Epoxide hydrolase in human and rat peroxisomes: implication for disorders of peroxisomal biogenesis

Kalipada Pahan, Brian T. Smith, and Inderjit Singh[1]

Department of Pediatrics, Medical University of South Carolina, Charleston, SC 29425

Abstract To understand the basis of excretion of excessive amounts of epoxydicarboxylic fatty acids (EDFA) in urine of patients with disorders of peroxisomal biogenesis (Pitt, J. J., and A. Poulos. 1993. *Clin. Chim. Acta.* **223:** 23–29), the activity of epoxide hydrolase (EH) was measured in cultured skin fibroblasts from control subjects and patients with peroxisomal disorders. EH activity was approximately 40% lower in fibroblasts that lack intact peroxisomes (Zellweger syndrome), whereas the activity in other peroxisomal disorders (X-adrenoleukodystrophy and rhizomelic chondrodysplasia punctata) with intact peroxisomes was similar to control. To identify the specific enzyme/organelle that represents the decrease in EH activity in Zellweger cells, we have analyzed this activity in different subcellular organelles from control and Zellweger skin fibroblasts. EH activity was enriched in peroxisomes from control fibroblast. EH activity in isolated mitochondria, microsomes, or cytosol from Zellweger fibroblast was similar to that of control fibroblast. These observations indicate that deficient activity of EH in cells from Zellweger patients is due to lack of peroxisomal EH activity. The peroxisomal EH is differentially induced to a higher degree by ciprofibrate, a hypolipidemic agent and peroxisome proliferator, than EH activity in other organelles and cytoplasm. The high specific activity of EH in peroxisomes and differential induction of EH activity in peroxisomes as compared to other organelles, and the excretion of EDFA in patients who lack peroxisomes suggests that peroxisomal EH may be responsible for the detoxification of EDFA, and that this enzyme in peroxisomes may be a different protein than the EH found in other organelles.–**Pahan, K., B. T. Smith, and I. Singh.** Epoxide hydrolase in human and rat peroxisomes: implication for disorders of peroxisomal biogenesis. *J. Lipid Res.* 1996. **37:** 159–167.

Supplementary key words human and rat liver • ciprofibrate • Zellweger syndrome

Peroxisomal disorders are a class of inherited metabolic and neurological diseases that are classified as *a)* a generalized dysfunction of peroxisome biogenesis as in Zellweger syndrome, infantile Refsum's disease, and neonatal adrenoleukodystrophy; *b)* a deficiency of a number of peroxisomal functions with intact peroxisomes as in RCDP (rhizomelic chondrodysplasia punctata); or *c)* disorders of single enzyme deficiency with intact peroxisomes (e.g., adult Refsum's disease, X-adrenoleukodystrophy) (1). These disorders are generally diagnosed on the basis of clinical features and assessment of a number of peroxisomal functions (1, 2). Zellweger syndrome, with its multiple and fatal abnormalities is considered as a dramatic example of the loss of peroxisomal function in humans. Although failure to form peroxisomal membranes would be a possible explanation for the apparent absence of this organelle, several studies have demonstrated the presence of different membrane proteins in Zellweger disease patients in the form of "ghosts" that lack the majority of matrix proteins (1–3). Recently, it has been reported that the elevated urinary excretion of epoxydicarboxylic fatty acids (EDFA) in patients with disorders of peroxisome biogenesis may be useful for the diagnosis of disorders of peroxisome biogenesis (4); however, the enzyme responsible for the normal metabolism of EDFA and the basis of excessive excretion of EDFA is not known. The lack of detoxification of EDFA in patients who lack peroxisomes suggests that this might be a peroxisomal function. The peroxisomes are now known to be involved in several vital metabolic processes including oxidation of fatty acids (e.g., unsaturated, very long chain, and branched chain) and synthesis of cholesterol, bile acids, and plasmalogens (1). Identification of epoxide metabolism in peroxisomes describes another important function for peroxisomes.

Epoxide hydrolases (EH) are a group of enzymes that catalyze the conversion of epoxides to less toxic and readily excretable dihydrodiols. They have been found in tissues of all mammalian species tested, with the highest levels being found in liver and kidney (5). Peroxisomes play a significant role in the metabolism of reactive oxygen species with the consumption of

Abbreviations: EH, epoxide hydrolase; EDFA, epoxydicarboxylic fatty acids; TSO, *trans*-stilbene oxide.

