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Metabolism of Monoepoxides of Methyl Linoleate: Bioactivation and Detoxification

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Leukotoxin (ltx) and isoleukotoxin (iltx) methyl esters, are metabolites of methyl linoleic acid, an essential fatty acid. They have been associated with acute respiratory distress syndrome. The observed toxicity of ltx and iltx is, in fact, due to the metabolism of the epoxides to their corresponding diols by soluble epoxide hydrolase (sEH). Herein, we demonstrate that ltx/iltx are toxic in a time-dependent manner to human sEH expressing cells with a LT_{50} of 10.6 ± 0.8 h and that ltx and iltx have K_M of 6.15 ± 1.0 and 5.17 ± 0.56 μM, respectively, and V_{max} of 2.67 ± 0.04 and 1.86 ± 0.06 μmol/min/mg, respectively, which can be inhibited by sEH inhibitors. We show that four major metabolites of ltx/iltx are formed in our system, including ltx/iltx free acid, ltxd/iltxd, free acid, and phosphotidylcholine and phosphotidylethanolamine containing the carboxylic acid forms of both ltx/iltx and ltxd/iltxd, but that the only metabolite associated with toxicity is the carboxylic acid form of ltxd/iltxd, suggesting the involvement of cellular esterases. We demonstrate that a serine esterase inhibitor provides some protection from the toxicity of epoxy fatty esters to sEH expressing cells as do intercellular free sulfhydryls, but that this protection is not due to glutathione conjugation. With these data, we have proposed an extension of the metabolic pathway for ltx/iltx in eukaryotic cells.

Key Words: **linoleic acid; leukotoxin; leukotoxin diol; fatty acids; soluble epoxide hydrolase; phospholipids; glutathione; esterase.**

Methyl linoleate, an essential fatty acid and the predominant polyunsaturated fatty acid in the Western diet (1), is a major component of membrane fatty acids (2) and endothelial cell trigylceride stores (3). In developed countries, nearly 40% of the energy in the diet comes from fat (4). Linoleic acid levels are elevated in humans and other animals when sustained on a diet with high vegetable oil levels (5, 6). Leukotoxin methyl ester, (12Z)9(10)-Z-EpOME, methyl ester, (ltx),[2] and isoleukotoxin methyl ester, (9Z)12(13)-*Z*-EpOME, methyl ester (iltx), are metabolites of linoleic acid, methyl ester (7–9), the carboxylic acid form of which have been associated with acute respiratory distress syndrome (ARDS) (10, 11). High levels of circulating ltx carboxylic acid (11.4–37 nmol/ml serum) have been associated with toxicity in patients suffering from multiple organ failure, of which ARDS is one part, following recovery from the primary shock of severe burns (>50% body surface). The patients suffer from pulmonary edema, cardiac failure, and coagulation abnormalities (10, 11). Injection of ltx, in rats, results in

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[2] Abbreviations used: ARDS, acute respiratory distress syndrome; BNPP, bis-(*para*-nitrophenyl)phosphate, BSA, bovine serum albumin; CDNB, 1-chloro-2,4-dinitrobenzene; Da, daltons; DCU, *N*,*N*′-dicyclohexyl urea; DEM, diethyl maleate; DMDO, dimethyldioxirane; DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; EEG, ethyl ester glutathione; ESI, electrospray ionization; FID, flame ionization detector; GBq, gigabecquerel; GC, gas chromatograph; GSH, glutathione; GST, glutathione-S-transferase; HP, Hewlett-Packard; HPLC, high performance liquid chromatography; hsEH, human soluble epoxide hydrolase; IC_{50}, median inhibitor concentration; iltx, isoleukotoxin methyl ester; iltxd, isoleukotoxin diol methyl ester; Lac Z, β-galactosidase; LC_{50}, median lethal concentration; LPATs, lysophospholipid acyl transferases; LT_{50}, median lethal time; LSC, liquid scintillation counter; ltx, leukotoxin methyl ester; ltxd, leukotoxin diol methyl ester; *m*-CPBA, *meta*-chloroperoxybenzoic acid; MTT, 3-(4,5-dimethylthiazol-2-*yl*)-2,5-diphenyltetrazolium bromide; OTFP, 3-n-octylthio-1,1,1-trifluoro-2-propanone; PMSF, α-phenyl methyl sulfonyl fluoride; PNPA, *para*-nitrophenyl acetate; sEH, soluble epoxide hydrolase; Sf-21, *Spodoptera frugiperda;* *t*-DPPO, *trans*-diphenylpropene oxide; Tn, *Trichoplusia ni;* UGT, UDP-glucuronosyl transferase; *vic*, *vicinal*.

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severe histological changes, including intravascular congestion and coagulation and alveolar exudation, edema, hemorrhage and emphysema (12). These changes are similar to those seen in human patients presenting with ARDS (12, 13). Ltx and iltx have also been shown to decrease cardiac function in dogs (14).

Soluble epoxide hydrolase (sEH) is an enzyme commonly found, among other places, in the liver, lungs, and kidneys of animals (15–18). It hydrates both ltx and iltx with a high rate (19). It has a high V_{max} and a low K_M for epoxystearic acid, 9,10-Z-EpO (20), a compound similar in structure to ltx and iltx. We have previously demonstrated that the observed toxicity of ltx and iltx is, in fact, due to the metabolism of the epoxides to their corresponding diols, leukotoxin diol methyl ester, (12Z)9,10-*threo*-DiHOME methyl ester (ltxd), and isoleukotoxin diol methyl ester, (9Z)12,13-*threo*-DiHOME, methyl ester (iltxd) by sEH (21). However, very little other metabolic work has been done. It is not known if the diol is the final toxic metabolite, nor is the nature of any detoxification pathway well established. Herein, we describe our findings with respect to metabolism of ltx and iltx by sEH as well as involvement with free sulfhydryls and metabolism by cellular esterases.

MATERIALS AND METHODS

Chemicals and instruments. [1-^{14}C] Linoleic acid (2.14 GBq/nmol) was purchased from NEN Life Sciences (Boston, MA); [1-^{14}C] oleic acid (1.96 GBq/nmol) was purchased from Amersham Life Sciences (Arlington Heights, IL). Nonradioactive fatty acids were purchased from Sigma (St. Louis, MO), as were all other chemicals except as noted. Solvents used were HPLC grade.

