TOXICOLOGICAL SCIENCES **50,** 30–35 (1999)

Soluble Epoxide Hydrolase in Rat Inflammatory Cells Is Indistinguishable from Soluble Epoxide Hydrolase in Rat Liver

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Received October 15, 1998; accepted March 10, 1999

Soluble epoxide hydrolase (sEH) is a ubiquitous mammalian enzyme for which liver and kidney are reported to have the highest activity. We have shown that the soluble epoxide hydrolase (sEH) activity present in rat neutrophils and macrophages is kinetically, immunologically, and physically indistinguishable from rat liver cytosolic sEH. Cytosol from rat liver or inflammatory cells and recombinant rat sEH were incubated with *trans*-diphenylpropene oxide (tDPPO), a selective substrate for sEH. The tDPPO hydration activity we observed in inflammatory cell cytosol was lower than that from liver. The Km for tDPPO hydration observed in rat inflammatory cell cytosol was the same as the Km for rat liver cytosol (10 μM). Recombinant rat sEH and cytosol from rat liver or inflammatory cells were incubated with the sEH inhibitors, chalcone oxide, 4-fluorochalcone oxide, and 4-phenylchalcone oxide. The IC_{50} values were 40, 8, and 0.4 μM, respectively, in all samples. Furthermore, sEH activity could be completely immunoprecipitated out of the samples, and the amount of antibody required to do so was apparently identical, regardless of the source of enzyme. SDS-polyacrylamide gel electrophoresis followed by Western blot analysis revealed a single sEH band with a molecular weight of 62 kDa. Isoelectric focusing followed by Western blot analysis revealed multiple bands containing tDPPO-hydrating activity. Although the inflammatory cell bands had the same pattern as those from liver cytosol, the recombinant sEH showed a different banding pattern. These multiple bands were not an artifact of the IEF gel selected. Furthermore, in a 2-dimensional IEF gel, the bands re-migrated to the same position. The presence of sEH in inflammatory cells suggests that this enzyme may have an important endogenous function.

Key Words: **epoxide hydrolase; neutrophil; macrophage; leukotoxin; inflammation; *trans*-diphenylpropene oxide; *cis*-stilbene oxide.**

Mammalian epoxide hydrolases (E.C. 3.3.2.3.) add water to epoxides and thereby produce vicinal diols. The 2 forms of epoxide hydrolase, the microsomal (mEH) and the soluble (sEH), are ubiquitous enzymes that have been identified in virtually every species and cell type examined. When comparing sEH activity across species, mouse liver has the highest activity, followed by human and rat liver, respectively (Hammock *et al.* 1997). In most tissues tested, the sEH is indistinguishable from the liver form, and sEH induced with clofibrate is indistinguishable from uninduced liver sEH (Hammock *et al.*, 1986). However, a variant of sEH with a different isoelectric point was isolated from mice treated with *N*-ethyl-*N*-nitrosourea (Nourooz-Zadeh *et al.*, 1992), and another variant with different substrate specificity, pI inhibitor sensitivity, and chromatographic properties was isolated from human epidermis (Winder *et al.*, 1993).

Neutrophils and macrophages are collectively called inflammatory cells because these are the first cell types to respond to tissue injury. They release a host of signaling molecules and cytotoxic enzymes and compounds (Sibille and Marchandise, 1993). Recently, an endogenous role for sEH in inflammation has been proposed. Linoleic acid is oxidized to its 9,10- or 11,12-epoxide (named leukotoxin and isoleukotoxin, respectively) by an oxidative burst or cytochrome P450 (Moghaddam *et al.* 1996). Leukotoxin was shown to be biosynthesized by neutrophils (Hayakawa *et al.* 1986). In most cases, the vicinal diol product of sEH is considered a detoxification product; however, in the case of leukotoxin, the diol appears to be more toxic in a number of systems, including mouse, cultured insect Sf-21 cells, and HeLa cells (Moghaddam *et al.* 1997). Because neutrophils were shown to produce the parent epoxide, we hypothesized that inflammatory cells also contained sEH and therefore could biosynthesize luekotoxin and isoleukotoxin diols as well. sEH activity in human leukocytes has been demonstrated (Seidegård, 1984), but because rodents provide models for toxicity studies in our laboratory, we aimed to fully characterize sEH activity in rodent inflammatory cells. Because sufficient numbers of inflammatory cells are difficult to isolate from mice due to their small body size, we limited our study of mouse inflammatory cell sEH to activity determination, and focused on the rat for the remainder of the experiments. The involvement of sEH in lipid metabolism, and potentially, in the biosynthesis of bioactive lipid mediators may implicate this enzyme as a target for therapeutic intervention. The purpose of this study was to establish whether liver

A preliminary account of this work was presented in poster format at the Society of Toxicology's annual meeting in Seattle, Washington in March, 1998, by A. J. Draper and B. D. Hammock.