[1]To whom correspondence should be addressed.

10–30% of the cellular oxygen (6) and contain H_2O_2 (various oxidases) and O_2^- (e.g., xanthine oxidase, cytochrome P450)-producing enzymes (7, 8). The oxidative stress observed in peroxisomes in ischemia-reperfusion (9) and endotoxemia (10) suggests that epoxide formation may be associated with dysfunction of peroxisomes. Reactive oxygen species produced in peroxisomes or in other organelles during normal or stress conditions may biotransform polyunsaturated fatty acids, prostaglandins, cholesterol, or xenobiotics to reactive epoxides.

To understand the biochemical basis of the excretion of excessive amounts of EDFA in peroxisomal biogenesis disorders, we have examined the subcellular distribution of EH in cultured skin fibroblasts from control and Zellweger patients (a disorder of biogenesis of peroxisomes) and in livers from humans and rats. By using *trans*-stilbene oxide (TSO) as a substrate for EH we show that epoxide hydrolase is enriched in peroxisomes, and the absence of peroxisomal epoxide hydrolase leads to a decrease in total cellular activity in Zellweger fibroblast. The EH activities in other subcellular organelles from Zellweger cells were similar to control, suggesting that excessive excretion of EDFA in Zellweger patients is due to the specific loss of peroxisomal EH activity. In rat liver, ciprofibrate, a peroxisome proliferator used as a hypolipidemic agent, differentially induced this activity in various subcellular organelles with the highest induction being observed in peroxisomes. This observation suggests that EH in peroxisomes may be a different protein than EH found in other cellular compartments.

MATERIALS AND METHODS

Materials

Nycodenz was obtained from Accurate Chemical and Scientific Corp., Westbury, NY. Cytochrome c, n-dodecane, and digitonin were purchased from Sigma Chemical Co., St. Louis, MO. [^{3}H]*trans*-stilbene oxide was purchased from American Radiolabeled Chemicals, Inc., St. Louis, MO. Fetal calf serum, trypsin, and tissue culture media were from GIBCO. Ciprofibrate was a gift from Dr. Albert Soria (Sterling-Winthrop Research Institute, Rensselaer, NY).

Ciprofibrate treatment

Male Sprague-Dawley rats (weighing 250–300 g) received ciprofibrate (0.025% by weight) supplemented with standard pellet diet (Wayne Rodent, Box 8604, Madison, WI) for 2 weeks.

Subcellular fractionation of cultured control and Zellweger skin fibroblasts

Skin fibroblasts were grown to confluency in 75-cm^2 dishes, and 20 or more confluent flasks were harvested by mild trypsinization and incubated for 1 h as a suspension in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum at 37°C. After centrifugation, cell pellets were washed with homogenizing buffer (0.25 M sucrose, 1 mM EDTA, 1 μg/ml antipain, 0.7 μg/ml leupeptin, 0.2 mM phenylmethylsulfonylfluoride, 0.1% ethanol, and 3 mM imidazole buffer, pH 7.4) and subfractionated by differential and isopycnic density gradient using Nycodenz as described previously (11). Subcellular fractions containing different organelles were identified by the following marker enzymes: catalase for peroxisomes (12), cytochrome c oxidase for mitochondria (13), and NADH-cytochrome c reductase for microsomes (14). Protein concentrations were measured by the procedures of Bradford (15).

Subcellular fractionation and isolation of peroxisomes from human and rat liver

Peroxisomes from rat and human liver were isolated according to the procedures described previously (16). Briefly, liver homogenates were subjected to differential centrifugation to obtain heavy mitochondrial, light mitochondrial (the "lambda" fraction), microsomal, and cytosolic fractions as described by Leighton et al. (17). Peroxisomes from the lambda fraction were prepared by isopycnic equilibrium centrifugation in a gradient consisting of 30 ml of 0–50% (w/v) Nycodenz with 4 ml of 55% (w/v) Nycodenz as a cushion in 39-ml tubes as described previously (16).

The peroxisomes from ciprofibrate-treated liver are relatively fragile, therefore caution should be exercised during experimental manipulations as the disrupted peroxisomes lose a major part of the matrix content and do not move in the gradient to the proper density.