Gas chromatographic analyses were performed on a Hewlett-Packard (HP) 5890A gas-chromatograph (GC) equipped with a flame ionization detector (FID) and 30 m × 0.25 mm i.d., 250 μm DB-5 column (J & W Scientific, Folsom, CA). High performance liquid chromatography (HPLC) analysis and purification employed a Perkin–Elmer system consisting of a series 410 BIO pump, a Reodyne 7125 injection port equipped with a 50-μl injection loop for analysis and a Whatman injection port with a 250-μl injection loop for purification. Absorbance was monitored at 215 nm using an analytical UV detector. Separation of radiolabeled epoxides was effected on a 4.6 mm × 22 cm silica column, 5-μm particle size. Thin-layer chromatography (TLC) was performed either on silica P_{254} plates or 19 channel plates coated with silica 250 with preabsorbant spotting area (Baker, Philipsburg, NJ). The radioactive TLC scanner was a Bioscan System 200 imaging scanner equipped with a Model 1000 bioscan Auto Changer and Bioscan System 200 Data Collection Software version 2.247. The liquid scintillation counter (LSC) used was a Wallac Model 1409. Spectrophotometry was performed on a V_{max} microplate reader (Molecular Devices, Menlo Park, CA). Curve fitting was performed using SigmaPlot (SPSS Inc., Chicago, IL) and Excel (Microsoft, Redmond, WA).

Cytotoxicity studies. Toxicity assays were performed in *Spodoptera frugiperda* (Sf-21) cells as previously described (22). Briefly, the cells were infected with baculovirus containing recombinant cDNA coding for either human soluble epoxide hydrolase (hsEH) or β-galactosidase (Lac Z), as a control enzyme at a multiplicity of infection of 0.1. Compounds were administered to cells (0.06–1.0 mM final concentration) 48 h postinfection, in dimethyl sulfoxide (DMSO) (final volume: 1%). Cells were incubated for an additional 24 h at 27°C and then assayed for viability by incubation for 2 h with 3-(4,5-dimethylthiazol-2-*yl*)-2,5-diphenyltetrazolium bromide (MTT). Alternately, a fixed concentration of compound was added to cells and aliquots were taken at 0, 1, 2, 4, 8, 12, 16, and 24 h and then assayed for viability. The cells were lysed with 500 μl of 250 mg sodium dodecyl sulfate in 1:1 dimethyl formamide: water, pH 4.5 over 12 h in the dark to dissolve the insoluble formazan product. Aliquots were then transferred to 96-well plates and the MTT hydrolysis was quantified at 560 nm. Viability is normalized to cells treated with vehicle control. Background absorbance for MTT in media corresponds to approximately 12% viability. Toxicity data are representative of at least three independent experiments.

Synthesis of radioactive compounds. [^{3}H]-*trans*-1,3-diphenylpropene oxide (*t*-DPPO) was synthesized as previously described (20). [^{14}C]Linoleic acid in ethanol (250 μl) was treated with excess diazomethane which was generated as previously described (23). The product of this, when visualized by the Bioscan radioactive TLC scanner was found to have a single detectable radioactive component which comigrated with an authentic sample of linoleic acid, methyl ester. The [^{14}C]linoleic acid, methyl ester in acetone, was then treated with a 0.5 molar ratio of dimethyl dioxirane (DMDO), generated as previously described (24). This reaction was shown to yield three radioactive components when analyzed with a plate scanner. These components comigrated with authentic methyl linoleate, a mixture of the monoepoxides of methyl linoleate, and the diepoxide of methyl linoleate, respectively. This mixture was separated by normal phase HPLC with 8% ether in hexane. The three peaks were collected by fractionation and quantified using LSC. The [^{14}C]linoleic acid, methyl ester collected from the HPLC was then oxidized with DMDO and the separation repeated. This was repeated until all [^{14}C]linoleic acid, methyl ester was oxidized. This was also sufficient to separate the ltx and iltx regioiosmers. There was no need for further purification as determined by TLC and HPLC analysis. Similar fractions were pooled and the solvent was evaporated. The samples were stored at −20°C under nitrogen until use. [^{14}C]-9(10)-*Z*-EpO, methyl ester was prepared similarly except that separation by HPLC was unnecessary because oxidation of [^{14}C]oleic acid, methyl ester by DMDO yielded a single product, which comigrated with authentic 9(10)-*Z*-EpO, methyl ester.

Synthesis of nonradioactive fatty acid epoxides and diols. The nonradioactive samples were synthesized as previously described (21, 25, 26) using methods similar to those described for the radioactive samples described above, except that fatty esters were oxidized with equimolar *meta*-chloroperoxybenzoic acid (*m*-CPBA) in methylene chloride (19, 27). Purified epoxides were hydrolyzed to the corresponding *vic*-diols in 1:1 v/v acetonitrile:5% aqueous perchloric acid. Synthesized compounds were purified by nitrogen pressurized flash chromatography on silica gel using equal volume step gradients of hexane/ethyl acetate mixtures (typically 5, 10, and 20% ethyl acetate). Fractionation was monitored by phosphomolybdic acid (4 g in 100 ml 4:1 H_20:ethanol) visualization of aliquots applied to silica gel TLC plates. Similar fractions were pooled and the solvent was evaporated. For regioisomers, separation was effected on a rotary TLC plate (Chromatron model 7924; Harrison Research) with 3% ethyl acetate in hexane. The compounds were analyzed for purity by GC-FID. The oven was initially held at 205°C for 10 min, ramped at 30°C/min to 228°C, then 2°C/min to 235°C, and finally at 20°C/min to 325°C. All compounds were greater than 96% pure and cochromatographed with authentic standards. The samples were stored at −20°C under nitrogen until use.

Soluble epoxide hydrolase preparation. Recombinant hsEH was produced in a baculovirus expression system (28) and purified by affinity chromatography (29). The preparations were at least 97% pure as judged by SDS–PAGE and scanning densitometry. No detectable esterase or glutathione transferase activity, which can interfere with this sEH assay, was observed (29). Protein concentration

was quantified using the Pierce BCA assay (Rockford, IL) using bovine serum albumin (BSA) as the calibrating standard.

