1 mM TSO (92%). Similarly, liver cEH purified 541-fold from fenofibrate induced Fischer 344 rats was shown to be a native 120 kDa dimer of two 61 kDa subunits. The enzyme expressed maximum activity of 205 n mole/min/mg at pH 7.4 toward the diagnostic substrate TSO with an apparent $K_m$ of 1.7 $\mu$M. In Western blots, polyclonal antibodies against rat liver cEH were shown to recognize a single 61 kDa protein band from liver cytosol of rat, mouse, guinea pig, Syrian hamster, and rabbit. This antibody precipitated neither human liver cEH_{TSO} or cEH_{CSO}. Antibodies against rat liver microsomal epoxide hydrolase reacted with cEH_{CSO} in the Western blot and on immunoprecipitation. Using antibodies against rat liver cEH, 24 positive clones were picked upon colony blot screening of a pEX 1/E. coli POP 2136 expression library. After Northern blotting against rat liver total RNA, clone 24 recognized a second mRNA-species besides the putative message for cEH_{TSO}, thus providing strong evidence for the multiplicity of rat liver cEH as well. The importance of these findings for the control of reactive metabolites of various origins is discussed.

Introduction

Epoxides are formed in vivo mainly by the microsomal cytochrome P-450-dependent monooxygenase system. Epoxides have been identified as intermediates of arachidonic acid (1) and steroid (2) metabolism as well as products of fatty acid peroxidation (3). Epoxides have also been identified as metabolites of numerous xenobiotic compounds containing olefinic or aromatic double bonds (4). Due to their electrophilicity, epoxides may bind covalently to cellular nucleophiles such as proteins and nucleic acids and thus elicit toxic, mutagenic or carcinogenic effects (5).

Enzymatic inactivation of reactive epoxides is provided in part by conjugation to glutathione (6) or the addition of water by epoxide hydrolases. Epoxide hydrolases (EC 3.3.2.3) constitute a family of enzymes comprising at least three major forms. The major forms have been characterized as distinct proteins on the basis of subcellular localization, molecular weight, optimum pH, substrate specificity, immunological properties, inhibition, and different response to known inducers (7). Purified microsomal epoxide hydrolase (mEH_b) has a molecular weight of about 50 kDa and hydrolyzes a wide variety of cyclic epoxides such as phenanthrene-9,10-oxide and benzo(a)pyrene-4,5-oxide (8) as well as mono- and cis-1,2-disubstituted oxiranes (9). Microsomal cholesterol epoxide hydrolase (mEH_{Ch}) is distinguished by its narrow substrate specificity for physiologically arising 5,6-epoxy-cholesterol and its derivatives (10).

In contrast, epoxides on cyclic systems are poor substrates for cytosolic epoxide hydrolase (cEH), which appears to be localized in peroxisomes as well as in the cytosol (11). This enzyme rapidly hydrolyzes aliphatic oxiranes, such as epoxides of cis- and trans-unsaturated fatty acids, including arachidonic acid, squalene oxides, and side chain epoxidized sterols (7). A diagnostic in vitro substrate for cEH is trans-stilbene oxide (TSO), which is neither metabolized by mEH_b, nor mEH_{Ch}. Conversely, benzo(a)pyrene-4,5-oxide and styrene oxide (STO) are used as selective substrates for mEH_b.

Most of our knowledge about cEH has been gained from studies on the purified enzymes from mouse and rabbit liver (12). These two species, together with the hamster, possess by far the highest TSO-hydrolyzing
activity of all investigated species (165–335 nmole/min × g liver and 106–129 nmole/min × g liver compared to human and rat liver with 15–22 nmole/min × g and 10–15 nmole/min × g, respectively) (13). The low specific cytosolic activity of rat and human liver cEH as well as the strong inducibility of the rat liver enzyme by hypolipidemic drugs with peroxisome proliferating activity suggested that these enzymes differ in properties and regulation and that rat liver cEH may actually be associated with peroxisomes and play a distinct role in the metabolic processes of this subcellular particle.

The different properties of cEH and mEHs, as well as the substantial differences among cEH activities in various species suggest that there are differences in detoxification capacities and protection of subcellular compartments from reactive epoxides. The largely unknown properties of human liver cEH make extrapolation of functional parameters and risk assessment with respect to environmental toxicants difficult. This investigation was therefore directed toward elucidating possible multiplicities of cEH in rats and humans, the properties of these isoenzymes, and their contribution to the cellular protection against reactive epoxides.