Separation of membrane and matrix of peroxisomes

Peroxisomes were incubated with digitonin (0.5 mg/ml) for 1 h at 4°C and then centrifuged at 50,000 rpm for 1 h in a Beckman 70 Ti rotor. Separation of membrane (residue) and matrix (soluble) was confirmed by measuring catalase activity. Catalase activity was present only in the matrix and not in the membrane.

Assay of epoxide hydrolase

EH activity was measured according to the method of Gill, Ota, and Hammock (18). Briefly, [^{3}H]*trans*-stilbene oxide suspended in ethanol (2 μl) was added to the enzyme assay medium to maintain the final concentration of 50 μM. The reaction was started by the addition of 5–50 μg of protein to a total reaction volume of 100

µl of 100 mM sodium phosphate buffer, pH 7.4. After incubation at 37°C for 10 min, the reactions were terminated by extracting the incubation mixture by rapid vortexing for 15–20 sec with 200 µl of n-dodecane. Reaction tubes were centrifuged and the aqueous layers were washed twice with n-dodecane. The amount of radioactivity in the aqueous phase is an index of ^{3}H-labeled diols hydrolyzed from TSO.

RESULTS

Epoxide hydrolase activity in cultured skin fibroblasts of control and patients with peroxisomal disorders

To understand the significance of an increased excretion EDFA, we examined the EH activity with TSO as the substrate as described in the Methods section in cell suspension of cultured skin fibroblasts of control subjects and patients with different peroxisomal disorders. This activity was significantly lower in Zellweger fibroblasts (approximately 60%) as compared to the control (**Table 1**). However, in peroxisomal disorders with normal cytochemical (catalase-containing) peroxisomes but with deficiency in single (X-adrenoleukodystrophy and Refsum disease) or multiple peroxisomal enzymes (rhizomelic chondrodysplasia punctata), epoxide hydrolase activity was found to be normal. This indicates that the decrease in epoxide hydrolase activity correlates only with the absence of peroxisomes. To further delineate this apparent enzymatic deficiency, we decided to study this activity in different subcellular organelles isolated from control and Zellweger fibroblasts.

Subcellular distribution of epoxide hydrolase in control and Zellweger fibroblasts

Subcellular organelles from control and Zellweger skin fibroblasts were prepared in Nycodenz gradients by a procedure described previously in our laboratory (11). The distribution of marker enzymes for different organelles and the activity of epoxide hydrolase are shown in **Fig. 1.** In control fibroblast, epoxide hydrolase activity was observed in peroxisomes, mitochondria, microsomes, and cytoplasm. The EH activity in peroxisomes was 10- to 20-fold higher than other organelles (**Table 2**) demonstrating that this enzyme is enriched in peroxisomes. In a gradient from Zellweger cells, the EH activity observed in mitochondria, microsomes, and cytoplasm was similar to that observed in these organelles from control cells. Consistent with the absence of peroxisomes in Zellweger cells, no activity was found in the peroxisomal region of the gradient from Zellweger cells. The high specific activity of EH in peroxisomes of control skin fibroblast as compared to other organelles indicates the importance of peroxisomal EH in detoxifying epoxide derivatives.

Subcellular localization of epoxide hydrolase activity in human and rat liver

As the majority of the metabolism of epoxide derivatives takes place in liver, we examined the distribution of EH in different subcellular compartments of human and rat liver. The distribution pattern of marker enzymes for different organelles (catalase for peroxisomes, cytochrome c oxidase for mitochondria, NADH cytochrome c reductase for microsomes) shows that these organelles were well resolved from each other in the gradient (**Fig. 2**). The activity of EH in both human and rat liver paralleled that of the peroxisomal marker, catalase. Striking differences were observed when the EH activity was compared with that of NADH cytochrome c reductase or cytochrome c oxidase, again suggesting that this EH activity is enriched in peroxisomes. The specific activities of EH in different subcellular organelles are summarized in Table 2. The specific activity in peroxisomes from human and rat liver ranged from 9 to 10 times higher than that in mitochondria and microsomes and from 3 to 4 times higher than that in cytosol. Consistent with the function of liver in the metabolism of epoxides, the liver EH had higher specific activity than cultured skin fibroblasts. The mitochondrial fractions from human liver had higher EH activity as compared to rat liver mitochondrial fractions, probably due to a high degree of contamination by peroxisomes as judged from the distribution pattern of catalase (Fig. 2). These human livers were procured for the purpose of transplantation by the liver transplant service but were not used for this purpose for various reasons. In general, these livers became available for these studies between 10 and 20 h of cold ischemia. We have previously observed that peroxisomes from tissues exposed to increasing periods of ischemia equilibrate in the lighter part of the gradient (mitochondrial regions)