Soluble epoxide hydrolase assays. Various concentrations of recombinant hsEH were incubated in a sodium phosphate buffer (100 μl, 100 mM, pH 7.4) containing 0.1 mg/ml BSA, placed in glass culture tubes, and kept on ice until the assay was initiated. Stock solutions of [^{14}C]-(12*Z*)9(10)-*Z*-EpOME, methyl ester, [^{14}C]-9(*Z*)12(13)-*Z*-EpOME, methyl ester and [^{14}C]-9(10)-*Z*-EpO, methyl ester (various concentrations, 5 nCi/μl) were prepared in ethanol and the assay was initiated by addition of the stock solution (1 μl) with a Hamilton repeating syringe into the buffer containing hsEH. Controls for nonenzymatic hydrolysis were performed by addition of substrate to buffer. Tubes were incubated in a shaking 37°C water bath for 5 min or as specified for each particular experiment. The reaction was stopped by addition of ether (100 μl) and sodium chloride. The sample was centrifuged and quick-frozen in a dry ice-acetone bath. This froze the aqueous layer, allowing the entire organic layer to be spotted on a preabsorbant TLC plate. Nonradioactive standards of ltx, iltx, ltxd, and iltxd were prespotted in the same lane as the sample. The plate was developed in 4:1 hexane:ethyl acetate. After the plate was thoroughly dried, the amount of epoxide and diol present were quantified by the Bioscan radioactive TLC scanner, as previously described (20), and visualized with phosphomolybdic acid and heat. In all assays the only detectable radioactive species comigrated with authentic nonradioactive standards and no other radioactive species were detected. Enzyme assays with *t*-DPPO were performed as previously described (20).

The Michaelis–Menten parameters for the epoxides were determined under steady-state conditions using eight substrate concentrations (1.0–50 μM, 5 nCi/μl). The final concentration of hsEH in buffer was 0.26 μg/ml. K_M and V_{max} values were determined by the method described by Wilkinson (30). Assay conditions for kinetic determination were as specified above. Appropriate times of incubation were determined, which allowed the enzymatic hydrolysis to be linear during the assay period for each particular substrate concentration.

Inhibition assays. Inhibition of hsEH by N,N′-dicyclohexyl urea (DCU) (31) was determined using racemic [^{14}C]leukotoxin as substrate. The enzyme (66 nM hsEH) was incubated with the DCU ([I] final concentrations from 1 to 10^4 nM) for 5 min in pH 7.4 sodium phosphate buffer at 30°C prior to substrate introduction ([*S*] = 50 μM). Activity was assessed by measuring the appearance of leukotoxin diol as described above. Assays were performed in triplicate. By definition, IC_{50} is the concentration of inhibitor that reduces enzyme activity by 50%.

Inhibition of hsEH by ltxd/iltxd was determined using racemic [^{3}H]-*t*-DPPO as substrate (20). The enzyme (12 nM hsEH) was incubated with the ltxd/iltxd ([I] final concentrations from 10 to 10^3 μM) for 10 min in pH 7.4 sodium phosphate buffer at 30°C prior to substrate introduction ([*S*] = 50 μM). The remaining activity was measured as described (20).

Metabolite studies. Sf-21 cells were grown and infected as described above. 48 h postinfection, either 220 mM [^{14}C]-ltx/iltx (0.8178 μCi), 125 mM ltxd/iltxd as a positive control or 50 μl DMSO as a vehicle control were added to the cells. At 0, 1, 2, 4, 8, 12, 16, and 24 h, 0.5 ml aliquots were removed. Cells and media were separated by centrifugation at 3000*g*. The cells were washed twice with fresh media and the media was pooled. A buffered solution of acetic acid (200 μl at pH = 4) was added to each cell or media sample and subsequently extracted three times with hexane:ethyl acetate (1:1; 500 μl for cell samples, 1000 μl for media samples). Samples were vortexed, centrifuged at 4000*g*, and the organic extracts were combined. Each sample was spiked with a standard mixture of methyl linoleate, and the methyl esters of ltx, iltx, ltxd, and iltxd and evaporated to dryness using a centrifugal vacuum evaporator. Aqueous and organic fractions were counted by LSC. Samples were reconstituted in 10 μl of hexane:ethyl acetate (1:1) and were loaded on to 20 × 20-cm silica gel plates. The plates were developed in solvent A (hexane:ethyl acetate (2:1)) and analyzed for radioactivity by the Bioscan radioactive TLC scanner and visualized with phosphomolybdic acid and heat. As will be shown in the results section, this resulted in several identifiable metabolites as well as a highly polar metabolite, tentatively identified as a phospholipid. All assays were performed in triplicate.

In order to verify the identity of this polar metabolite and to rigorously establish the amount of radioactivity in all possible pools, the above experiment was repeated with a few minor changes. In order to remove the extra variable of toxicity, only Lac Z expressing Sf-21 cells were used. Either 0.22 mM [^{14}C]-ltx/iltx (0.055 μCi) or 0.06 mM [^{14}C]-ltxd/iltxd were added to the cells, 48 h postinfection. CO_2 traps, consisting of 0.6 ml syringes, with plungers removed, were filled with 0.5 ml saturated KOH, and both ends sealed. Holes were made around the top of the syringe to allow free circulation of air within the cell flask. The syringes were suspended from the top of the spinner flasks containing the cells, and the whole apparatus sealed with three layers of Teflon tape. At 2 and 24 h the KOH solution was analyzed by LSC, correcting for quench, and aliquots were removed from the cell suspensions. Cells and media were separated by centrifugation at 2000*g*. The cells were washed twice with fresh media and the media was pooled. A buffered solution of acetic acid (200 μl at pH = 4) was added to each cell or media sample and subsequently extracted three times with chloroform:methanol (2:1) as described (32). Samples were mixed, centrifuged at 4000*g* and the organic extracts were combined. Samples were counted by LSC. Each sample was spiked with a standard mixture of methyl linoleate, and the methyl esters of ltx, iltx, ltxd, and iltxd and evaporated to dryness using a centrifugal vacuum evaporator. Samples were reconstituted in chloroform:methanol (2:1) and applied to TLC plates. Plates were developed in solvent B (petroleum ether:diethyl ether: acetic acid (80:20:1)) for fatty acid analysis, analyzed for radioactivity by plate scanner, and visualized with phosphomolybdic acid and heat. Alternately, plates were developed in solvent C (chloroform: methanol:acetic acid:water (100:67:7:4)) for phospholipid analysis, analyzed for radioactivity by plate scanner, and stained with iodine or ninhydrin and heat. All assays were performed in triplicate.

