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Inhibition of Soluble and Microsomal Epoxide Hydrolase by Zinc and Other Metals

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Inhibition of xenobiotic-metabolizing enzymes by metals may represent an important mechanism in regulating enzyme activity. Fourteen cations were evaluated for inhibition of microsomal epoxide hydrolase (mEH) (mouse, rat, and human liver), soluble epoxide hydrolase (sEH) (mouse, rat, and human liver), and recombinant potato sEH. Of the metals tested, Hg^{2+} and Zn^{2+} were the strongest inhibitors of mEH, while Cd^{2+} and Cu^{2+} were also strong inhibitors of sEH (I_{50} for all ~20 μM). Nickel (divalent) and Pb^{2+} were moderate inhibitors, but Al^{2+}, Ba^{2+}, Ca^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, and Mn^{2+} were weak inhibitors of both mEH and sEH (less than 50 % inhibition by 1 mM metal). Six anions (acetate, bromide, chloride, nitrate, perchlorate, and sulfate) were tested and found to have no effect on the inhibition of sEH or mEH by cations. The kinetics and type of inhibition for zinc inhibition of sEH and mEH were examined for mouse, rat, human, and potato. Zinc inhibits mEH in a competitive manner. Inhibition of human and potato sEH was noncompetitive, but interestingly, zinc inhibition of mouse sEH was very strong and uncompetitive. Inhibition by zinc could be reversed by adding EDTA to the incubation buffer. Additionally, mouse liver microsomes and cytosol were incubated with these chelators. Following incubation at 4°C, samples were dialyzed to remove chelator. Both mEH and sEH activity recovered was greater in samples treated with chelator than in control incubations. Similar treatment with the protease inhibitor Nα-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) did not affect enzyme activity recovered. During systemic inflammation, hepatic metallothionien is induced, and liver metal concentrations increase while serum metal concentrations are decreased. The inhibition of microsomal and soluble epoxide hydrolase by metals may represent a mechanism of down-regulation of enzyme activity during inflammation.

Key Words: **epoxide hydrolase; metals; zinc.**

Mammalian epoxide hydrolases (E.C. 3.3.2.3.) add water to epoxides and thereby produce vicinal diols. The two forms of epoxide hydrolase, the microsomal (mEH) and soluble (sEH), are ubiquitous enzymes that have been identified in virtually every cell type examined (Hammock *et al.* 1997). 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 by cytochrome P450 enzymes (Moghaddam *et al.* 1996). In most cases, diol formation constitutes a detoxification pathway, however, in the case of leukotoxin, the diol appears to be more toxic in a variety of cell systems examined (Moghaddam *et al.* 1997). Neutrophils have been shown to biosynthesize leukotoxin (Hayakawa *et al.* 1986), and we recently showed that neutrophils and macrophages contain the sEH necessary for diol formation (Draper and Hammock, in press). In producing the cytotoxic leukotoxin diol and other oxidized lipids such as dihydroeicosatrienoic acids (DHETs), sEH may play a role in inflammation and host defense. Additionally, sEH is involved in the cytochrome P450-dependent pathway of arachidonic acid metabolism and in the hydration of the oxygenated steroids squalene oxide and lanosterol epoxide (Hammock *et al.*, 1997).

Hepatic metallothionien is induced in inflammation, following which serum zinc content declines while hepatic zinc content increases (Gaetke *et al.*, 1997), thus we became interested in the effect that increased hepatic zinc may have on epoxide hydrolase enzymes. There is some indication in the literature that zinc, copper, and iron affect epoxide hydrolase activity, but it was not well characterized (Gill and Hammock 1980). Rat mEH was also shown to be inhibited by zinc and mercury (Parkki, 1980). Furthermore, a series of sulfhydryl-binding agents were shown to inhibit sEH (Mullin and Hammock, 1982). A sulfhydryl may serve as a binding site for metals as well. Additionally, another epoxide hydrolase, leukotriene A_4 hydrolase, was recently shown to be inhibited by metal cations (Wetterholm *et al.* 1994). This study was undertaken, then, to characterize the inhibition of mammalian mEH and sEH by zinc and other metal cations.

MATERIALS AND METHODS

Preparation of cell fractions. Male F344 rats and male Swiss-Webster mice were sacrificed by CO_2 asphyxiation. Liver cytosol and microsomes were prepared by differential centrifugation as previously described (Draper and Hammock, in press). Protein concentrations were determined with a commercially available kit (BCA protein assay, Pierce Chemical Co., Rockford, IL).

A preliminary account of this work was presented in poster format at the 38th annual meeing of the Society of Toxicology (Poster Session, 1999).

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Recombinant mouse and potato sEH were prepared using the baculovirus expression system as previously described (Beetham *et al.* 1993). Human liver cytosol and microsomes were purchased from Xenotech, LLC (Kansas City, KS). Because human liver microsomes and cytosol were prepared in buffer containing EDTA, the protein was filtered through a microcon-10 filter (molecular weight cutoff of 10,000 kDa, Millipore, Medford, MA) and resuspended in buffer lacking EDTA.