TABLE 1. Epoxide hydrolase activity in cultured skin fibroblasts of control and diseases with peroxisomal disorders

Cell lines	Epoxide Hydrolase
	nmol/min/mg protein
Control-I	6.46 ± 1.32
Control-II	6.15 ± 1.56
X-ALD	5.42 ± 1.68
Refsum	6.52 ± 0.92
RCDP	5.15 ± 1.12
Zellweger-I	3.42 ± 0.65
Zellweger-II	3.76 ± 1.02

Epoxide hydrolase activity was measured using [H^3]TSO as substrate in cultured skin fibroblasts of control, X-linked adrenoleukodystrophy (X-ALD), Refsum, rhizomelic chondrodysplasia punctata (RCDP) and Zellweger suspended in Hank's balanced salt solution (HBSS). The results are expressed as mean ± SD of three different experiments.

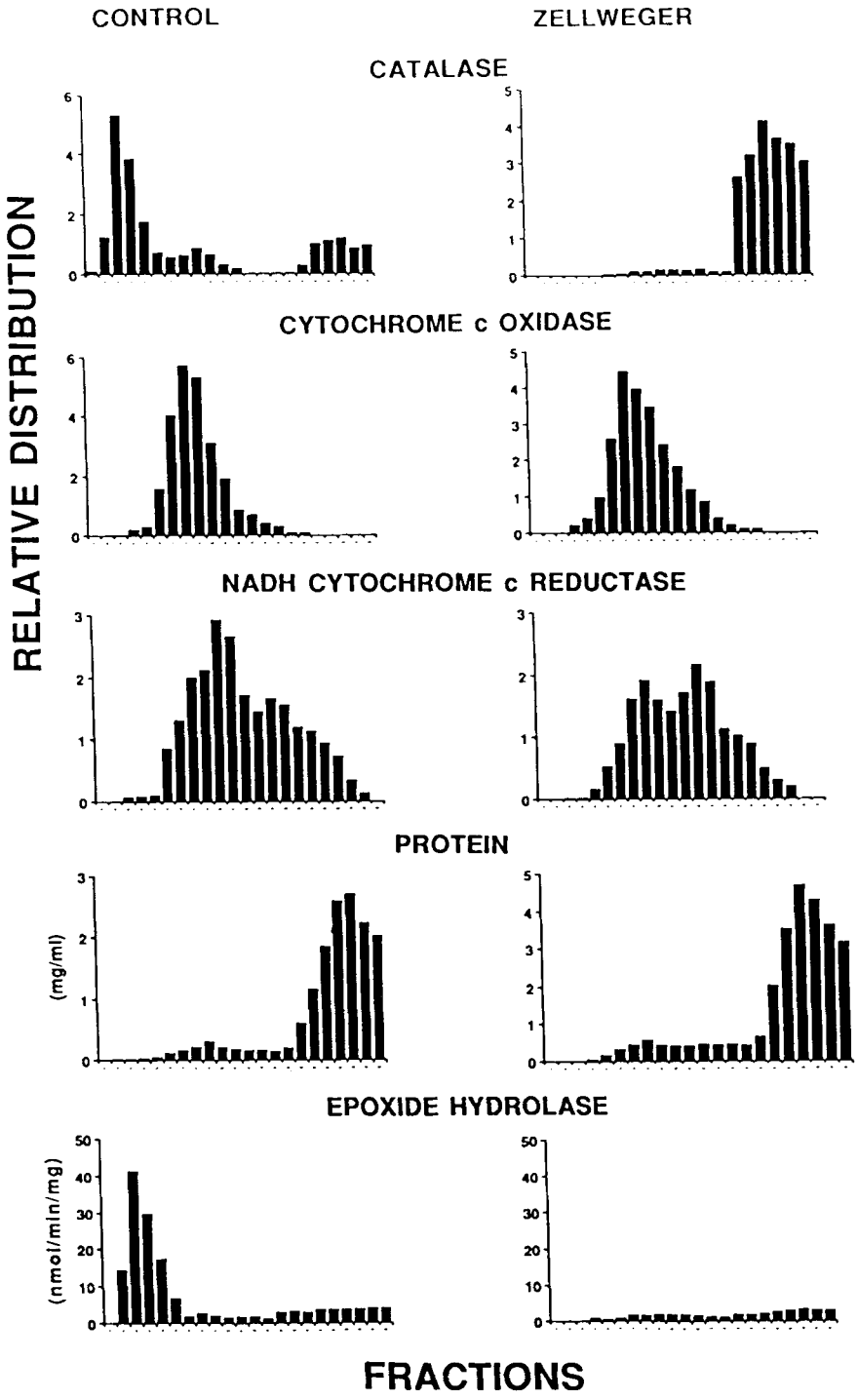

Fig. 1. Subcellular distribution of epoxide hydrolase in control and Zellweger fibroblasts. Control and Zellweger fibroblasts were fractionated by differential density gradient centrifugation as described in the text. The distribution of subcellular organelles in the gradients was identified by their marker enzymes: catalase for peroxisomes, cytochrome c oxidase for mitochondria, and NADH cytochrome c reductase for the endoplasmic reticulum. The activity of epoxide hydrolase in the gradient fractions was measured as described in the text. The gradient profiles of each tissue are average of two gradients.

TABLE 2. Specific activities of epoxide hydrolase in different subcellular organelles of skin fibroblasts and liver tissues

	Epoxide Hydrolase				
	Homogenate	Mitochondria	Microsomes	Peroxisomes	Cytosol
			nmol/min/mg protein		
Human liver	53.4 ± 11.2	41.5 ± 6.1	31.7 ± 3.0	319.0 ± 55.2	80.5 ± 14.3
Rat liver	50.1 ± 7.5	29.0 ± 5.3	30.2 ± 4.0	317.0 ± 20.7	75.8 ± 25.0
Human fibroblast	3.22 ± 0.45	2.45 ± 0.3	1.87 ± 0.12	37.5 ± 5.2	3.62 ± 0.4
Zellweger fibroblast;	1.92 ± 0.25	1.69 ± 0.3	1.79 ± 0.2	0	2.96 ± 0.35

The enzyme activities were measured as described in the text. The results obtained from three different experiments are expressed as mean ± SD.

in increasing amounts (9). Similarly, large amounts of peroxisomes (catalase) were shifted in the lighter part (mitochondrial regions) of these human gradients.

