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THE REGULATION OF CYTOSOLIC EPOXIDE HYDROLASE IN MICE*

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Summary: The concentration of cytosolic epoxide hydrolase in untreated and clofibrate-treated mouse liver extracts was estimated by immunoblotting. Clofibrate treatment of mice was found to increase liver cytosolic epoxide hydrolase concentration by two fold, showing that the increase in cytosolic epoxide hydrolase in mouse liver after clofibrate treatment is primarily due to induction. The induced and uninduced cytosolic epoxide hydrolase, and epoxide hydrolase in the cytosolic and mitochondrial fractions were compared and found to be identical or very similar. Cytosolic epoxide hydrolases in kidney and liver were similar in molecular weight and antigenic properties.

The oxidative metabolism of olefinic compounds generates epoxides which can then be hydrolyzed to vicinal diols by epoxide hydrolases (EHs, EC 3.3.2.3) (1). Because of their ability to hydrolyze potentially mutagenic and toxic epoxides the epoxide hydrolases may serve an important protective function in mammals (2). Liver EH activity, although concentrated mainly in the microsomal (mEH) and cytosolic (cEH) fractions, is also found in mitochondria, peroxisomes, plasma membranes, Golgi apparatus and nuclear membranes (3-6). The mEH and cEH are distinct enzymes (3,7-10). However, the mEH is similar to the EH in plasma membranes, Golgi apparatus and nuclear membranes (11), and the cEH is apparently similar to EH in the mitochondrial fraction and peroxisomes (mtEH) (5,12,13).

The mEH and cEH are differentially induced by phenobarbital and clofibrate, respectively (14,15). The increase in liver mEH activity by phenobarbital treatment is due to increased mEH concentration resulting from

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increased levels of translatable mEH mRNA (15). However, it is not known whether the increase in cEH activity upon clofibrate treatment is due to an increase in cEH concentration or an increase in cEH specific activity, although it has been suggested from cEH purification studies that the increased activity is from both increased cEH concentration and increased specific activity (9,10). Also it is not known if the cEH in different tissues (3) are structurally related to liver cEH.

In this paper we have estimated cEH concentrations in control and clofibrate-treated mouse liver by immunoblotting, compared mtEH with liver cEH. Similarly we have compared some properties of kidney EH with liver EH.

MATERIALS AND METHODS

Materials. Swiss-Webster mice were from colonies maintained at the Univ. California, Riverside. Goat-anti-rabbit-Ig-alkaline phosphatase was from Miles Lab., while Goat-anti-rabbit-^{125}I-IgG was from New England Nuclear. All other chemicals were the best grade commercially available.

Preparation of enzyme fractions and antibodies. Mice were treated with clofibrate (14), and mitochondrial, microsomal and cytosolic fractions were prepared as described (13). The purification of cEH and preparation of antisera to the purified cEH was performed as described earlier (8,12). Protein concentration was estimated according to Bradford (16).

Gel electrophoresis and electroblotting. Proteins were separated on 10% acrylamide gels using discontinous buffer system (17). The separated proteins were electrophoretically tranferred to nitrocellulose sheets overnight in blotting buffer (15.6 mM Tris-120 mM glycine, pH 8.3 containing 20% methanol) (18).