Inhibition of mEH and sEH activity by metal cations. All substrates were synthesized in the laboratory. Microsomal epoxide hydrolase was measured as the rate of hydration of [^{3}H]-*cis*-stilbene oxide (Gill *et al.*, 1983). Soluble epoxide hydrolase was measured as the rate of hydration of [^{3}H]-*trans*-diphenylpropene oxide (tDPPO) (Borhan *et al.*, 1995). Both activities were determined by liquid scintillation spectroscopy. Briefly, cytosol or microsomes from mouse, rat, human liver, or recombinant enzyme was incubated at 37°C (30°C for potato sEH) in 100-μl incubation mixtures containing Tris buffer (90 mM, pH 7.4 for tDPPO, 90 mM, pH 9.0 for cSO) and tDPPO or cSO (0.5 to 50 μM final concentration, added in 1 μl DMF or ethanol). Metal salts were obtained from Sigma, and the salts used were: $ZnSO_4$, $MnSO_4$, $CdSO_4$, $NiSO_4$, $CoCl_2$, $Pb(NO_3)_2$, $HgCl_2$, $CaCl_2$, $MgCl_2$, $FeCl_3$, $FeSO_4$, $CuSO_4$, $AlCl_3$, and $BaCl_2$. Metal salts were dissolved in Tris buffer and diluted appropriately before addition of protein. Incubations were stopped after 2–10 min with the addition of 60 μl methanol and 200 μl isooctane. Zero-time and zero-protein incubations served as blanks. Incubations with no metal served as 100% values. Incubations were vortexed vigorously to extract the substrate into the isooctane (the diol metabolite remains in the aqueous phase). A known aliquot (50–70 μl) of the aqueous phase was removed and added to 1 ml scintillation cocktail for scintillation counting. Extraction efficiency was ~91% for tDPPO diol and ~65% for stilbene diol. Addition of metal salts did not change the partition characteristics of this assay. Studies described in this manuscript were designed such that substrate metabolism was <15%.

Kinetic data analysis. Kinetic data were analyzed using the Enzyme Kinetics software package from Trinity Software (Campton, NH). This program weights data toward high substrate concentration (weighting factor = 4). Ki, Km, and Vmax values were calculated from non-linear Michaelis-Menten kinetics. Data are shown in Lineweaver-Burk plots from which values can be more easily extracted.

Dialysis of mEH and sEH with chelators and protease inhibitor. All chelators and Nα-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) were obtained from Sigma Chemical Co. Mouse liver microsomes or cytosol (400 μg protein) were diluted into 1 ml 25-mM HEPES buffer (pH 8.0) containing no chelator or 3 mM EGTA, dipicolinic acid, 1,10-phenanthroline, 1,7-phenanthroline, or TLCK. Samples were incubated at 4°C for 24 h. Following incubation, samples were transferred into pre-rinsed dialysis tubing and dialzyed with gentle stirring for 24 h at 4°C. Buffer used for dialysis was the same as that used for enzyme activity determination (90 mM Tris, pH 7.4 for cytosol, pH 9.0 for microsomes). Following dialysis, 100-μl aliquots were analyzed as described above for cSO or tDPPO activity.

RESULTS

Kinetics of Substrate Metabolism

The kinetics of cSO hydration by mEH in mouse, rat, and human liver microsomes were analyzed with Enzyme Kinetics software (Trinity Software) using non-linear regression, as described in Materials and Methods. Microsomes were diluted in Tris buffer (90 mM, pH 9.0) and incubated with increasing concentrations of cSO. Rates of cSO hydration were determined as described in Materials and Methods. As shown in a Lineweaver-Burk plot (Fig. 1) mouse, rat, and human mEH have variable Km values (15, 2.4, and 4.0 μM respectively),