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sEH can be used as a model to develop potentially therapeutic inhibitors for the inflammatory cell sEH. If the sEH in inflammatory cells is substantially different than the sEH in liver and kidney, this intervention could be selective.

MATERIALS AND METHODS

Preparation of cell fractions. Male F344 rats and male Swiss-Webster mice were obtained from Charles River Laboratories (Cambridge, MA) at 7 weeks of age and were maintained on a 12-hr light/dark cycle at 25°C. The animals were fed standard chow *ad libitum.* Animals were allowed to equilibrate to their surroundings for 1 wk prior to use. Neutrophils and macrophages were isolated from animals by published methods (Germolec *et al.* 1995). Briefly, animals were treated 14 h prior to use with a 3–15-ml intraperitoneal injection of 1% glycogen in phosphate-buffered saline (PBS) to elicit neutrophils. Animals were euthanized by CO_2 asphyxiation. Peritoneum was lavaged 5× with 10 ml PBS containing 0.5 mM EDTA. Lungs were lavaged 10× with 5 ml PBS containing 1 U/ml of heparin. Peritoneal and lung lavage fluid were filtered through sterile gauze and washed once in PBS. Histopaque 1083 (Sigma) was used to separate neutrophils from other cells by differential centrifugation. Red blood cells were lysed from the pellet with hypotonic saline. Neutrophils were washed once in PBS. Erythrocytes were lysed from filtered lung lavage using an ammonium chloride lysing buffer. Macrophages were washed a final time in PBS. All cells were counted and viability was assessed using trypan blue exclusion.

Cells were lysed using a probe sonicator, or by freezing fractions at −80°C. The cell lysate was centrifuged at 100,000 g for 65 min and the supernatant (cytosol) was removed for further study. Rat liver cytosol was also prepared by centrifugation of liver homogenate 100,000 g for 65 min. Protein concentrations were determined with a commercially available kit (BCA protein assay, Pierce Chemical Co., Rockford, IL). Recombinant rat sEH was prepared using the baculovirus expression system as previously described (Beetham *et al.*, 1993). Cytosol prepared from cell homogenate was used without further purification.

Determination of epoxide hydrolase activity. All substrates were synthesized in the laboratory as previously reported (Borhan *et al.*, 1995; Gill *et al.*, 1982). Soluble epoxide hydrolase activity was determined with the substrate [^{3}H]-*trans*-diphenylpropene oxide (tDPPO). Microsomal epoxide-hydrolase activity was determined with the substrate [^{3}H]-*cis*-stilbene oxide (cSO). The rates of hydration of tDPPO and cSO were determined by liquid scintillation spectroscopy following differential extraction. Briefly, cytosol from various rat cell types was incubated at 37°C in 100-ml incubation mixtures containing sodium phosphate buffer (90 mM, pH 7.4) and tDPPO or cSO (1–50 μM, added in 1 μL ethanol). Incubations were stopped after 5 min with the addition of 60 μl methanol and 200 μl isooctane for tDPPO, 250 μl isooctane for cSO. Zero-time and zero-protein incubations served as blanks. Incubations were vortexed vigorously to extract the substrate into the isooctane (the diol metabolite remains in the aqueous phase). A known aliquot of the aqueous phase was removed and added to 1 ml scintillation cocktail for scintillation counting. Extraction efficiency was ~91% for tDPPO and 65% for cSO (Borhan *et al.*, 1995). In all experiemnts descibed in this manuscript, total substrate metabolism did not exceed 15%.

Inhibition of sEH activity by known sEH chemical inhibitors. All inhibitors were synthesized in the laboratory as previously reported (Mullin and Hammock 1982). Cytosol (containing equal tDPPO activity) was incubated with tDPPO as described above. Protein was preincubated with inhibitor (added in 1 μl DMF) for 15 s before the addition of tDPPO. Three inhibitors of clearly different potencies were selected (Mullin and Hammock 1982). Incubations with no inhibitor (DMF only) served as 100% values.