Phospholipase A_2 hydrolysis. Cellular fractions from the above experiment were dried under nitrogen to a lipid residue and resuspended in 1 ml of sodium phosphate buffer (pH 8.0) containing 5 mM calcium chloride. To this solution was added 50 units of porcine pancreatic phospholipase A2 (Sigma). The solution was mixed and incubated at 37°C for 1 h. After incubation, the lipids were extracted with 1 ml of ethyl acetate after acidification with acetic acid to pH 4.0. All assays were performed in triplicate.

Esterase assays. Four known esterase inhibitors (3-*n*-pentylthio-1,1,1-trifluoro-2-propanone (PTFP), 3-*n*-octylthio-1,1,1-trifluoro-2-propanone (OTFP), bis-(*para*-nitrophenyl)phosphate (BNPP) and phenyl methyl sulfonyl fluoride (PMSF)) were assayed for toxicity (0.06–1 mM final concentration for all) and for their ability to inhibit esterases in the Sf-21 cell system. Esterase activity assays were performed using *para*-nitrophenyl acetate (PNPA), as previously described (33). However, only PMSF was found to be nontoxic to these cells at an effective dose (40% of esterase activity). To assess the effect of esterase inhibition on epoxy fatty ester toxicity, cells were preincubated with PMSF (1 mM for 2 h) followed by exposure to various concentrations of the free acid or methyl, ethyl, *n*-propyl, isopropyl, or *n*-butyl esters of epoxy stearate (9,10-EpO). All assays were performed in triplicate.

Glutathione assays. Glutathione transferase levels were measured using 1-chloro-2,4-dinitrobenzene (CDNB) (34). We were unable to detect any glutathione transferases in whole Sf-21 cell homogenate under conditions where we could easily see conjugation of 0.03 μmol/min/mg protein. Glutathione (GSH) levels were measured as free sulfhydryls using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (35).

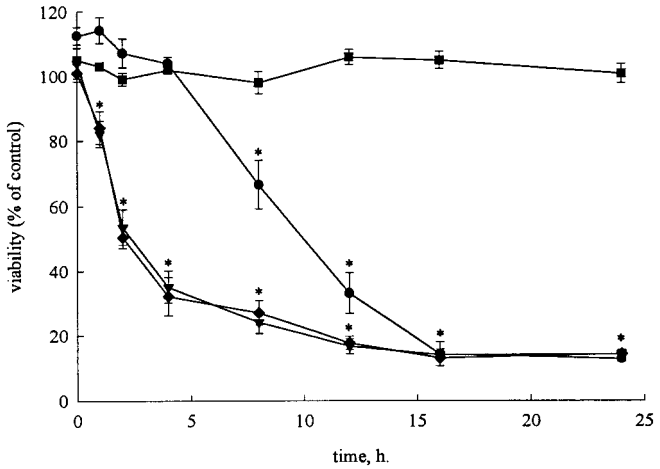

FIG. 1. Time-dependent toxicity of ltx/iltx and ltxd/iltxd. Sf-21 cells expressing either hsEH (● ltx/iltx, ▼ ltxd/iltxd) or Lac Z (■ ltx/iltx, ◆ ltxd/iltxd) were exposed to compounds for 24 h. Viability was measured by MTT as described under Materials and Methods. Values represent mean ± SD from at least four independent experiments. *Significantly different ($P \leq 0.01$) from control cells (treated with DMSO).

To assess the effect of cellular GSH levels on ltx/iltx induced toxicity cells were preincubated for 2 h with either DMSO (vehicle control), 0.125 mM diethyl maleate (DEM), or 10 mM ethyl ester glutathione (EEG), a glutathione analog that can cross the cell membrane (36), before addition of 0.125 mM ltx/iltx. All assays were performed in triplicate.

Glutathione conjugate formation. Cells (20×10^6) were washed 3× with 1× phosphate buffered saline and resuspended in sodium phosphate buffer (pH 8.0) containing GSH (1 mM, 5 mM, or 10 mM final concentration). The assay was initiated by addition of 25 μl of 100 mM [^{14}C]-ltx/iltx (final concentration: 0.5 mM, 7.5 nCi). Cells were incubated in a shaking water bath at 28°C for 24 h and aliquots taken at 0, 2, 4, 8, 16, and 24 h. Aliquots were extracted with hexane:ethyl acetate (1:1) after acidification with acetic acid to pH 4.0. Organic and aqueous phases were counted. Alternately, 0.5 mM ltx/iltx was incubated with acetonitrile:sodium phosphate buffer (pH 8.0) (2:1) containing GSH (1, 5, or 10 mM final concentration). Assays were run under nitrogen, stirring vigorously at room temperature, and aliquots taken and extracted as described above, 9,10-epoxy stearate was added as an external standard and the amount of ltx/iltx present was quantified by GC-FID using a standard curve. All assays were performed in triplicate.

RESULTS

Time-dependent toxicity of ltx/iltx and ltxd/iltxd. While previous reports have shown that ltx, ltxd, and their regioisomers are toxic in a concentration-dependent manner (21), time-dependence of toxicity has not been established (Fig. 1). Since toxicity appears to be equivalent between regioisomers, we used mixtures of these isomers. Sf-21 cells expressing either hsEH or Lac Z were incubated with 0.125 mM ltx/iltx or ltxd/iltxd for 24 h. Similar to the results from the dose-dependence studies, ltx/iltx was not toxic unless hsEH was present. The LT_{50} for ltx/iltx in hsEH expressing cells was 10.6 ± 0.8 h. The LT_{50} for ltxd/iltxd in hsEH or Lac Z expressing cells was 2.6 ± 0.7 h.