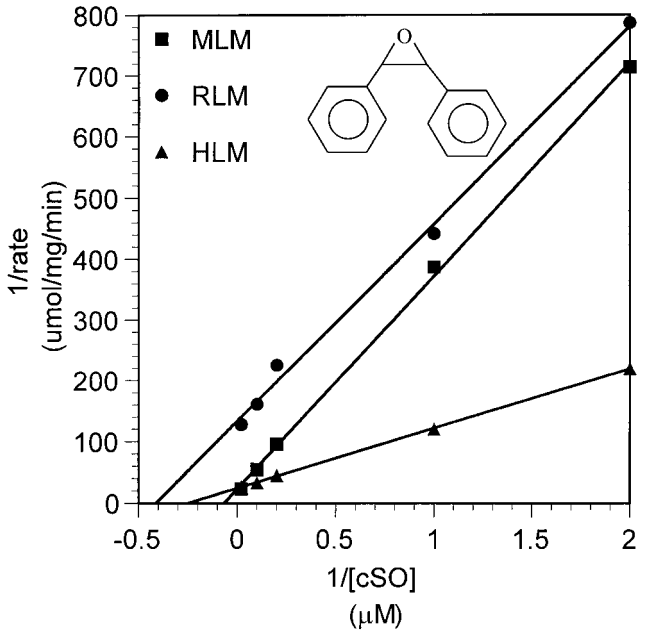

FIG. 1. Determination of Km for mEH hydration of cis-stilbene oxide. [^{3}H]-*c*SO (50, 10, 5, 2, or 1 μM) was incubated with mouse, rat, or human liver microsomes. Stilbene diol formation was monitored after differential extraction, as described in Materials and Methods. Each point represents the mean of 2 separate determinations. Inset shows structure of cSO.

and Vmax values (43, 7.7, and 40 nmol/mg/min respectively) for this substrate. The kinetics of tDPPO hydration by sEH in mouse, rat, and human liver cytosol were also analyzed. Cytosol aliquots were diluted in Tris buffer (90 mM, pH 7.4) and incubated with increasing concentrations of tDPPO. Rates of tDPPO hydration were determined as described in Materials and Methods. As shown in a Lineweaver-Burk plot (Fig. 2) mouse, rat, and human sEH have very similar Km values (7.8, 10, and 8.1 μM, respectively), but variable Vmax values (61, 7.8, and 40 nmol/mg/min, respectively) for this substrate. At the buffer pH values used, there is expected to be very little contribution of mEH to tDPPO hydration or sEH to cSO hydration (<0.1%), nor have other hydrolases been shown to contribute to the hydration of these substrates (Borhan *et al.*, 1995).

Screening of Metals as mEH and sEH Inhibitors

Metal salts were dissolved in assay buffer (90 mM Tris, pH 9.0 for mEH and pH 7.4 for sEH) and added to dilutions of mouse, rat, or human liver microsomes or cytosol. The rate of cSO or tDPPO hydration was measured as described in Materials and Methods. As shown in Figure 3, zinc, cadmium, mercury, and copper were potent inhibitors of sEH, while all other metals examined inhibited less than 50%. sEH activity is routinely measured in phosphate buffer, but because some of the metals precipitated out as phosphate salts, it was necessary to perform these analyses in Tris buffer. Consequently, Tris

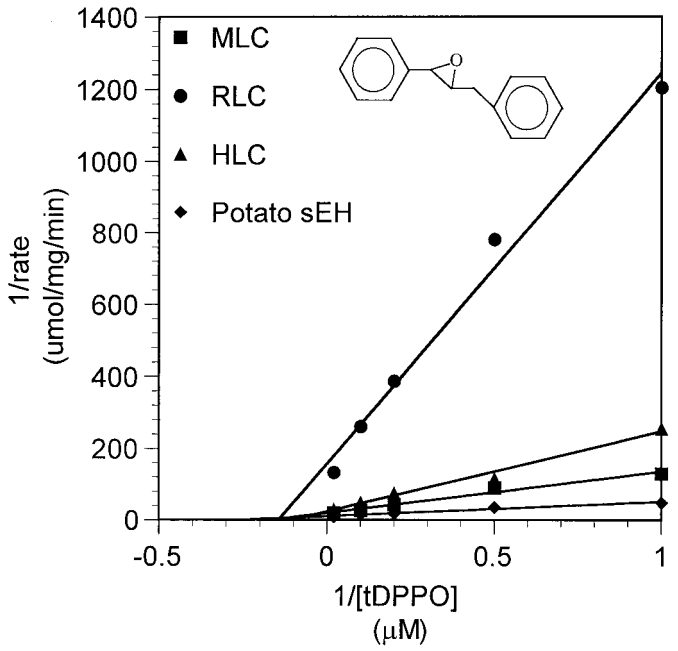

FIG. 2. Determination of Km for sEH hydration of trans-diphenylpropene oxide. [^{3}H]-*t*DPPO (50, 10, 5, 1, or 0.5 μM) was incubated with mouse, rat, or human liver cytosol. tDPPO diol formation was monitored after differential extraction, as described in Materials and Methods. Each point represents the mean of 2 separate determinations. Inset shows structure of tDPPO.

buffer (90 mM, pH 7.4) was used for all the sEH studies described herein. Similarly, inhibition of mEH by metals was determined as shown in Figure 4. As with sEH, zinc and mercury were potent inhibitors of mEH.

To examine the possible inhibitory effects of anions, mouse liver cytosol, and microsomes were incubated with tDPPO or cSO in the presence of Zn^{2+} and Ca^{2+} salts of acetate, bromide, chloride, nitrate, perchlorate, and sulfate anions. As shown in Figure 5, the anions caused no change in the inhibition of sEH or mEH by zinc or calcium.