Purification of Rat and Human Liver cEH

Cytosolic epoxide hydrolase was purified to apparent homogeneity from fenofibrate-induced rat liver (14). Throughout the purification, enzyme activity was monitored with the substrates STO, CSO, TSO, and TESO, and there was no evidence from the elution profiles for activities other than STO, TSO, or TESO hydrolases, which coincided in all chromatograms. Also, the purification factors of 423, 541, and 485 for STO, TSO, and TESO hydrolase activity, respectively, were of the same order of magnitude. Additionally, cEH activity of untreated and induced animals expressed identical elution behavior in the corresponding chromatography steps. Further comparing the specific activity toward TSO of purified cEH from induced animals (173 nmole/min × mg) with 62 nmole/min × mg for the estimated 30% pure enzyme from controls indicated that the two enzyme forms were identical. There was no evidence for a multiplicity of cEH in rat liver at the protein level.

Investigation of human liver cEH from cytosol revealed that CSO, which is regarded a diagnostic substrate for mEHs, was hydrolyzed at the considerable rate of about 1.3 nmole/min × mg, which is slightly higher than the turnover by monkey (Macaca fascicularis) and more than 10 times higher than the hydration of CSO by rat liver cytosol (13). Isoelectric focusing confirmed the existence of two distinct human liver cytosolic epoxide hydrolases for the hydration of CSO (cEHCSO) and TSO (cEHTSO), and the specificities of both proteins were mutually exclusive. cEHTSO was very unstable and could only be partially purified by anion exchange chromatography and gel filtration (Fig. 1).

In contrast, cEHCSO was purified 85-fold to apparent homogeneity from 60 g of liver rom a 23-year-old male organ donor who had died in an accident (15) (Fig. 2).

**Figure 1.** Fractionation of human liver cytosolic epoxide hydrolase by anion-exchange chromatography. Cytosolic protein, 8 mg, was fractionated on a Mono Q column (FPLC) at a flow rate of 1 mL/min, and eluates were assayed for epoxide hydrolase activity toward CSO and TSO.
Biochemical Properties of Purified Rat and Human Liver cEH

Native rat liver cEH elutes as a protein of molecular weight 120 kDa during gel filtration on Sephadex G-150 and yields a single band after SDS-PAGE corresponding to a molecular weight of 60 kDa. This suggests a native dimer of two closely related, if not identical, subunits (14) (Fig. 2).

Similarly, gel filtration of human liver cytosol on Superose-12 revealed a native molecular weight of approximately 130 kDa for TSO-hydrolyzing activity, which is close to 140 kDa reported by Wang et al. (16) for purified human liver cEH,

Table 1. Effect of different modulators on the activity of human liver cytosolic epoxide hydrolase.

| Modulator     | Microsomal with | cEH<sub>Cso</sub> | cEH<sub>Tso</sub> with CSO* with TSO* |
|---------------|----------------|-------------------|-----------------------------------|
| TCPO          | 20            | 5                 | 9.5                               |
| Benzip        | 75            | 9                 | 91                                |
| 1-Benzylimidazole | 80        | 4                 | 60                                |
| Chalcone      | 107           | 4                 | 52                                |
| STO           | 54            | 1                 | 8                                 |

* Activities of cEH<sub>Cso</sub> and cEH<sub>Tso</sub> were determined after separation by anion exchange chromatography as described by Schiadt et al. (15). Incubations contained the individual compounds at a concentration of 1 mM.

for rat liver cEH (14) and human liver cEH<sub>Tso</sub>. A relationship of cEH<sub>Cso</sub> to the classical cytosolic epoxide hydrolases may be deduced from a similar response of cEH<sub>Cso</sub> to the inhibitors benzip, 1-benzylimidazole, and chalcone, whereas inhibition of cEH<sub>Cso</sub> by TCPO or STO would rather argue for a mEH<sub>b</sub>-like isoenzyme (Table 1).

Unlike purified mouse liver cEH, which readily hydrates CSO at a rate of 136 nmole/min × mg, approximately one-seventh the rate of TSO-hydrolysis (17), rat liver cEH does not metabolize CSO at a detectable level and hydrates TSO at a rate of 173 nmole/min × mg in the presence of 26 μM substrate. The corresponding apparent K<sub>m</sub> and V<sub>max</sub> values were determined from Lineweaver-Burk plots as 1.7 μM and 205 nmole/min × mg, respectively.

Purified human liver cEH<sub>Cso</sub>, on the other hand, converts CSO at a rate of 145 nmole/min × mg, like mouse liver cEH, but does not accept TSO as a substrate. Partially purified cEH<sub>Tso</sub> does not metabolize STO, in contrast to purified mouse (17) and rat liver cEH as well as human liver cEH<sub>Tso</sub> (16) and cEH<sub>Cso</sub>.