Immunoprecipitation of tDPPO activity. Solutions (1 mg/ml) of rabbit anti-rat sEH, rabbit non-immune IgG and goat anti-rabbit IgG were prepared in 20 mM potassium phosphate buffer (pH 7.4). Recombinant rat sEH, rat liver cytosol, or inflammatory cell cytosol were incubated with 125 μg rabbit IgG (rabbit anti-rat sEH with remainder made up with non-immune IgG) for 2 h at room temperature in a shaking water bath. An excess of goat anti-rabbit IgG (175 μg) was added, and shaking at room temperature was continued for 2 h. Samples were then centrifuged at 100,00 g for 20 min at room temperature and 40 μl of supernatant (in triplicate) was removed. Sixty μL of 90 mM $NaPO_4$ (pH 7.4) was added to each aliquot of supernatant and tDPPO hydration activity was assayed as described above.

SDS–PAGE Western blot. Recombinant rat sEH, rat liver cytosol and inflammatory cell cytosol were diluted 1× with sample buffer containing SDS (sodium dodecyl sulfate, 10%), bromophenol blue (0.1%), and β-mercaptoethanol (10%), and samples were set in a boiling water bath for 5 min. Samples were applied to a 8–16% acrylamide gradient gel (Novex, San Diego, CA), and the gel was run at 40 mA until the dye front had migrated to the bottom of the gel. Tank buffer was 0.2% SDS in Tris-glycine buffer pH 8.6. A semi-dry blotting technique was used to transfer samples from the SDS–PAGE to the nitrocellulose membrane. Blotting buffer was 20% methanol in Tris-glycine buffer. The Western blot was performed using polyclonal anti-rat sEH antibodies prepared in this laboratory. The nitrocellulose membrane was blocked with dry milk suspended in PBS, and the blot was developed using metal-enhanced 3,3′-diaminobenzidine (Boehringer-Mannheim, Indianapolis, IN). In addition, samples were analyzed on a non-gradient acrylamide gel (10% acrylamide).

Isoelectric focusing. Proteins were filtered through a Microcon-10 filter (Millipore, Medford, MA) to de-salt and eliminate small peptides. Ten to 60 μg protein was loaded on a Pharmacia PAG-plate IEF gel (pH 3.5–9) and gel was run according to instructions provided by the manufacturer. Following focusing, the gel was cut into 0.5-cm sections and the gel slices were eluted into 150 μl of 90 mM sodium phosphate buffer at 4°C overnight. tDPPO activity was determined on 100 μl of eluate as described above. To determine the pH gradient of the gel, the gel was cut into 0.5-cm sections and eluted into 200 μl of 20 mM potassium chloride at room temperature for 30 min. A standard pH meter was used to determine pH of eluate. Narrow-range IEF (pH 4–6.5) was performed as described for wide-range IEF. Western blotting was also performed on these IEF gels by carefully removing gel from backing and blotting under the same conditions outlined above. The two-dimensional IEF-IEF gel was done by excising the lane containing rat liver cytosol and re-applying the entire lane, perpendicular and face-down, to a new IEF gel with additional lanes of rat liver cytosol run on both sides of the gel as a control. The second gel was run exactly as the first and a Western blot for sEH was performed. In addition, to confirm IEF results using commercial Pharmacia IEF gels, samples were also applied to IEF gels (pH 3–7) obtained from Novex (San Diego, CA) and run according to the manufacturer's instructions.

RESULTS

Rat inflammatory cells were isolated, cytosol fractions prepared and tDPPO hydration activity determined as described in Materials and Methods. Macrophages and neutrophils had comparable levels of activity, which was approximately one-tenth of that found in liver cytosol when expressed as specific activity (Fig. 1). This sEH activity in mouse inflammatory cells was not high enough to do further characterization without use of a large number of animals, thus the rat was used as a model for the remaining experiments. In addition, as shown in Fig. 1, rat alveolar and peritoneal macrophages had the same level of sEH activity. Under the cSO incubation conditions used, with which we could measure 20 pmol/mg/min of rat liver microsomal mEH activity, no activity was observed in inflammatory cell microsomal preparations. Comparison of sEH from rat