Plant sEH enzymes have some sequence similarity with the mammalian enzymes. However, the plant sEH is missing the N-terminal third of the mammalian sEH and is a monomer while mammalian sEH exists as a dimer (Stapleton *et al.*, 1994). In order to determine to which part of the mammalian sEH the metals may be binding (the common half, or the mammalian-specific half), the inhibition of potato sEH by metals was analyzed. As shown in Figure 6, even the most potent metal inhibitors of sEH and mEH do not strongly inhibit recombinant potato sEH.

Kinetics of Metal Inhibition of mEH and sEH

Various concentrations of substrate were incubated with various concentrations of metals as described in Materials and Methods. The rate of cSO and tDPPO hydration was determined as described in Materials and Methods. Ki values and type of inhibition for zinc inhibition of sEH and mEH enyzmes were determined with Enzyme Kinetics software. Dixon plots of zinc inhibition of recombinant mouse sEH and mouse liver microsomal mEH (Fig. 7) are shown. A component of mouse liver cytosol, but not human liver cytosol, buffered the inhibition by zinc. In order to determine kinetic constants, recombinant mouse sEH was used. The Ki value and type of inhibition by zinc for all samples examined are listed in Table 1.

Effect of chelators on enzyme Activity

Mouse liver microsomes were incubated with cSO in the presence of increasing concentrations of zinc sulfate. After 5 min of incubation, 1 μl of 100 mM EDTA (1 mM final concentration) was added to some samples to examine the effect of chelation on zinc inhibition. The rate of product formation is given by the slope of the lines in Figure 8. The rate in the absence of zinc is 383 pmol/mg/min and the presence of parallel lines after EDTA addition to all zinc-containing samples indicates that EDTA completely removes zinc inhibition of mEH up to the highest concentration tested.

Finally, to investigate the effect of low concentrations of metals that may be in the buffer, aliquots of mouse liver microsomes or cytosol were incubated overnight with chelators or a protease inhibitor. Following incubation at 4°C, samples were dialyzed in assay buffer and cSO and tDPPO hydration

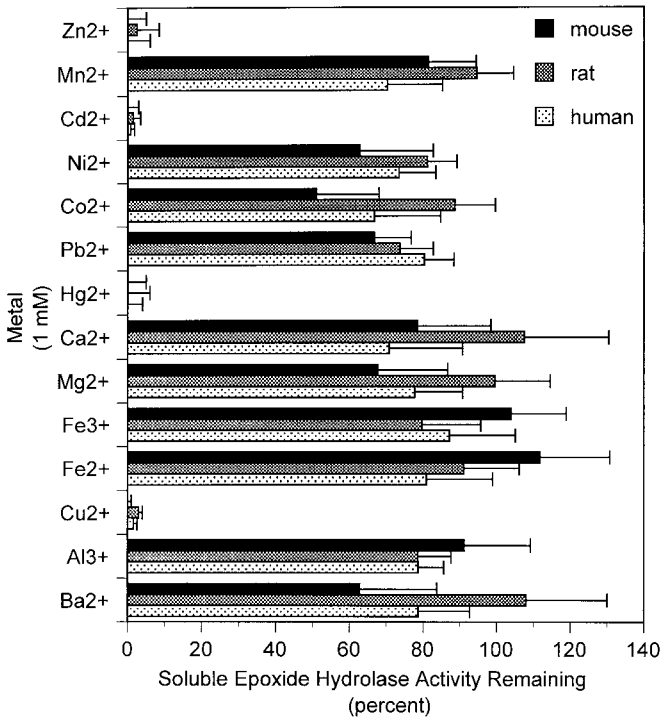

FIG. 3. Inhibition of mouse, rat, and human sEH by metal cations. [^{3}H]-*t*DPPO (50 μM) was incubated with mouse, rat, or human liver cytosol in the presence or absence of 1 mM metal cations. tDPPO diol formation was monitored after differential extraction, as described in Materials and Methods. Each bar represents the mean (±SD) of 3 separate determinations.