Epoxide hydrolase detection and estimation. cEH activity was assayed by the hydrolysis of trans-stilbene oxide (TSO) as described (19). The visualization and estimation of concentration of epoxide hydrolase bands on nitrocellulose sheets was performed by minor modifications of published methods (20,21). Briefly, the remaining binding sites on nitrocellulose sheet with electroblotted proteins were blocked with 0.5% Tween in phosphate buffer saline (PBS) and then the nitrocellulose sheet was incubated with cEH rabbit antiserum diluted (1:1000) in PBS containing 0.05% Tween 20 (PBST). Subsequently the nitrocellulose sheet was washed with PBST and then incubated for 2 hr with either goat-anti-rabbit-^{125}I-IgG (0.03 µg/ml) or goat-anti-rabbit-IgG-alkaline phosphatase diluted (1:1000) in PBST. When goat-anti-rabbit-^{125}I-IgG was used, the nitrocellulose sheet was dried and subjected to autoradiography. Appropriate areas from the nitrocellulose sheet were then cut and counted in a gamma counter. Backgrounds were determined using identically sized slices of nitrocellulose sheet that were obtained from a portion of sheet where no darkening of the corresponding autoradiographic film was seen. When goat-anti-rabbit-IgG-alkaline phosphatase was used to detect cEH antibody, the nitrocellulose sheet was stained for alkaline phosphatase by incubating in a staining solution containing 5-bromo-4-chloroindoxyl phosphate and nitrotetrazolium blue until the bands were visualized (5-10 min). The intensity of the bands was estimated by reflectance scanning.

RESULTS AND DISCUSSION

Different amounts of pure cEH and cytosolic proteins from livers of clofibrate-treated and untreated mice were subjected to electrophoresis, electroblotted to nitrocellulose, and cEH detected by autoradiography. The cEH concentration was observed to give a linear relationship with ^{125}I counts (Fig. 1). In untreated mice the level of cEH was 0.42% of cytosolic protein, while in clofibrate-treated mice cEH concentration was 0.87%. The corresponding levels of cEH activity in these cytosolic fractions, as detected by the hydrolysis of TSO, was 6.0 and 12.2 nmol/min/mg protein, respectively. Based on the specific activity of purified cEH (8), the concentration of cEH observed in cytosol accounts for all of the TSO hydrolase activity detected in the untreated cytosol. This estimate of cEH in crude cytosol is also very close to that estimated by purification of cEH (8).

The data presented here shows that increased cEH activity is due to an increase in cEH concentration rather than an increase in its specific activity. Thus clofibrate treatment does not appear to increase cEH activity as a result of increased specific activity resulting from change(s) in structure of the enzyme as proposed (9). However, the possibility of minor changes in

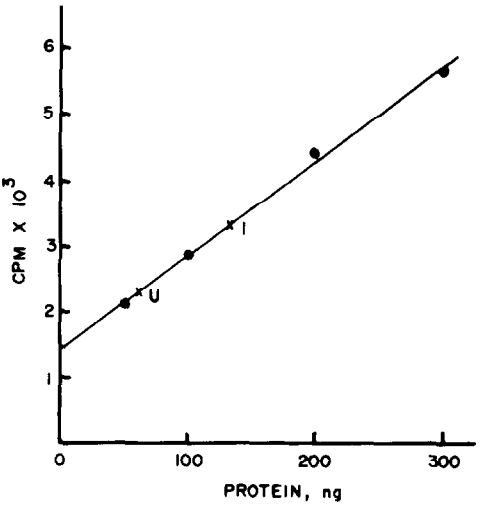

Fig. 1. Estimation of cEH in mouse liver cytosol by immunoblotting. Purified cEH (50, 100, 200 and 300ng) and cytosolic proteins (15µg) from untreated (U) and clofibrate-treated (I) mice livers were resolved in 10% SDS-PAGE, and electroblotted onto a nitrocellulose sheet. The visualization and estimation of cEH concentration was as described under Materials and Methods.

amino acid composition and sequence, and specific activity cannot be totally excluded, and must await further characterization of the protein. Whether this cEH increase by clofibrate is due to an increase in enzyme synthesis or decreased enzyme degradation is not known. However, as cEH is a stable enzyme and little degradation is observed in crude cytosol upon prolonged storage (Gill et al., unpublished), most of the increase in cEH concentration is probably due to an increase in protein synthesis. It is also likely that the increase levels of cEH activity after clofibrate treatment are possibly due to increased levels of cEH mRNA. If this occurs the induction of cEH would be similar to the induction of mEH activity by phenobarbital, which occurs due to an increase in mEH concentration and translatable mEH mRNA (15). The mechanism of induction of cEH by clofibrate and other peroxisomal profilerators is not known. Whether this induction is under the control of a cytosolic receptor (22) requires further investigation.