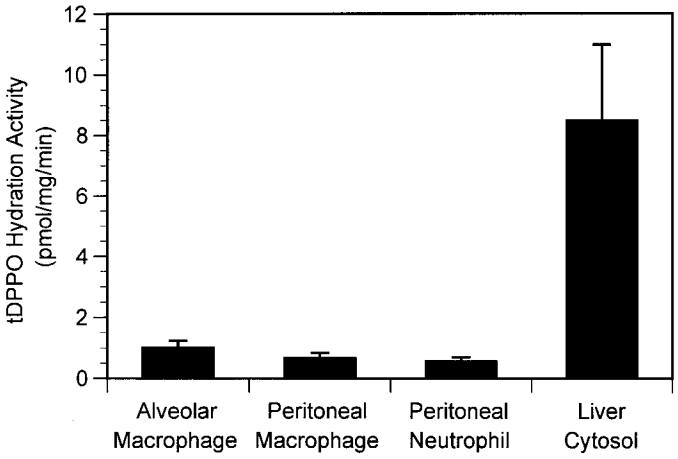

FIG. 1. tDPPO hydration (sEH) activity in rat inflammatory cells and rat liver cytosol. [^{3}H]tDPPO (50 μM) was incubated with cytosolic fractions from alveolar macrophages, peritoneal macrophages and peritoneal neutrophils and with rat liver cytosol. tDPPO diol formation was monitored after differential extraction as described in Materials and Methods. Each bar represents the mean (±SD) of 5 separate cell isolations (5 rats each). sEH activity in mouse inflammatory cell preparations was also measured, but was one-tenth the activity in rat inflammatory cells. The number of animals required in order to characterize that activity was prohibitive.

liver, rat inflammatory cells, and recombinant rat sEH was accomplished using 5 techniques as follows.

Michaelis-Menten Kinetics

The kinetics of tDPPO hydration was assessed for rat liver cytosol and cytosol prepared from rat inflammatory-cell preparations. As shown in the Lineweaver-Burk plot in Figure 2, the *x*-intercept, and thus the Km, of each preparation is approximately equal, ~10 μM. The Vmax values, given by the reciprocal of the *y*-intercept, are 9 nmol/mg/min for liver, 7 nmol/mg/min for alveolar macrophage, and 5 nmol/mg/min for peritoneal macrophages and neutrophils. These values are within experimental error introduced by the fact that these are not pure preparations of she, which would require the use of a prohibitory number of animals.

Chemical Inhibition

The sensitivity of sEH activity (tDPPO hydration) in cytosol from rat liver, rat inflammatory cells, and recombinant rat sEH to known chemical inhibitors was assessed under saturating conditions. The IC_{50} for inhibition of rat liver cytosol sEH by chalcone oxide, 4-fluorochalcone oxide, and 4-phenylchalcone oxide were determined from the data presented in Figure 3.The IC_{50} values were found to be 40 μM, 8 μM, and 0.4 μM, respectively, as expected from weak, moderate, and potent inhibitors (Mullin *et al.*, 1982). As shown in Table 1, regardless of the source of sEH, 40 μM chalcone oxide, 8 μM 4-fluorochalcone oxide, and 0.4 μM 4-phenylchalcone oxide caused approximately 50% inhibition. Because 4-phenylchalcone oxide is a near stoichiometric inhibitor of sEH (1 molecule of 4-phenylchalcone oxide causes the slow tight-binding competitive inhibition of one molecule of sEH), it was important to use identical sEH activities in these experiments.

Immunoprecipitation

Aliquots of cytosol from rat liver, inflammatory cells, and recombinant rat sEH constituting equal tDPPO hydration activity were used for immunoprecipitation. sEH activity was immunoprecipitated from cytosolic fractions by incubation with specific rabbit anti-rat sEH antibodies followed by incubation with goat anti-rat secondary antibody. In all cases, sEH activity could be completely precipitated, and the amount of anti-sEH antibody required was the same in all cases, regardless of the source of activity (Fig. 4).

SDS–PAGE Western blot

Cytosol from rat liver, alveolar macrophages, elicited peritoneal neutrophils, and recombinant sEH were applied to an SDS–polyacrylamide gel, and a Western blot was done. As shown in Figure 5, the major band recognized by the anti-rat sEH antibody was at 62 kDa. Secondary bands were also observed in the rat liver cytosol and recombinant protein lanes, which are thought to be either breakdown products or the result of differential post-translational modification. In addition, samples were analyzed on a non-gradient acrylamide gel (10%