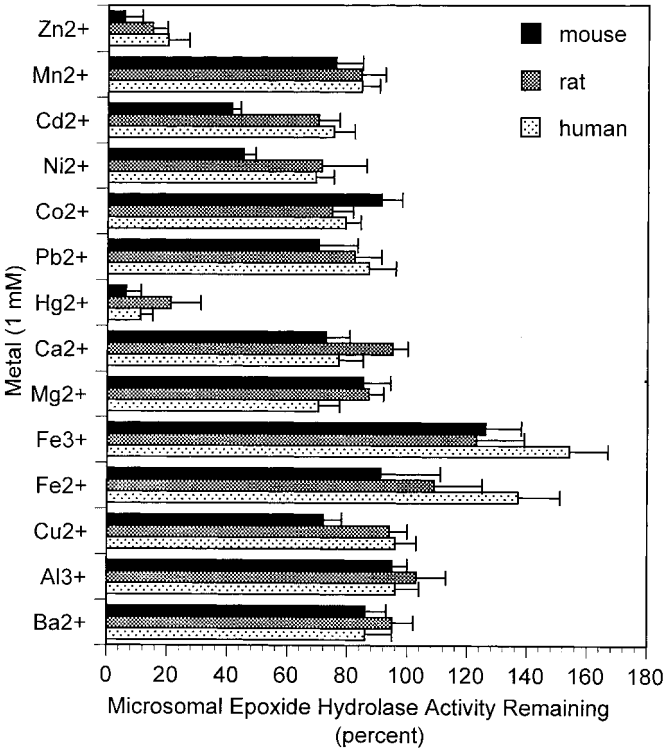

FIG. 4. Inhibition of mouse, rat and human mEH by metal cations. [^{3}H]-*c*SO (50 μM) was incubated with mouse, rat, or human liver microsomes in the presence or absence of 1 mM metal cations. Stilbene diol formation was monitored after differential extraction, as described in Materials and Methods. Each bar represents the mean (±SD) of 3 separate determinations.

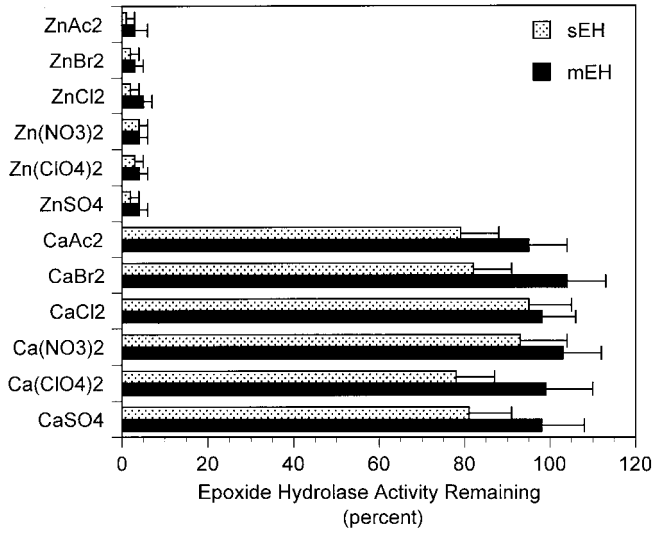

FIG. 5. Inhibition of mouse liver sEH and mEH by zinc and calcium salts. [^{3}H]-*t*DPPO (50 μM) was incubated with mouse cytosol and [^{3}H]-*c*SO (50 μM) was incubated with mouse liver microsomes in the presence or absence of 1 mM metal salts. tDPPO and cSO diol formation were monitored after differential extraction, as described in Materials and Methods. Each bar represents the mean (±SD) of 3 separate determinations.

activity were determined and compared with control. As shown in Figure 9, EGTA, dipicolinic acid, and 1,10-phenanthroline preserved mEH and sEH activity. 1,7-Phenanthroline also preserved activity, although this compound is not a metal chelator. Finally, the protease inhibitor, TLCK, did not preserve mEH or sEH activity more than untreated controls.

DISCUSSION

This report shows that mouse, rat, and human soluble and microsomal epoxide hydrolases can be inhibited by zinc and other metals. As expected from early reports (Gill and Hammock 1980; Pakki, 1980), of the metals tested, zinc and mercury were the strongest inhibitors of microsomal epoxide hydrolase (>90% inhibition with 1 mM metal). In addition to zinc and mercury, copper and cadmium also strongly inhibited the soluble epoxide hydrolase (>90% inhibition with 1 mM metal). All three mammalian species were remarkably similar in their sensitivity to the metals, but the mechanism of inhibition appears to be species-specific. Inhibition of mEH by zinc was either mixed or competitive, while inhibition of sEH by zinc was non-competitive for human sEH, but noncompetitive for mouse sEH. Based on these results, we conclude that the metal binding site is different from the enzyme active site for sEH, but may be close to or in the active site for mEH. One mechanism by which metal cations may inhibit epoxide hydrolase is by binding to a sulfhydryl group on the surface of the enzyme. The noncompetitive nature of the zinc inhibition of mouse sEH suggests that the sulfhydryl is more exposed upon