Intraperoxisomal localization of epoxide hydrolase and effect of ciprofibrate treatment on rat liver epoxide hydrolase

Peroxisomes are made of a granular matrix surrounded by a single limiting membrane. The matrix proteins are released as soon as the limiting membrane is disrupted. To understand the intraperoxisomal organization of EH, the activity for TSO-hydrolysis was studied in the matrix and membrane isolated after digitonin treatment of the purified peroxisomes. The membrane and matrix from digitonin-permealized peroxisomes were separated by centrifugation as described under Materials and Methods. The activity for the hydrolysis of TSO by EH was observed mainly in the peroxisomal matrix, but not in the membrane proteins (**Table 3**).

Ciprofibrate and clofibrate, hypolipidemic drugs known to cause proliferation of peroxisomes and increase in the peroxisomal enzyme activities in rats and mice, were also found to cause substantial increase in the EH activity in cellular homogenates (19–21). We examined the effect of ciprofibrate on EH activity observed in subcellular compartments. Livers from control and ciprofibrate-treated rat livers were fractionated by differential and isopycnic gradient centrifugation as described under Materials and Methods. In our studies, ciprofibrate induced EH activity by 9.5-fold in peroxisomes, 6.0-fold in cytosol, 3.0-fold in mitochondria, and 3.5-fold in microsomes (**Fig. 3**). Second highest induction to peroxisomes was observed in cytoplasm. The peroxisomes from ciprofibrate-treated liver are relatively more fragile as compared to peroxisomes from control liver; therefore, part of the activity observed in cytoplasm of ciprofibrate-treated liver may be the contribution of peroxisomal EH released from disruption of peroxisomes during experimental manipulations (e.g., homogenization, centrifugal force during centrifugation). Nevertheless, the differential induction of EH activity in different cellular compartments/organelles indicates that the same enzyme protein may not be responsible for the activity in these cellular compartments.