Immunological Properties of Rat and Human Liver cEH

With polyclonal antibodies against purified rat liver cEH, a single band was obtained after Western blotting of purified rat liver cEH and cytosol from control as well as clofibrate-induced animals. Strong induction of this enzyme by the hypolipidemic compound was clearly demonstrated (Fig. 3A). The antibody also recognized proteins with a molecular weight of about 60 kDa (corresponding to rat liver cEH) from liver cytosols of mouse, Syrian hamster, and New Zealand white rabbit (Figs. 4C,E,F). The reaction with guinea pig liver cytosol (Fig. 4D) was very weak, and there was no cross-reactivity with liver cytosol from green monkey (Fig. 4G). The two low molecular weight proteins in Syrian hamster cytosol that were distinguished by the antibody may be products of cEH-proteolysis.

Immunoprecipitation with antiserum against rat liver cEH demonstrated essentially 100% removal from rat liver and kidney cytosol of TSO hydrolyzing activity,
whereas human liver cEH_TSO was not precipitated at all, and precipitation of cEH_CSO did not exceed 20% of the initial activity even at the highest antiserum concentration (14,15). However, essentially all human liver cytosolic CSO hydrolase as well as STO hydrolase activity was precipitated with a polyclonal antibody against purified rat liver mEH_b, while the TSO-hydrolyzing activity remained unaffected.

Although the immunological relationship of rat liver mEH_b and cEH_CSO was confirmed by Western blotting, the small degree of similarity was underlined by the weak response of about one-tenth the signal intensity at 7.7 times the protein concentration (Fig. 3B).

From these immunological investigations one can conclude that rat, mouse, hamster, and rabbit liver as well as rat kidney TSO-hydrolase activities reside on structurally related proteins. On the other hand, human liver cEH_TSO and cEH_CSO as well as green monkey and guinea pig liver cytosolic TSO-hydrolase appear to be structurally completely different from rat liver cEH. A distant, but definite structural relationship of rat liver mEH_b with human liver cEH_CSO could be demonstrated.

**cDNA Cloning of Rat Liver cEH**

A cDNA expression library was constructed using purified poly-A mRNA from tiadenol-induced rat liver in the plasmid pEX1/E. coli POP 2136 system according to Haymerle et al. (18) and screened by a colony blot hybridization procedure with polyclonal antibodies against rat liver cEH (19). Twenty-four positive clones were confirmed upon rescreening, and the cDNA inserts of the four biggest clones, ranging between 0.8 and 1.2 kb, were verified by cross-hybridization using Southern and Northern blotting against three independent total RNA preparations from untreated, Aroclor 1254- and tiadenol-induced rat livers (Fig. 5).

In Northern blotting, all clones recognized the same mRNA, which is believed to be the coding mRNA for rat liver cEH. Additionally, clone 24 picked up a second mRNA species, somewhat smaller than the putative mRNA for cEH. This result may indicate for the first time the existence of a second isoenzyme of cEH in rat liver, which may be related to human liver cEH_CSO. However, this activity has not been allocated as yet to a distinct rat cytosolic protein, although minute CSO-hydrolyzing activities of about 0.1 nmole/min × g have been described in rat liver (13). Recently, a cytosolic hepoxilin epoxide hydrolase with molecular weight 53 kDa and marginal activity toward STO was isolated from rat liver (20) and may correspond to the newly identified mRNA.
MULTIPICLITY OF RAT AND HUMAN CYTOSOLIC EPOXIDE HYDROLASES

Figure 5. Hybridization of cDNA inserts from positive cEH clones with total rat liver RNA. Total RNA from control (lane A), Aroclor 1254-induced (lane B), and Thioacetamide-induced (lane C) rat liver was subjected in four identical sets (I-IV) to denaturing 0.7% agarose gel electrophoresis, transferred to nitrocellulose sheets and hybridized with [32P]-labeled cDNA probes from clones 7 + 14 (I); 14 + 15 (II); 14 + 24 (III); and 7, 14, 15, 24 (IV). Hybridization was visualized by autoradiography.