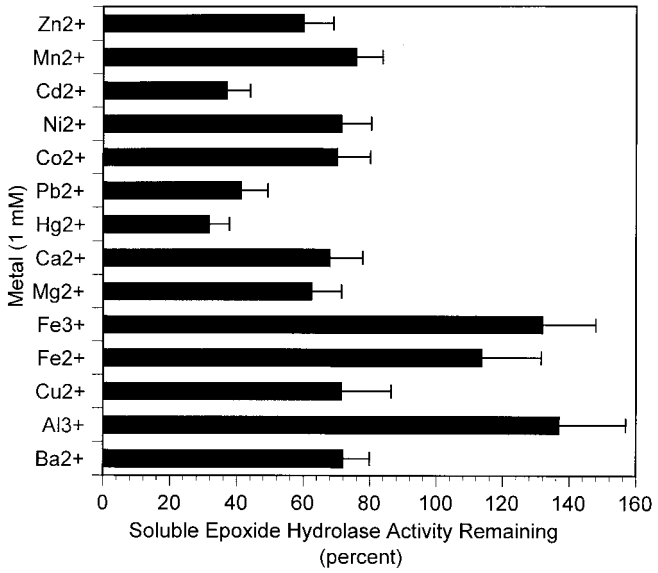

FIG. 6. Inhibition of recombinant potato sEH by metal cations. [^{3}H]-*t*DPPO (50 μM) was incubated with recombinant potato sEH in the presence or absence of 1 mM metal cations. tDPPO diol formation was monitored after differential extraction, as described in Materials and Methods. Each bar represents the mean (±SD) of 3 separate determinations.

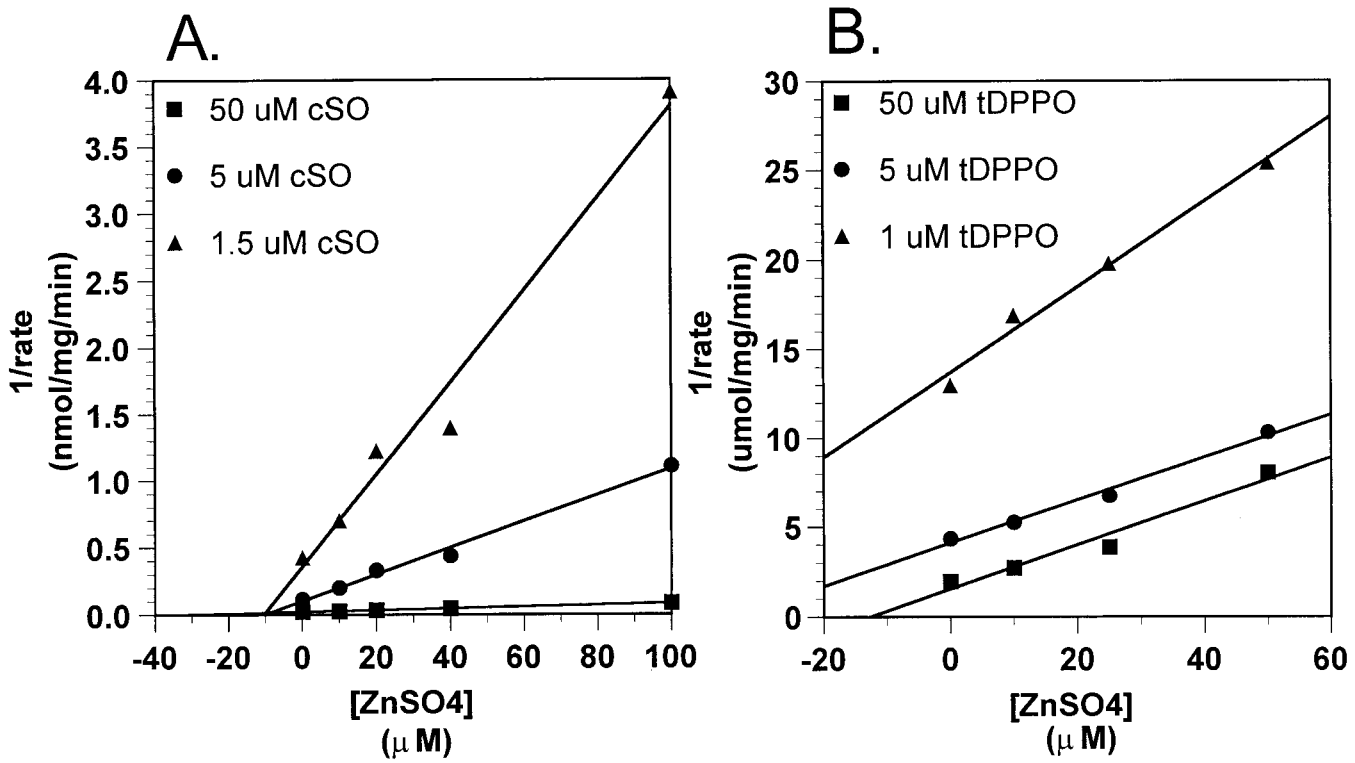

FIG. 7. Determination of kinetics and type of inhibition for zinc inhibition of mouse mEH and sEH. [^{3}H]-*t*DPPO (50, 5, or 1 μM) or [^{3}H]-*c*SO (50, 5, or 1.5 μM) were incubated with mouse liver microsomes or recombinant mouse sEH in the absence or presence of zinc sulfate (0 to 100 μM), as described in Materials and Methods. Each point represents the mean of 2 separate determinations.

substrate binding, whereas the sulfhydryl in rat and human sEH must be more readily accessible in the absence of substrate. Sulfhydryl reagents have been shown to inhibit mouse liver cytosolic sEH, the more nonpolar reagents stronger inhibitors, with 100 μM sodium *p*-hydroxymercuriphenylsulfonate causing 98% inhibition (Mullin and Hammock, 1982). The Greek letter α can be used to describe the relative competitive and