Apparent kinetic constants for hsEH. To allow a comparison between time to toxicity and ltx/iltx activation rate by hsEH, we measured the kinetic constants for recombinant hsEH produced in Sf-21 cells with ltx and iltx. Synthesis and separation of [^{14}C] labeled ltx and iltx were performed as described above. Various concentrations (1.0–50 μM) of the two compounds were incubated with recombinant hsEH at 37°C for 5 min. Results are linearized in a Lineweaver–Burk plot (Fig. 2). For comparison, we also measured the kinetic constants of 9,10-epoxy stearate, methyl ester, a ltx analog lacking the double bond, and *trans*-diphenylpropene oxide (*t*-DPPO), an exogenous substrate for sEH (20). The results are presented in Table I. The kinetic constants for murine sEH have previously been measured using 9,10-epoxystearate, methyl ester and *t*-DPPO (20). Our results show that K_M values for ltx and iltx are in the same range as the K_M for *t*-DPPO (5.17–6.15 μM), but the V_{max} for *t*-DPPO is two- to threefold greater than for ltx or iltx. This can be seen as well in the k_{cat}/K_M ratio, which is threefold greater for *t*-DPPO, indicating that it is a better substrate than ltx or iltx. The similarity in the k_{cat}/K_M ratio for ltx and iltx indicates that the enzyme is not highly regioselective. Surprisingly, the K_M and the V_{max} for 9,10-epoxy stearate, methyl ester, were much lower than for the other substrates. However, the V_{max} values for both 9,10-epoxystearate, methyl ester and *t*-DPPO were in the same range as values previously seen for specific activity of these compounds with both crude and pure hsEH and murine sEH (19, 20). However, Halarnkar *et al.* found that while the relative rates of

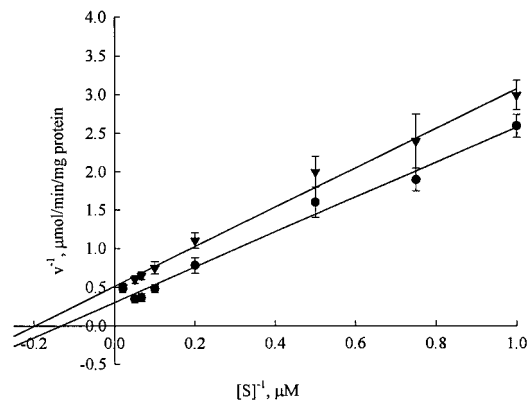

FIG. 2. Lineweaver–Burk plot of ltx and iltx. Recombinant hsEH was incubated with various concentrations of ltx (●) or iltx (▼) at 37°C for 5 min. The K_M and V_{max} of ltx and iltx were calculated to be 6.15 ± 1.0 μM, 2.67 ± 0.04 μmol/min/mg protein and 5.17 ± 0.56 μM, 1.86 ± 0.06 μmol/min/mg protein, respectively. These values agree well with the K_M and V_{max} obtained by fitting the data to the Michaelis–Menten equation by the method described by Wilkinson (30); results are mean ± SD.

TABLE I

Apparent Kinetic Constants for hsEH

Substrate	$K_M(\mu M)$	V_{max} (μmol min^{-1} mg^{-1})	k_{cat}[a] (s^{-1})	k_{cat}/K_M (s^{-1} μM^{-1})
Methyl leukotoxin	6.15 ± 1.0	2.67 ± 0.04	2.78	0.452
Methyl isoleukotoxin	5.17 ± 0.56	1.86 ± 0.06	1.93	0.37
Methyl epoxy stearate	1.2 ± 0.9	1.12 ± 0.1	1.16	0.97
t-DPPO	5.80 ± 0.7	7.8 ± 1.3	8.11	1.40

Note. The values are means ± SD of at least three experiments done in triplicate for each substrate concentration at 37°C as described under Materials and Methods.

[a] Calculated based on a molecular weight of 62.5 kDa.

hydrolysis of ltx and iltx with purified human liver were in the same range as our values, the specific activity for iltx was higher than that of ltx (19), the reverse of our data. Nonetheless, these results also suggest that ltx and iltx are good substrates for hsEH and that hsEH may play a large role in their metabolism *in vivo.*

Inhibition of hsEH. As a further test of the hypothesis that ltx and iltx are substrates for hsEH, we examined metabolism of ltx and iltx with hsEH inhibited. We have previously seen that hsEH expressing cells pretreated with 60 μM *N, N′*-dicyclohexyl urea (DCU), an hsEH inhibitor, are protected from all toxicity induced by 1 mM ltx/iltx *in situ* (31). We therefore repeated these experiments *in vitro.* Recombinant hsEH was preincubated with DCU and activity assayed with ltx. The IC_{50} for DCU under these conditions was 44 ± 2 nM, comparable to the IC_{50} previously seen with DCU, using *t*-DPPO as a substrate (31).

One recognized mode of enzymatic regulation is a negative feedback loop resulting from product inhibition. Ltxd/iltxd inhibition of sEH could be a modulating factor in toxicity. Therefore, the inhibitory potential of ltxd/iltxd was tested and found to have no effect at concentrations as high as 1 mM (data not shown).

In situ metabolism of ltx/iltx. In order to determine whether or not ltxd/iltxd was the final toxic metabolite, Sf-21 cell cultures expressing either hsEH or Lac Z were incubated with 0.22 mM [^{14}C]-ltx/iltx for 24 h. Total radioactivity was quantified by LSC (Table II), and >95% of the radioactivity was found in the organic extractions of the cellular and media fractions. Radioactive metabolites were identified and quantified by means of a plate scanner. 100 ± 5% of total radioactiv-

TABLE II

Radioactivity Associated with [^{14}C]-Ltx/iLTX, Methyl Ester and [^{14}C]-Ltxd/Iltxd, Methyl Ester after Incubation with Sf-21 Cells for 2 or 24 h

	[^{14}C]-ltx/iltx, methyl ester, % radioactivity			[^{14}C]-ltxd/iltxd, methyl ester, % radioactivity		
	Cells	Media	Both	Cells	Media	Both
2 h						
Organic	43.8 ± 2.1	51.0 ± 2.3	94.8 ± 4.4	27.5 ± 0.7	67.9 ± 1.8	95.4 ± 2.5
Aqueous	<1.0	1.8 ± 0.6	1.8 ± 0.6	<1.0	2.9 ± 0.7	2.9 ± 0.7
Protein	<1.0	<1.0	<1.0	<1.0	1.8 ± 0.3	1.8 ± 0.3
KOH			<1.0			<1.0
Total			97.0 ± 5.0			100.1 ± 3.5
24 h						
Organic	25.4 ± 2.8	69.2 ± 3.2	94.6 ± 6.1	26.3 ± 0.9	68.5 ± 1.1	94.8 ± 2.0
Aqueous	<1.0	2.9 ± 0.4	2.9 ± 0.4	<1.0	2.1 ± 0.4	2.1 ± 0.4
Protein	<1.0	1.4 ± 0.4	1.4 ± 0.4	<1.0	2.3 ± 0.5	2.3 ± 0.5
KOH			<1.0			<1.0
Flasks			<1.0			<1.0
Total			98.9 ± 6.9			99.2 ± 2.9