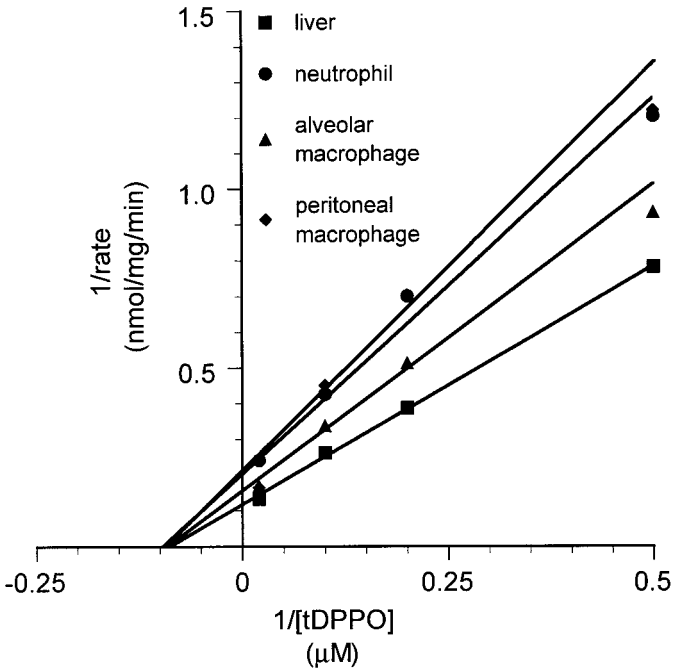

FIG. 2. Michaelis-Menten kinetics of tDPPO hydration (sEH) activity in rat inflammatory cells and rat liver cytosol. [^{3}H]tDPPO (1–50 μM) was incubated with cytosolic fractions from alveolar macrophages, peritoneal macrophage, and peritoneal neutrophils and with rat liver cytosol. tDPPO diol formation was monitored after differential extraction as described in Materials and Methods. Each point represents the mean of 2 separate determinations.

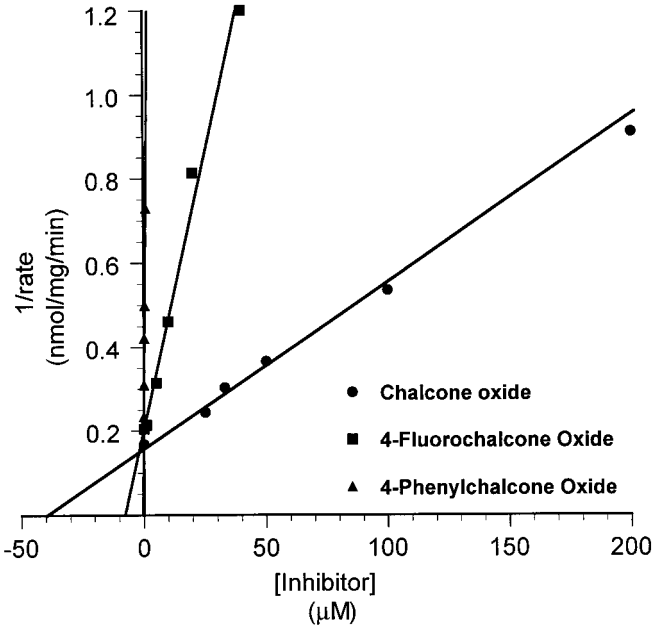

FIG. 3. Determination of IC_{50} values for inhibition of tDPPO hydration (sEH) activity in rat liver cytosol by chalcone oxide, 4-fluorochalcone oxide and 4-phenylchalcone oxide. [^{3}H]tDPPO (50 μM) was incubated with rat liver cytosol in the presence and absence of increasing concentrations of chalcone oxide, fluorochalcone oxide and phenylchalcone oxide. tDPPO diol formation was monitored after differential extraction as described in Materials and Methods. The IC_{50} was determined from these data. Each datum point represents the mean of three separate determinations.