DISCUSSION

The studies described here clearly demonstrate that Zellweger cells have lower EH activity as compared to control, and this deficiency is due to absence of peroxisomes and peroxisomal EH activity. Epoxides are a group of highly reactive molecules of both exogenous and endogenous origin. Some of the most potent carcinogens and mutagens known become active only when transformed to their epoxides (22). Being highly electrophilic, they are able to react easily with nucleophilic groups such as lipids containing double bonds (e.g., unsaturated fatty acids), DNA, RNA, and proteins. A number of xenobiotics, including many clinical drugs, are metabolized to their epoxides by cytochrome P450-dependent monooxygenases (23). Human diets also sometimes contain epoxides or their diene precursors such as aflatoxins. Furthermore, a number of epoxides such as epoxides of prostaglandins, leukotrienes, arachidonic acid, cholesterol, and unsaturated fatty acid are formed biosynthetically (24). To our knowledge, EH is currently the only known enzyme that catalyzes the conversion of epoxides to less toxic, more polar, and readily excretable dihydrodiols.

Earlier investigations of subcellular localization of EH activity by differential centrifugation and immunocytochemical techniques have resulted in conflicting conclusions. The same TSO-hydrolase activity has been reported to be present in heavy and light mitochondrial fractions (25, 26), in cytosol (27), in microsomes (28), and in peroxisomes (29). Moreover, it was suggested that the peroxisomal and cytoplasmic activities are the contribution of the same enzyme (26, 30). The excretion of excessive amounts of EDFA (4) cannot be explained by the observed partial deficiency of EH activity in skin fibroblasts of Zellweger syndrome, a peroxisomal biogenesis disorder, if the same EH is present in various subcellular compartments. Our studies on subcellular distribution of EH activity in both fibroblast and liver samples provide strong evidence that EH is enriched in