Table 2. Classification of cytosolic epoxide hydrolases from different species according to physical, biochemical, and immunological properties.

| Class | Species               | Activity toward* | Cross-reactivity with antiserum against* | Reference |
|-------|-----------------------|------------------|-----------------------------------------|-----------|
| I     | Mouse liver           | TSO 939-1500; CSO 136-180; STO 475-622 | MW 59000; pI 5.5 | cEH +; mEHb-; (17,23,24) |
|       | Rat liver             | 173              | 1567; 61000; 5.5 | +; -; (14) |
|       | Rabbit liver          | 0.69-0.93 9; 0.11-0.15 4 | 1580; 57000; 6.0 | +; -; (25)* |
|       | Hamster liver         | 2.10-2.20 9; 0.11-0.15 4 | NM; 57000; - | +; -; (19)* |
| II    | Guinea pig liver      | 0.87-0.99 9; 0.15-0.21 4 | NM; 57000; 7.4 | -; -; (13)* |
|       | Monkey liver          | 0.36-0.44 9; 0.94-0.96 4 | NM; 58000; NM | -; -; (19)* |
|       | Human liver cEH TSO   | 1.02 9; 0.32     | ND; NM | -; -; (15) |
|       | Human liver cEH FSO   | 150              | NM; 58000; 5.1-6.1 | -; -; (15) |
| III   | Human liver cEH CSO   | ND               | 145; 49000; 9.2 | -; +; (15) |
|       | Human liver cEH FNSO  | 14               | NM; 50000; - | -; +; (16) |

*Activities are given in nmole/min/mg.
*(+) Indicates weak but clearly detectable cross-reactivity; (-) indicates very faint reactivity.
*ND, not detectable; NM, not measured.
*(+) Activities were determined from cytosol.
(See also Figure 4.)*
*(+) Activity after anion exchange chromatography of cytosol.

Conclusions

Two forms of human liver cEH, cEH CSO, and cEH TSO have been identified, and cEH CSO as well as rat liver cEH have been purified to apparent homogeneity. An attempt has been made to assign these enzyme forms to three classes (Table 2) according to their physical, biochemical, and immunological properties, taking into account the known properties of cEH from other species.

Class I cEH isoenzymes are characterized by immunological cross-reactivity with either cEH-antiserum against any member of this group and diagnostic substrate specificity toward TSO, STO, and with some limitations, towards CSO. Mouse, rat, rabbit, and hamster liver cEH meet these requirements. Class II comprises
Table 3. Metabolism of methylated styrene oxides by rat and human liver cytosol and microsomes.*

| Substrate                | Microsomes | Cytosol | Microsomes/cytosol |
|--------------------------|------------|---------|--------------------|
|                          | Rat        | Human   | Rat                | Human   |
| Styrene oxide            | 4.300      | 40.7    | 0.300              | 5.600   |
| trans-2-Methyl styrene oxide | NM*       | 86500.0 | NM                 | 2100.0  |
| cis-2-Methylstyrene oxide | 0.260     | 5.4     | 0.010              | 0.664   |
| trans-1,2-Dimethyl styrene oxide | ND | 0.238 | ND                     | ND           |
| cis-1,2-Dimethyl styrene oxide | 0.08      | 0.665   | ND                 | 0.034   |
| 2,2-Dimethyl styrene oxide | 0.007    | 0.130   | 0.001              | 0.122   |

* Activities were determined at 0.5 mM substrate concentration as described by Milbert et al. (26) and are given in nmole/min/mg.

** NM, not measured; ND, not detectable.

† Ratio cannot be formed because one of the values is zero.

guinea pig, monkey, and human liver cEH_TSO as well as human cEH_TSO, which have diagnostic substrate specificity for TSO and low turnover rates with CSO (13). No cross-reactivity is observed among members of this class with antiserum against cEH or mEH from either species of group I. Class III isoenzymes, which are represented by human liver cEH_CSO and cEH_TNO, have lower molecular weights than the TSO-hydrolyses of classes I and II and express an immunological relationship with antibodies against mEH of either source.

From this classification it appears that the structural and functional multiplicity of cEH increases with phylogenetic differentiation. The possible impact of this cEH differentiation on the cellular protection against reactive epoxides was investigated with differentially methylated styrene oxides and microsomes or cytosol as enzyme source (Table 3). Human enzymes of either source metabolized all model substrates more efficiently than the corresponding rat liver enzymes. The ratio of microsomal/cytosolic hydration for a certain substrate is clearly shifted toward two to seven times lower values in humans. This finding can be explained by the presence of cEH_CSO, which provides mEH-like detoxification capacities particularly for cis-substituted epoxides such as cis-2-methyl styrene oxide, cis-1,2-dimethylstylene oxide, and 2,2-dimethyl styrene oxide.

In conclusion, a multiplicity of human as well as rat liver cEH could be demonstrated at the enzyme level for the human, and at the mRNA level for rat liver cEH. However, due to human liver cEH_CSO, this multiplicity appears much more effective in humans than in rats for the protection of different subcellular compartments against reactive epoxides, and human liver seems to be far more efficient in detoxification of reactive epoxides by epoxide hydrolase. Rat liver may therefore not be an appropriate model for epoxide hydrolase-related risk assessment in humans.

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