TABLE 1
Inhibition of Epoxide Hydrolases by Zinc

Protein source	Ki[a] (μM)	Type of inhibition
Mouse liver microsomes	7.6 ± 1.2	Mixed
Rat liver microsomes	12 ± 2.4	Competitive
Human liver microsomes	61 ± 6.5	Competitive
Potato sEH (recombinant)	620 ± 42	Noncompetitive
Mouse sEH (recombinant)	0.58 ± 0.11	Uncompetitive
Human liver cytosol	23 ± 4.9	Noncompetitive

Note. Protein was incubated with tDPPO for sEH activity determinations and cSO for mEH activity determinations. Activity was determined following a differential extraction of the diol metabolites, as described in Materials and Methods.

[a] The Ki value and type of inhibition was determined by analyzing data with Enzyme Kinetics software.

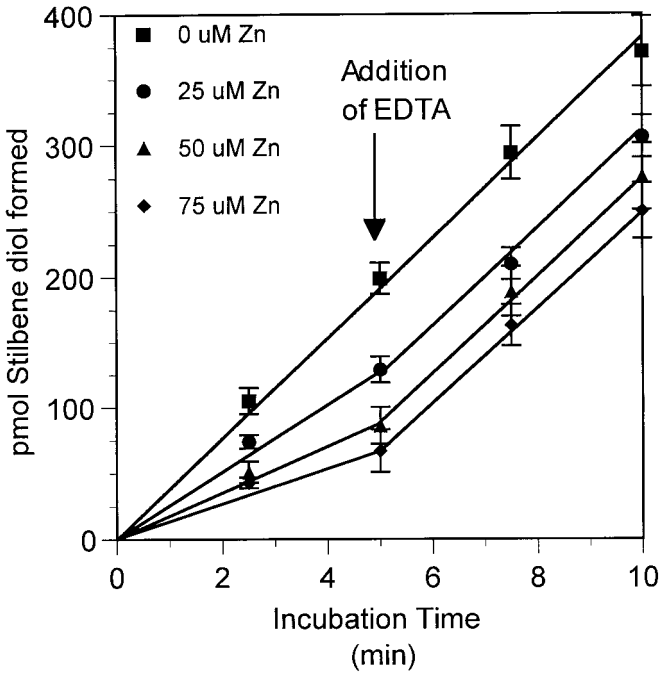

FIG. 8. Effect of EDTA on the inhibtion of mouse mEH by zinc. [^{3}H]-*c*SO (50 μM) was incubated with mouse liver microsomes in the presence or absence of zinc sulfate (25, 50, or 75 μM). EDTA (1 mM final concentration) was added to incubations at 5 min, and stilbene diol formation was monitored after differential extraction, as described in Materials and Methods. Each point represents the mean (±SD) of 3 separate determinations.

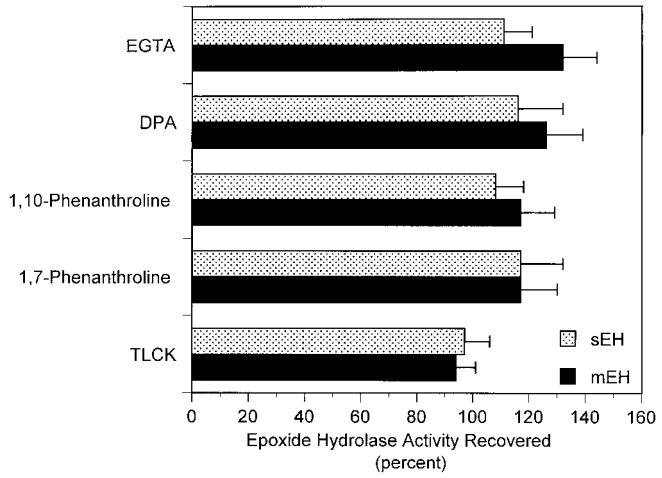

FIG. 9. Effect of incubation of mouse liver microsomes and cytosol with chelators or a protease inhibitor on the recovery of mEH or sEH activity. Mouse liver cytosol or microsomes were incubated for 24 h with chelators or a protease inhibitor. Samples were subsequently dialyzed for 24 h against buffer, and remaining sEH or mEH activity was measured, as described in Materials and Methods. Each point represents the mean (±SD) of 3 separate determinations.

noncompetitive nature of mixed-type inhibition. For purely competitive inhibitors, α approaches infinity, while for noncompetitive inhibitors, $\alpha = 1$. The mixed-type zinc inhibition of mouse liver mEH is largely competitve, with an α value greater than 10^5.