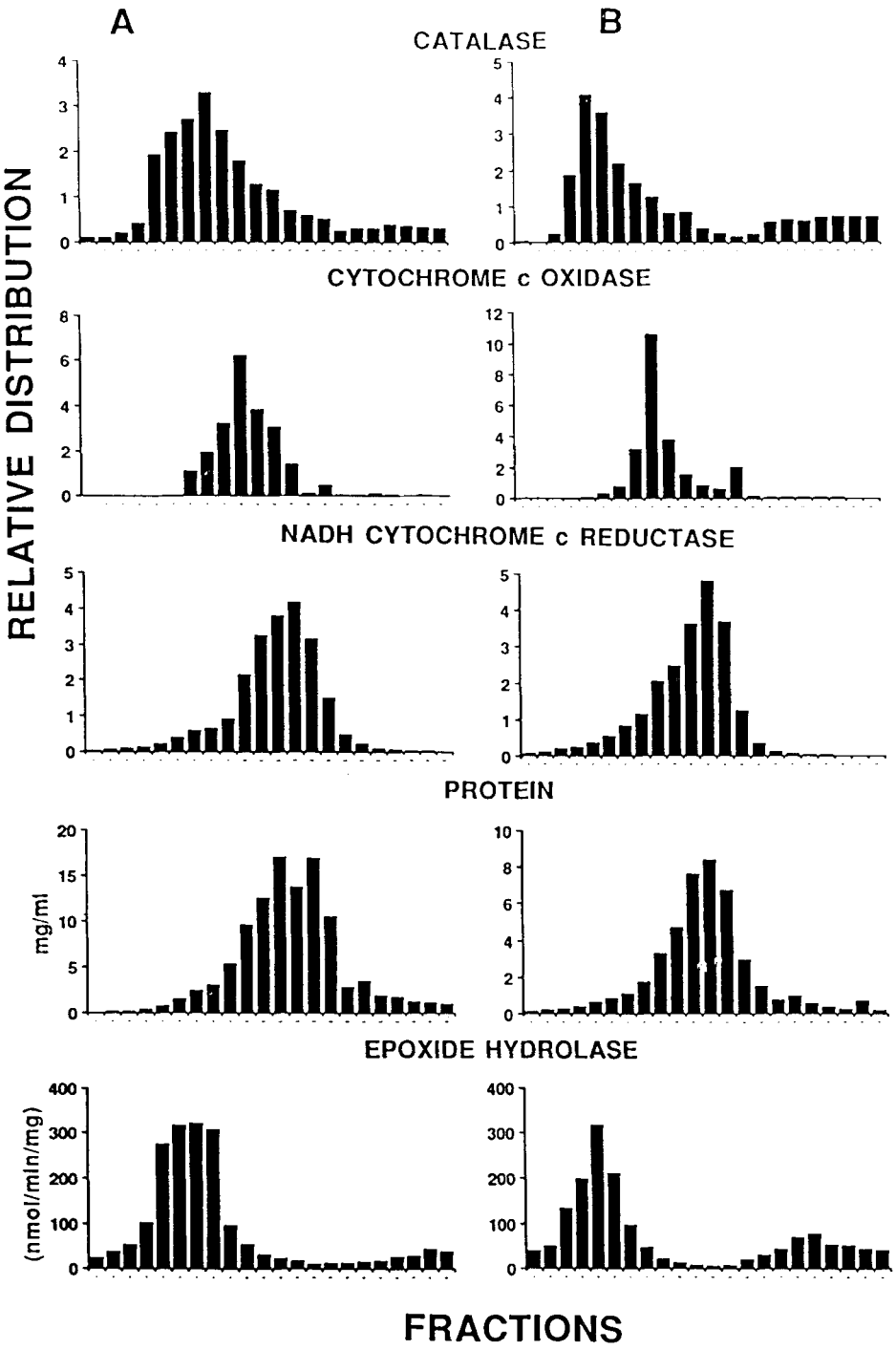

Fig. 2. Subcellular localization of epoxide hydrolase activity in human and rat liver. Human liver (A) and rat liver (B) were fractionated by differential and density gradient centrifugation as described in the text. The gradient profiles of each tissue are the average of two gradients.

peroxisomes. Excessive excretion of EDFA in disorders of peroxisome biogenesis that lack peroxisomal EH activity but with normal EH activities in other cellular compartments (e.g., mitochondria, microsomes and cytoplasm) suggests that peroxisomal EH activity may be responsible for the detoxification of fatty acid epoxides (EDFA). Furthermore, the higher specific activity and the possible specificity of peroxisomal EH towards EDFA as compared to other EH activities in the cell suggest that peroxisomal EH is a different protein than the EH observed in other cellular compartments. The induction of EH activity in peroxisomes as compared to

TABLE 3. Intraorganellar distribution of epoxide hydrolase in peroxisomes isolated from human and rat liver

	Epoxide Hydrolase		
	Peroxisomes	Matrix	Membrane
	nmol/min/mg protein		
Human liver	319.0 ± 55.2	346.0 ± 42.1	4.6 ± 1.1
Rat liver	317.0 ± 20.7	332.0 ± 27.5	5.2 ± 2.1

The activity of epoxide hydrolase was measured as described in the text. The results are expressed as mean ± SD of three different experiments.

the activities in other cellular compartments in ciprofibrate-treated liver also support the conclusion that the peroxisomal EH is a different protein than other EH in the cell.

There is now ample evidence regarding the exclusive or predominant role of peroxisomes in β-oxidation of very long chain fatty acids, branched fatty acids, dicarboxylic fatty acids, prostaglandins, and xenobiotics as well as the α-oxidation of 3-methyl-substituted fatty acids such as phytanic acid (1–3, 16). The peroxisomal EH may be responsible for conversion of fatty acid epoxides, produced from unsaturated fatty acids as a result of oxidative stress, to fatty acid diols prior to their β-oxidation in peroxisomes. The fatty acid epoxides may be produced by oxygen radicals with unsaturated fatty acids and/or unsaturated fatty acid-containing lipids, or they may be formed by the addition of water to double bonds of polyunsaturated fatty acids and cyclization of the hydroxy groups. The addition of one molecule of water to each of the double bonds of linoleic acid will give rise to 9,12-dihydroxystearic acid which in turn by cyclization of the hydroxy groups and ω-oxidation may produce EDFA. These fatty acid epoxides may be transported to peroxisomes for their further metabolism. Theoretically, for their ω-oxidation to dicarboxylic acids, these unsaturated fatty acids, prior to or after their conversion to epoxide derivatives, must come in contact with microsomes (31). The inability of the normal activities of EH in cytoplasm and microsomes to detoxify the EDFA in Zellweger syndrome suggest that peroxisomal EH may be responsible for detoxification of fatty acid epoxides and loss of this activity in Zellweger results in excessive excretion of fatty acid epoxides.