Pure cEH and cytosolic, microsomal and mitochondrial proteins from clofibrate treated and untreated mice were separated on 10% SDS polyacrylamide gel, electroblotted to nitrocellulose, and the cEH detected by autoradiography using cEH antiserum and goat-anti-rabbit-^{125}I-IgG (Fig 2). The mitochondrial fraction was observed to contain a protein whose molecular weight and antigenic properties are similar to cEH. A minor band corresponding to cEH is also seen in the microsomal fraction. Detection of a number of bands in the micro-

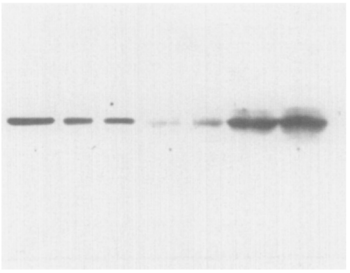

Fig. 2. Comparison of cEH in different subcellular fractions of mouse liver. Purified cEH (lane 1), and cytosolic (lanes 2 and 3), microsomal (lanes 4 and 5) and mitochondrial (lanes 6 and 7) fractions were analyzed as described under Materials and Methods. Lanes 2, 4 and 6 are from untreated mouse liver and 3, 5 and 7 from clofibrate-treated mouse liver.

somal and mitochondrial fraction, but not in the cytosolic fraction, could potentially be due either to minor impurities of these mitochondrial and microsomal proteins in purified cEH used for obtaining antiserum or common antigenic determinants in cEH and some mitochondrial and microsomal proteins. Similar results were obtained using goat-anti-rabbit-Ig-alkaline phosphatase detection of cEH (data not shown).

The mouse liver cEH and mtEH appear to be identical. They have similar or identical molecular weight, antigenic properties, isoelectric points and substrate specificities (12,13). However, the EH in the cytosolic and mitochondrial fractions appear to be differentially regulated as the relative level of distribution of EH in these two fractions varies with different mouse strains (Kaur and Gill, unpublished). If mtEH in the mitochondrial fraction is synthesized in the cytosol and transported to the mitochondria and peroxisomes, the mtEH apparently does not undergo permanent chemical modification (e.g. glycosylation) during transport to these organelles. Additionally, although not detected by the methods used above, it is possible that minor changes in the amino acid sequence, could provide signal sequences for transport. We also could not detect a precursor form for the EH in the mitochondrial fraction in the cytosol. This, however, does not exclude synthesis of pre-mtEH or the co-translational transport of mtEH.

Mouse liver and kidney extracts and different subcellular fractions were analyzed for the presence of protein cross reacting with cEH antiserum (Fig. 3). Kidney extracts, cytosol and mitochondrial fractions contained a protein

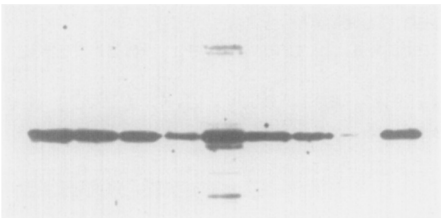

Fig. 3. Epoxide hydrolases in mouse clofibrate kidney and liver. Purified cEH (lane 1), crude liver homogenate (lane 2), liver cytosol (lane 3), liver microsomes (lane 4), liver mitochondria (lane 5), crude kidney homogenate (lane 6), kidney cytosol (lane 7), kidney microsomes (lane 8) and kidney mitochondria (lane 9) were analyzed as described under Materials and Methods.

which cross reacted with liver cEH antiserum and has similar or identical molecular weight. These results show that cEH in liver and kidney are similar proteins and that kidney cEH and mtEH are similar to the liver proteins in molecular weight and antigenic properties.

Under all the conditions used here and elsewhere (12,13) there has been no evidence for any structural polymorphism of cEH. This apparent lack of structural polymorphism further suggests that changes in cEH upon clofibrate treatment are due primarily to increased cEH levels rather than increase in specific activity of cEH, although the latter possiblility cannot be excluded.

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