Note. Lac Z expressing cells were incubated with either 0.22 mM [^{14}C]-ltx/iltx, methyl ester or 0.06 mM [^{14}C]-ltxd/iltxd, methyl ester for 24 h. DMSO solutions (33.5 μl, (ltx/iltx) 0.05 ± 0.003 μCi, (ltxd/iltxd) 0.15 ± 0.006 μCi) of lipid were added to initiate the reaction. Aliquots were taken at various time points. Cells and media were separated and extracted as described under Materials and Methods. Fractions were counted by LSC. Experiments were performed in triplicate, values are mean ± SD.

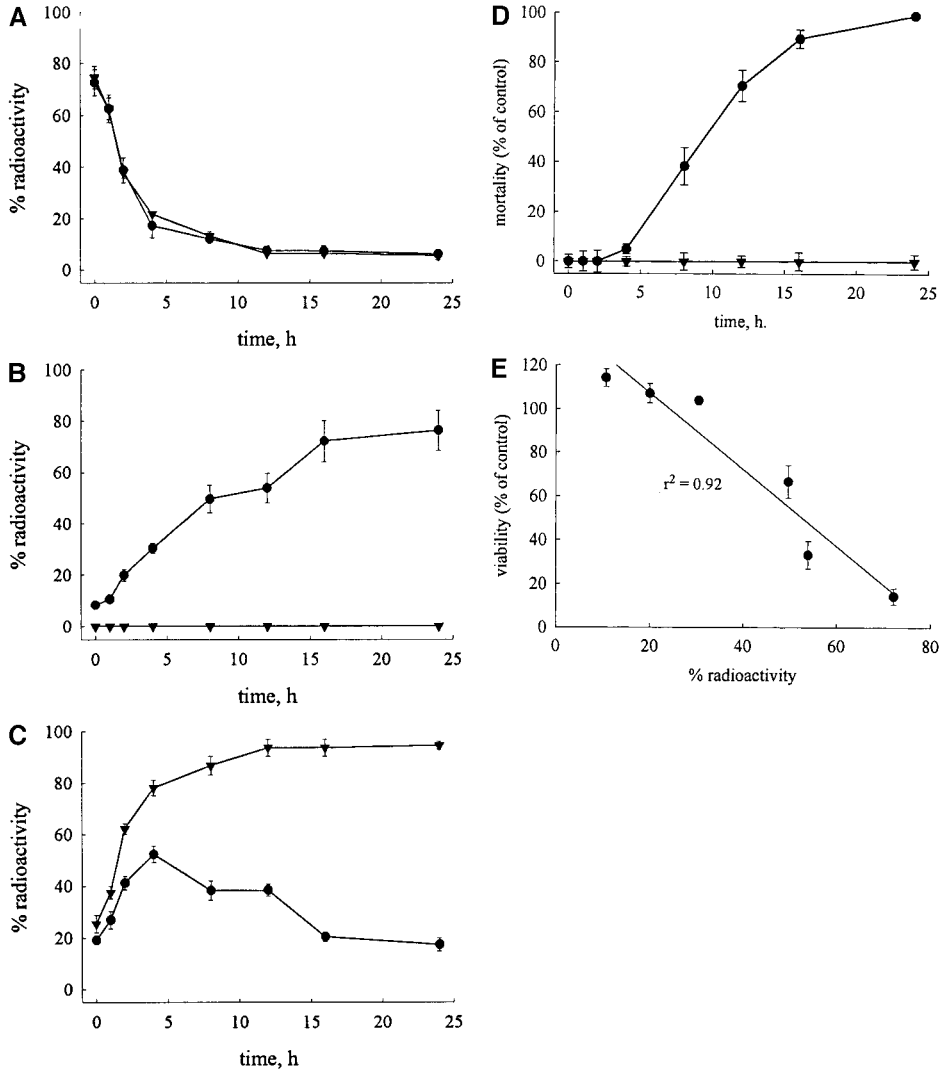

FIG. 3. Major metabolites of [^{14}C]-ltx/iltx in media. Sf-21 cells expressing either hsEH (●) or Lac Z (▼) were treated with 0.220 mM [^{14}C]-ltx/iltx for 24 h. After removing cells, media were extracted at the times indicated, radioactive components separated on TLC, analyzed by radioactive TLC scanner, and quantified by LSC (98.3 ± 5.6% of total radioactivity was recovered, 50–70% of that was in the media fractions). Two major metabolites were seen in the media. Shown here is the percentage of radioactivity for compounds in the media fractions: (A) methyl ltx/iltx (Rf: 0.78), (B) ltxd/iltxd, free acid (Rf: 0.08), and (C) ltx/iltx, free acid (Rf: 0.37). (D) Mortality over time. (E) The correlation of viability with diol production in hsEH-expressing cells. Assays were performed in triplicate; results are mean ± SD. If not shown, the standard deviation of the replicate is smaller than the point on the graph.

ity was recovered which corresponded to four major metabolites in combined media and cell extracts. Subsequently the media and cells were analyzed separately. Two major metabolites (Rf: 0.04 in solvent B) were found in the cellular fraction. These metabolites migrated in a manner consistent with phospholipids and stained with ninhydrin, indicating the presence of a free amine. Two major metabolites (Rf: 0.37, 0.08, in solvent B) were found in the media fraction of hsEH expressing cells; these cochromatographed with authentic standards of ltx/iltx, free acid, and ltxd/iltxd, free acid, respectively. No ltxd/iltxd methyl esters were found as metabolites in any experiment. Only one major metabolite (Rf: 0.37 in solvent B) was found in the media fraction of Lac Z expressing cells; this compound cochromatographed with authentic ltx/iltx, free acid. Notably, we did not see a peroxy fatty acid metabolite which would have been apparent with UV visualization. Two of the three metabolites were found in both hsEH and Lac Z expressing cells treated with ltx/iltx; but the ltxd/iltxd, free acid metabolite was only found in cells expressing hsEH (Figs. 3A–3C). Toxicity was only found in cells expressing hsEH (Fig. 3D) and diol production correlated well with toxicity (Fig. 3E). These data are consistent with the hypothesis that the diol is the toxic metabolite. While the cells were