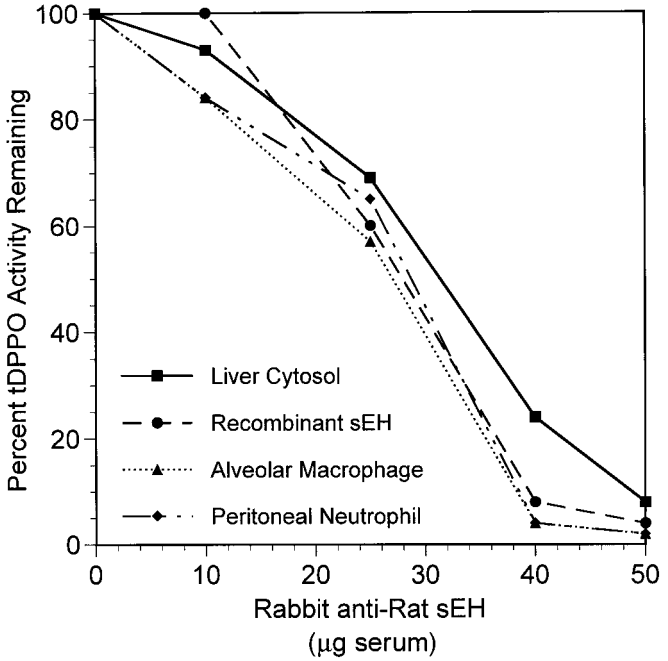

FIG. 4. Immunoprecipitation of tDPPO hydration activity from rat cytosol preparations by a specific anti-rat sEH antibody. Rat cytosol preparations from inflammatory cells and liver constituting equal tDPPO hydration activities were incubated with a polyclonal anti-rat sEH antibody for 2 h at room temperature. A secondary antibody (goat anti-rabbit IgG) was added, and incubations continued for another 2 h at room temperature. Samples were centrifuged at 10,000 g for 20 min, and supernatants were analyzed for tDPPO hydration activity as described in Materials and Methods. Each point represents the mean of 2 separate determinations.

acrylamide) and the same results were obtained (data not shown).

Isoelectric Focusing

Finally, isoelectric focusing was used to determine the pI of sEH from rat liver cytosol, rat inflammatory cells, and recombinant sEH. A wide-range gel resulted in a single, albeit broad, peak of tDPPO hydration activity corresponding to the published pI of 5.6 (Fig. 6) (Hammock *et al.*, 1986). Eighty percent of the sEH activity was recovered for rat liver cytosol and recombinant rat sEH. Because of the low activity in the inflammatory cell fractions, recovery was difficult to determine. The data presented are representative of 3 separate gels that were run under identical conditions. On the narrow range IEF gel, however, not only were multiple peaks of tDPPO hydration activity recovered, but the pI of the recombinant sEH appeared to be slightly higher than the pI of the sEH in rat liver and inflammatory cells (Fig. 7). As shown in Figure 8, a Western blot of the IEF gel revealed that the anti-rat sEH antibody recognized more than one band in each sample. While rat liver cytosol and inflammatory cells had 2 major bands at pI 5.4 and 5.6, the recombinant sEH had at least 6 distinguishable

TABLE 1
Inhibition of tDPPO Hydration Activity by Chalcone Oxides[a]

Enzyme source	Chalcone oxide[b]	4–Fluorochalcone oxide	4-Phenylchalcone oxide
Recombinant rat sEH	52.8± 9.9[c]	42.0± 8.7	41.0±4.2
Rat liver cytosol	51.7±16.6	47.0±13.7	48.3±3.5
Rat peritoneal neurophil	49.0± 9.3	41.8±10.3	46.8±9.3
Rat alveolar macrophage	46.5± 7.0	49.5±10.6	48.3±5.9

[a]Protein was incubated with inhibitor and tDPPO as described in Materials and Methods.
[b]Concentration of inhibitor used: chalcone oxide 40 μM, 4-fluorochalcone oxide 8 μM, 4-phenylchalcone oxide 0.4 μM.
[c]Percent activity remaining after inhibition with chalcone oxide, 4-fluorochalcone oxide or 4-phenylchalcone oxide ± standard deviation of four separate determinations.

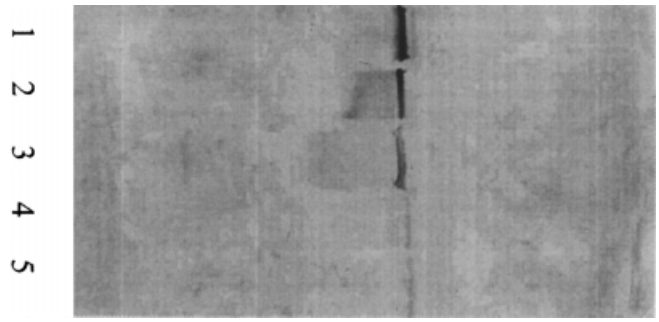

FIG. 5. SDS–PAGE Western blot of soluble epoxide hydrolase in rat liver, rat inflammatory cells and recombinant rat sEH. SDS–PAGE and Western blot were performed as described in Materials and Methods. Lane 1: alveolar macrophages, lane 2: peritoneal neutrophils, lane 3: rat liver cytosol, lane 4: recombinant rat sEH, and lane 5: standard molecular weight markers. Blot shown is representative of 4 replicates done as well as results from 10% acrylamide gels.

bands, with the major band corresponding to the major peak of tDPPO activity recovered at pI 5.9. This IEF-Western blot was repeated on IEF gels from 2 different vendors to be sure that it was not merely an artifact of the gel used. Results were the same on both gels (data not shown). To investigate further whether these multiple bands are artifacts, rat liver cytosol was subjected to isoelectric focusing in 2 dimensions. As shown in Figure 9, the bands re-migrate to the same position upon re-application.