Recently oxidative stress conditions have been implicated in many disease processes like ischemia-reperfusion injury (9), endotoxemia (10), atherosclerosis (32), Alzheimer's disease (33), aging (34), etc. Reactive oxygen intermediates produced in these diseases may alter membrane lipid composition with the accumulation of lipid peroxides. Unsaturated or polyunsaturated fatty acids of membrane lipids may produce fatty acid epoxides after oxidant-insult. In atherosclerotic conditions, low density lipoproteins (LDL) and very low density lipoproteins (VLDL) rich in unsaturated and polyunsaturated fatty acids (35) may be converted to their epoxide derivatives. Ciprofibrate and clofibrate, hypolipidemic agents, are also known to lower lipoprotein level in atherosclerosis (36). The highest induction of peroxisomal EH among other EH in the cell by ciprofibrate suggests that peroxisomal EH may be involved in decreasing the levels of lipids or lipoproteins by ciprofibrate. Therefore, peroxisomal EH may be considered as an important enzyme in detoxification of at least fatty acid epoxides in various pathophysiological conditions associated with oxidative stress.

Mammalian peroxisomal matrix proteins studied so far are synthesized on free polysomes and are translo-

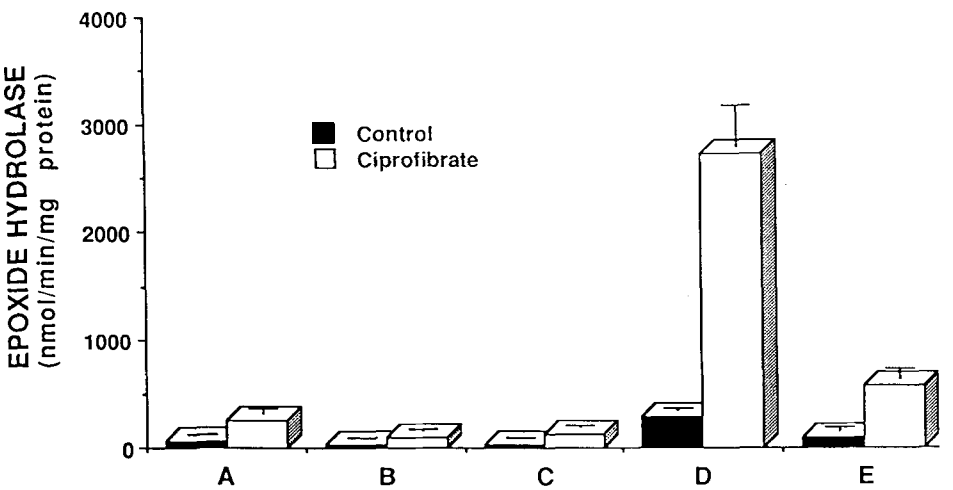

Fig. 3. Effect of ciprofibrate on rat liver epoxide hydrolase. Specific activity of epoxide hydrolase in homogenate (A), mitochondrial (B), microsomal (C), peroxisomal (D), and cytosolic (E) preparations from control and ciprofibrate-treated rats (see Materials and Methods). Results are mean ± SD of six values.

cated post-translationally into existing peroxisomes by a unique conserved tripeptide sequence of S/A/C-K/H/R-L (37) emphasizing the importance of leucine at the end position. The gene for cytoplasmic EH has been cloned recently from rats and mice (38, 39). These proteins contain SKI (serine-lysine-isoleucine) at the C-terminal, a tripeptide similar to known peroxisomal targeting signal (PTS-1). Gould and associates (40) have demonstrated that the presence of leucine at the end position of PTS-1 is absolutely essential for targeting of proteins to peroxisomes. These observations suggest that although SKI tripeptide is similar to the PTS-1, the protein with SKI tripeptide may not be targeted to peroxisomes. These observations provide additional support to earlier conclusions that peroxisomal EH may be a different protein than the EH found in other organelles. Therefore, peroxisomal EH may differ from other cellular EH at least with respect to the targeting signal sequence as peroxisomal proteins are synthesized in the mature form and post-translationally targeted to peroxisomes possibly without any other post-translational modification. At present, it is not known whether different EH are the product of different genes or the product of alternate splicing of the same gene.

In summary, we report that the increased excretion of epoxides in Zellweger patients is due to the absence of EH in peroxisomes. The EH in peroxisomes may be responsible for detoxification of fatty acid epoxides, produced from unsaturated fatty acids as a result of oxidative stress, and peroxisomal EH may be a different protein than EH found in other subcellular compartments.

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