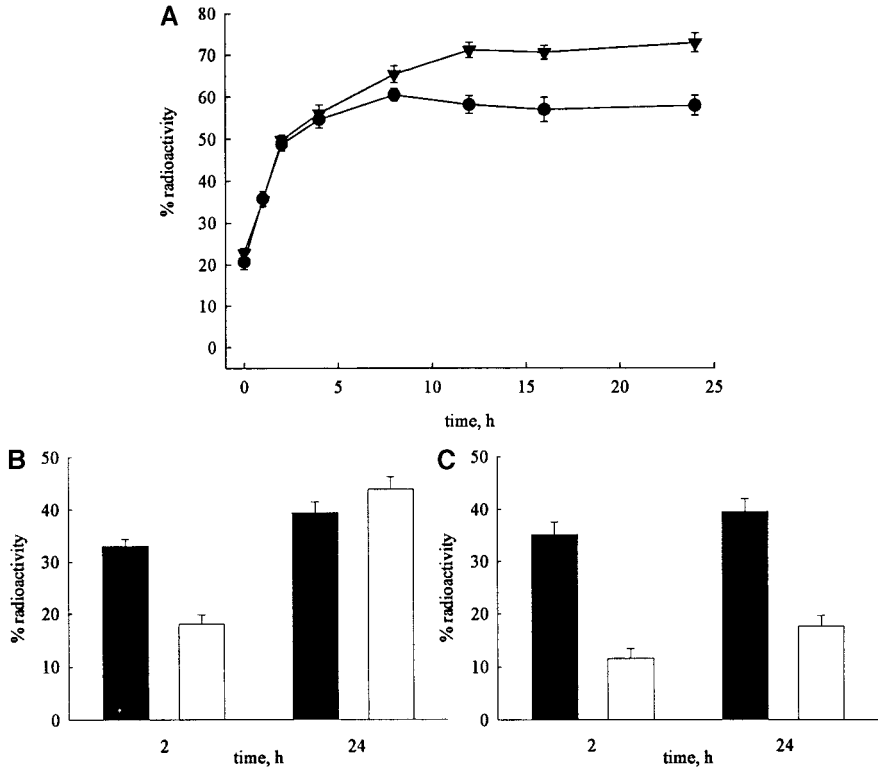

FIG. 4. Analysis of putative phospholipids found from [^{14}C]-ltx/iltx. Sf-21 cells expressing either hsEH (●) or Lac Z (▼) were treated with 0.22 mM [^{14}C]-ltx/iltx for 24 h. Cells and media were extracted and radioactive components were separated on TLC, analyzed by plate scanner, and quantified by LSC (98.3 ± 5.6% of total radioactivity was recovered, 25–45% of that was in the cellular fractions). Analysis on TLC with solvent B showed several minor, but (A) one major metabolite at the origin which increased over 24 h. After development in solvent C, this metabolite was found to have two major components which cochromatographed with phosphotidylcholine and phosphotidylethanolamine. Lac Z expressing cells were treated with either (B) 0.22 mM [^{14}C]-ltx/iltx or (C) 0.06 mM [^{14}C]-ltxd/iltxd for 24 h. Cell extracts were analyzed at 2 and 24 h for percentage of radioactivity associated with phosphotidylcholine (black bars) and phosphotidylethanolamine (white bars). Upon treatment with a phospholipase, cell extracts released 75.2 ± 6.1% of their radioactivity as ltx/iltx, free acid or ltxd/iltxd, free acid, respectively. All experiments were done in triplicate, results are mean ± SD.

treated with methyl esters, the majority of the [^{14}C]-ltx/iltx and all of the [^{14}C]-ltxd/iltxd analyzed was in the free acid form, indicating the presence and activity of cellular esterases. The percentage of radioactivity in each fraction was similar for assays where aliquots were taken at 0, 1, 2, 4, 8, 12, 16, and 24 h as well as in assays where aliquots were taken at 2 and 24 h.

The majority of the radioactivity in the cell pellets was associated with metabolite 1 (Rf: 0.04 in solvent B) (Fig. 4A), the remainder corresponded to the carboxylic acid forms of ltx/iltx or ltxd/iltxd. To investigate the identity of this polar metabolite, 2 and 24 h cellular fractions of Lac Z-expressing cells treated with either [^{14}C]-ltx/iltx or [^{14}C]-ltxd/iltxd were spotted on TLC plates, developed in solvent C, and stained with either ninhydrin and heat or iodine in order to separate and visualize the phospholipids (Figs. 4B and 4C). Two major metabolites were found corresponding to authentic standards of phosphotidylcholine (Rf: 0.25 in solvent C) and phosphotidylethanolamine (Rf: 0.76 in solvent C), respectively. Both metabolites stained with iodine, but only the metabolite which comigrated with phosphotidylethanolamine stained with ninhydrin, indicating the presence of a free amine. Upon incubation of the Lac Z expressing cellular fractions with phospholipase A2, cells which had been incubated with [^{14}C]-ltx/iltx methyl ester released 73.1 ± 3.4% of the radioactivity associated with the phospholipids as ltx/iltx, free acid. Cells which had been incubated with [^{14}C]-ltxd/iltxd methyl ester released 76.2 ± 4.9% of the radioactivity associated with the phospholipids as ltxd/iltxd, free acid. (data not shown). Lac Z-expressing cells incubated with ltx/iltx had twice as much ltx/iltx incorporated into phosphotidylcholine as phosphotidylethanolamine at 2 h, but at 24 h the ratio was 1:1. Lac Z-expressing cells incubated with ltxd/iltxd, on the other hand, incorporated ltxd/iltxd into phosphotidylcholine:phosphotidylethanolamine at a 3:1 ratio at 2 h and a 2:1 ratio at 24 h. Additionally, a greater proportion of the radioactivity associated with the cellular fraction of ltx/iltx-treated Lac Z-expressing cells was

incorporated into phospholipids. Given the higher rate of incorporation of epoxyeicosanoids than dihydroxyeicosanoids into phospholipids (37, 38) this may indicate that ltx/iltx is a better acyl transferase substrate or imply that 220 mM ltx/iltx is a saturating concentration for phosphotidylcholine uptake.