DISCUSSION

Rat neutrophil and macrophage cytosol have been shown to contain the same soluble epoxide hydrolase as found in rat liver cytosol. sEH plays an important role in the hydration of epoxides on xenobiotics. Because inflammatory cells are located at each area where xenobiotic exposure takes place, these are often the first cells to be exposed to xenobiotic agents. The ability of these cells to metabolize xenobiotics, then, may be an important factor in host defense. In addition, sEH has been shown to play a role in the biosynthesis of bioactive metabolites of both arachidonic and linoleic acids. Furthermore, neutrophils have been shown to biosynthesize leukotoxin (9,10-epoxy-12-octadecenoate) (Hayakawa *et al.* 1986). The additional presence of sEH in neutrophils shows that these cells may biosynthesize the more bioactive leukotoxin diol. Interestingly, the rice plant has also been shown to biosynthesize

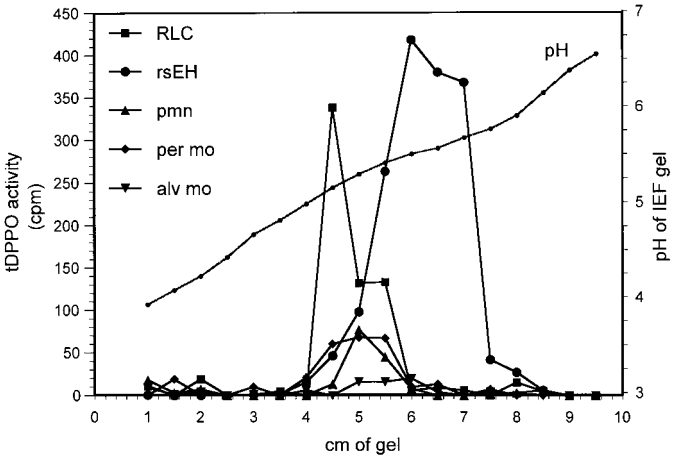

FIG. 7. Determination of pI for tDPPO hydration activity on narrow-range isoelectric focusing gel. Cytosol from rat liver, inflammatory cells, and recombinant rat sEH were run on a narrow (pH 4–6.5) range isoelectric focusing gel as described in Materials and Methods. Gel was cut and bands were eluted in buffer overnight. tDPPO activity was determined on eluate. Data shown are representative of 3 replicates.

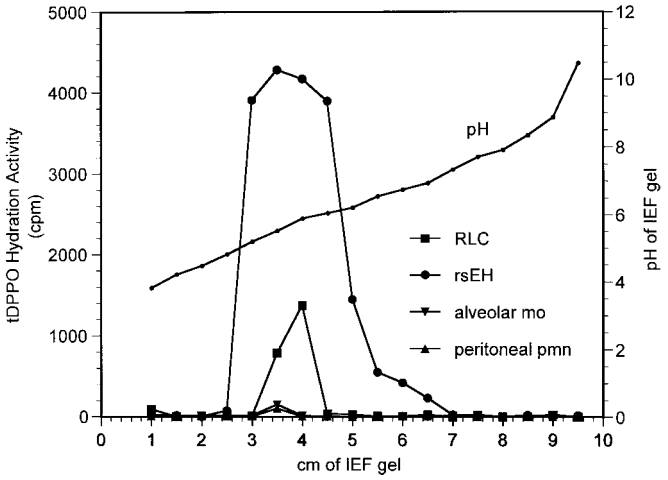

FIG. 6. Determination of pI for tDPPO hydration activity on wide-range isoelectric focusing gel. Cytosol from rat liver, inflammatory cells and recombinant rat sEH were run on a wide (pH 3.5–9) range isoelectric focusing gel as described in Materials and Methods. Gel was cut and bands were eluted in buffer overnight. tDPPO activity was determined on eluate. Data shown are representative of 2 replicates.