Plant sEH differs from mammalian sEH in that the plant enzyme is missing about a third of the mammalian sequence and exists as a monomer, as opposed to the mammalian dimer (Stapleton, 1994). In order to investigate the metal binding site further, we examined the sensitivity of the plant sEH to metals. Zinc inhibition of recombinant potato sEH was much weaker (Ki = 620 μM) than zinc inhibition of mammalian sEH (Ki = 0.58 to 23 μM). Furthermore, unlike the mouse sEH, which was inhibited by zinc noncompetitively, inhibition of potato sEH was noncompetitive.

Metal chelators were examined to determine whether metals, present at low levels in our buffers, were affecting our routine measurements of epoxide hydrolase activity. Upon incubation with chelators followed by dialysis, the activity of epoxide hydrolase recovered was increased insignificantly by the presence of chelators. In order to test the hypothesis that the chelators were working as protease inhibitors, we tested a protease inhibitor that is not a metal chelator, TLCK. This compound did not improve epoxide hydrolase-activity recovery over control. Additionally, 1,10-phenanthroline, a zinc chelator, preserved epoxide hydrolase activity. Because 1,10-phenanthroline oxide is a substrate for mEH (ref), we used 1,7-phenanthroline to test the hypothesis that 1,10-phenanthroline was preserving activity by binding to the active site of mEH. The results suggest that both 1,10-phenanthroline and 1,7-phenanthroline preserve epoxide hydrolase activity to some extent, but we cannot rule out the possibility that they are acting as bidentate and monodentate chelators, respectively.

Leukotriene A_4 hydrolase is an apparently unrelated epoxide hydrolase that catalyzes the biotransformation of leukotriene A_4 to leukotriene B_4. This enzyme has been shown to be a zinc metalloenzyme with dual enzyme activities (Wetterholm *et al.*, 1991). The peptidase activity requires zinc in the catalytic site, while the epoxide hydrolase activity seems to require zinc only for enzyme structure (Medina *et al.*, 1991). Many divalent cations were shown to inhibit leukotriene A_4 hydrolase (Wetterholm *et al.*, 1994). Our initial studies showed that the metals tested for inhibition by Wetterholm *et al.* also inhibited mEH with approximately the same I_{50} values (data not shown). Consequently, we investigated whether mEH or sEH may be a metalloenzyme. Atomic absorption showed only a trace amount of zinc in our purified recombinant enzyme preparations (less than 1 atom zinc per 100 molecules of enzyme). Furthermore, purified sEH did not have the peptidase activity exhibited by leukotriene A_4 hydrolase. Finally, a comparison of protein sequence with the BLAST program did not reveal any significant sequence similarities. We have not been able to explain mechanistically the reason for similar I_{50} values for metals between mEH and leukotriene A_4 hydrolase.

Menza *et al* (1994) describe inhibition of phospholipase A_2 by zinc as a result of zinc replacing calcium in a specific calcium-binding site. In order to investigate this possibility for sEH and mEH, microsomes or cytosol were incubated with cSO or tDPPO in the presence of zinc and increasing concentrations of calcium or magnesium (data not shown). Because neither the presence of calcium or magnesium affected zinc inhibition kinetics, we concluded that zinc was not displacing either of these cations.

Recently, an endogenous role for soluble epoxide hydrolase in lipid metabolism has been suggested (Moghaddam *et al* 1996). In addition to forming DHETs from arachidonic acid cytochrome P450 metabolites, sEH catalyzes the formation of leukotoxin diol from leukotoxin (9,10-epoxy-12-octadecenoate), and in all cell systems examined, leukotoxin diol is more toxic than leukotoxin (Moghaddam *et al*, 1997). Leukotoxin has been shown to be produced by neutrophils (Hayakawa *et al*, 1986), and we have recently shown that neutrophils and macrophages contain the sEH necessary to hydrate leukotoxin to its diol (Draper and Hammock, in press). Because of this previous work, we have suggested that leukotoxin diol may be involved in inflammation. A paradox in this work has been that high levels of leukotoxin epoxide are found in the serum and alveolar fluid of patients with both severe burns and adult respiratory distress syndrome (Kosaka *et al.*, 1994; Ozawa *et al.*, 1988). If hepatic sEH levels are not down-regulated, we would expect that all serum leukotoxin would be hydrated to its diol. However, it has long been known that hepatic metallothionien is upregulated during inflammation, which causes an increase in hepatic zinc with a concomitant decrease in serum zinc (Gaetke *et al.*, 1997). Serum zinc is protein-bound to

albumin and β_2-macroglobulin and normal levels are 85 to 110 μg/dl. Cellular zinc is expected to be protein-bound as well, to metallothionien (Goyer, 1996). However, metallothionien was shown to donate these zinc ions to other enzymes (Udom and Brady, 1980). Although we have yet to determine whether sEH activity is down regulated during inflammation, the results of this study, together with the information known about hepatic zinc concentrations during inflammation, suggests the intriguing possibility that inhibition by metals is a mechanism of hepatic sEH inhibition during inflammation.

ACKNOWLEDGMENTS

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