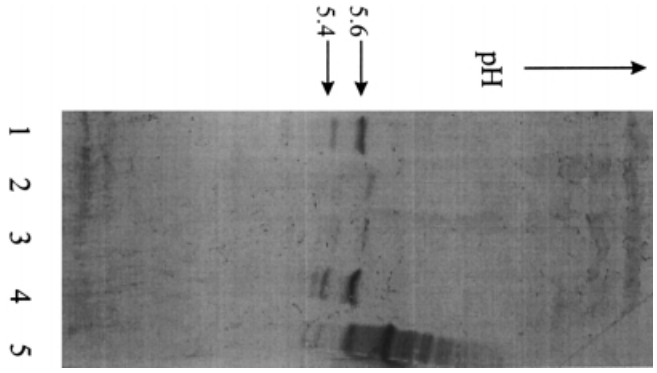

FIG. 8. Isoelectric focusing-Western blot of sEH in rat liver, inflammatory cells and recombinant rat sEH. Isoelectric focusing was carried out as in Figure 6, and Western blot was done on gel as in Figure 4. Lane 1: Peritoneal neutrophil, lane 2: peritoneal macrophage, lane 3: alveolar macrophage, lane 4: rat liver cytosol, lane 5: recombinant rat sEH. Data shown are representative of 4 replicates.

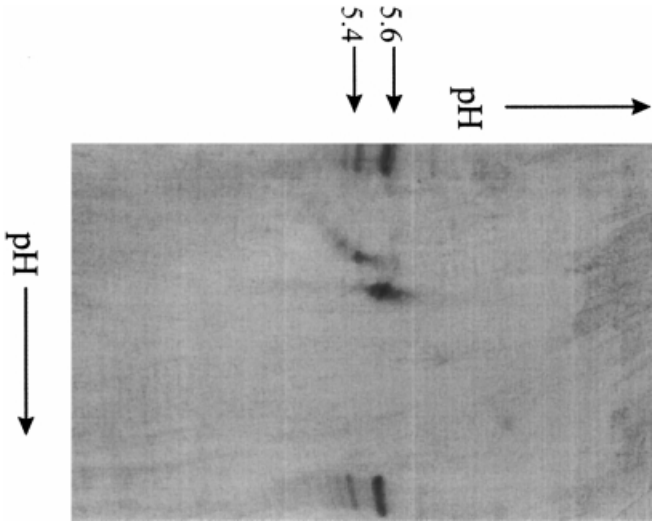

FIG. 9. 2-Dimensional isoelectric focusing-Western blot of sEH in rat liver cytosol. Isoelectric focusing was carried out as in Figure 6. Following focusing, the entire lane containing rat liver cytosol was excised and placed on a fresh IEF gel perpendicular to the lanes. Isoelectric focusing was carried out under identical conditions in the second dimension. Lanes marked S are standards of rat liver cytosol run in the second gel only. Western blot was done on gel as in Figure 4.

leukotoxin, which also has anti-fungal activity against rice blast disease (Kato *et. al*., 1983).

In the experiments described herein, tDPPO and *cis*-stilbene oxide (cSO) were used as marker substrates for soluble and microsomal epoxide hydrolase respectively. Although sEH activity in rat inflammatory cells was readily measured, mEH activity was undetectable. Human leukocytes were shown to contain both sEH and mEH activity as measured with *trans*-stilbene oxide (tSO) and cSO, respectively. The activities were extremely low, however, and measurement required a large number of cells and highly radiolabeled substrate (Seidegård *et al.* 1984). In a separate study, mEH activity was found in human leukocytes, but not shown to have any relation to tobacco exposure (Heckbert *et al.* 1992).

When determining the isoelectric point of the sEH from various sources, the appearance of multiple bands was unexpected. When the protein was eluted out of the gel, each band resulted in tDPPO hydration activity. Although there were multiple bands on IEF-Western blots for each source of sEH, the banding pattern was the same for rat inflammatory cells as for rat liver. However, the recombinant rat sEH had a different banding pattern. We are currently investigating the cause of these multiple bands, and undertaking studies to determine the difference between the recombinant and native protein. Because the molecular weight, kinetics, and inhibitory potential of sEH are the same regardless of the source of enzyme, we believe that the multiple bands are the result of differential secondary post-translational modification, such as glycosylation, phosphorylation, or interaction with a free thiol group on the surface of the protein.

ACKNOWLEDGMENTS

AJD is the recipient of an NIEHS postdoctoral fellowship ES05808 and the Amgen-American Liver Foundation Fellowship. This work is also supported by NIEHS Grant R01-ES02710, NIEHS Center for Environmental Health Sciences Grant 1P30-ES05707 and the NIEHS Superfund Basic Research Program P42-ES04699.

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