Inhibition of cellular esterases and mediation of epoxide toxicity. We have previously shown that Sf-21 cells contain low levels of cellular esterases (39). We have also shown that viability of cells treated with the epoxystearate series: 9,10-EpO; 9,10-EpO, methyl ester; 9,10-EpO, ethyl ester; 9,10-EpO, *n*-propyl ester; 9,10-EpO, isopropyl ester; 9,10-EpO, *n*-butyl ester; viability increases with increasing ester size (40) Thus, to test the hypothesis that the free acid epoxide was the most potent protoxin, we attempted to inhibit cellular esterases and challenge the cells with the series listed above. Because epoxystearate has toxicity equivalent to that generated by ltx in this system (40), and is much easier to modify synthetically, we decided it was appropriate to continue using the epoxystearate series. We first tested four esterase inhibitors for toxicity in the system (0.06–1 mM, final concentration) and for their ability to inhibit Sf-21 cell esterases. Only PMSF-treated cells did not display acute toxicity at concentrations sufficient to inhibit 40% of esterase activity (1 mM PMSF), while the others were toxic at or below 0.25 mM (data not shown). We preincubated Sf-21 cells expressing hsEH or Lac Z with 1 mM PMSF for 2 h, then incubated with 0.06–1 mM 9,10-EpO, 9,10-EpO, methyl ester, 9,10-EpO, ethyl ester, 9,10-EpO, *n*-propyl ester, 9,10-EpO, isopropyl ester, or 9,10-EpO, *n*-butyl ester for 24 h. Viability at the highest concentration (1 mM) is shown (Fig. 5). Strangely, with esterase inhibition, viability was increased between 10–20% for hsEH expressing cells treated with the free acid, *n*-propyl ester, isopropyl ester, and *n*-butyl ester forms of the epoxide, but not for the methyl ester and ethyl ester forms. The corresponding series of diol esters gave the same results with and without PMSF (data not shown).

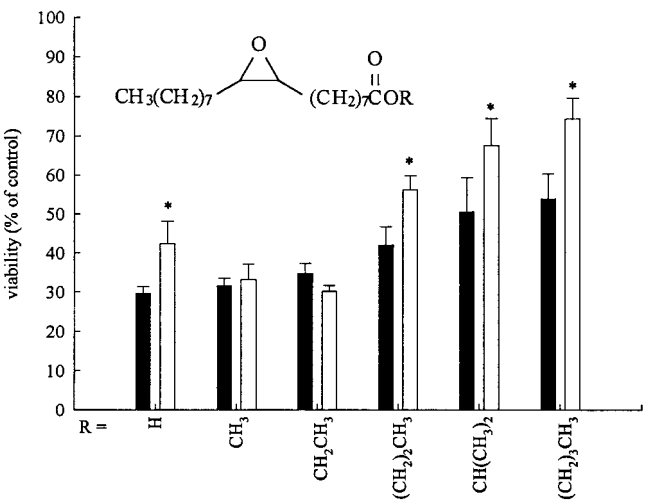

FIG. 5. Modulation of epoxy fatty ester toxicity by PMSF. Sf-21 cells expressing hsEH were pretreated with DMSO (black bars) or 1 mM PMSF (white bars) for 2 h and then treated with 1 mM compound for 24 h. Viability was measured by MTT. Assays were performed in triplicate; results are mean ± SD. *Significantly different ($P \leq 0.05$) from control cells (treated with DMSO). The corresponding series of diol esters gave the same results with and without PMSF (data not shown).

TABLE III

Free Sulfhydryl Levels in Sf-21 Cells before and after Treatment

Enzyme	Compound, (mM)	Free sulfhydryl levels (nmol/mg protein) 0 h	24 h
hsEH	ltx/iltx[a] 0.125	43.2 ± 7.4	3.9 ± 0.4
	ltxd/iltxd[a] 0.125	45.2 ± 6.2	2.7 ± 0.9
	DEM, 0.125	41.8 ± 4.0	10.2 ± 1.6
	EEG, 10.0	43.6 ± 5.3	61.1 ± 6.2
	DMSO	40.5 ± 9.2	31.9 ± 4.0
Lac Z	ltx/iltx[a] 0.125	44.3 ± 7.8	11.8 ± 1.5
	ltxd/iltxd[a] 0.125	44.4 ± 5.6	3.9 ± 1.3
	DEM, 0.125	41.4 ± 8.2	12.7 ± 3.0
	EEG, 10.0	46.3 ± 7.1	64.0 ± 5.4
	DMSO	42.9 ± 5.6	43.2 ± 7.6

Note. As measured by DTNB (34). Means are representative of at least three experiments conducted as described under Materials and Methods. With the exception of DMSO-treated cells, means at 0 and 24 h are significantly different.

[a] Mixture of the two regioisomers.

The effect of cellular sulfhydryl levels on ltx/iltx toxicity. Numerous epoxide containing compounds are known to affect cellular sulfhydryl levels. To determine if cellular sulfhydryl balance was implicated in ltx-induced toxicity, we measured free sulfhydryl levels (Table III) in Sf-21 cells. Under normal conditions, Sf-21 cells have 30–54 nmol free sulfhydryls/mg protein as measured by DTNB. We then tested the hypothesis that ltx/iltx would lower free sulfhydryl levels in the cells. The results are shown in Table III. Ltx/iltx (0.125 mM) depleted free sulfhydryl levels in Sf-21 cells to 10% of normal in hsEH expressing cells and 27% of normal in Lac Z-expressing cells over 24 h. Significantly, the Lac Z-expressing cells were still alive. Treatment with 0.125 mM diethyl maleate (DEM), a known GSH depletor, also decreased free sulfhydryl levels to 25% of normal, while not affecting cell viability. Conversely, 10 mM ethyl ester glutathione (EEG), a GSH analog, increased intercellular sulfhydryl levels to 140% of normal.

We then tested the hypothesis that increased free sulfhydryl levels would protect the cells from the tox-

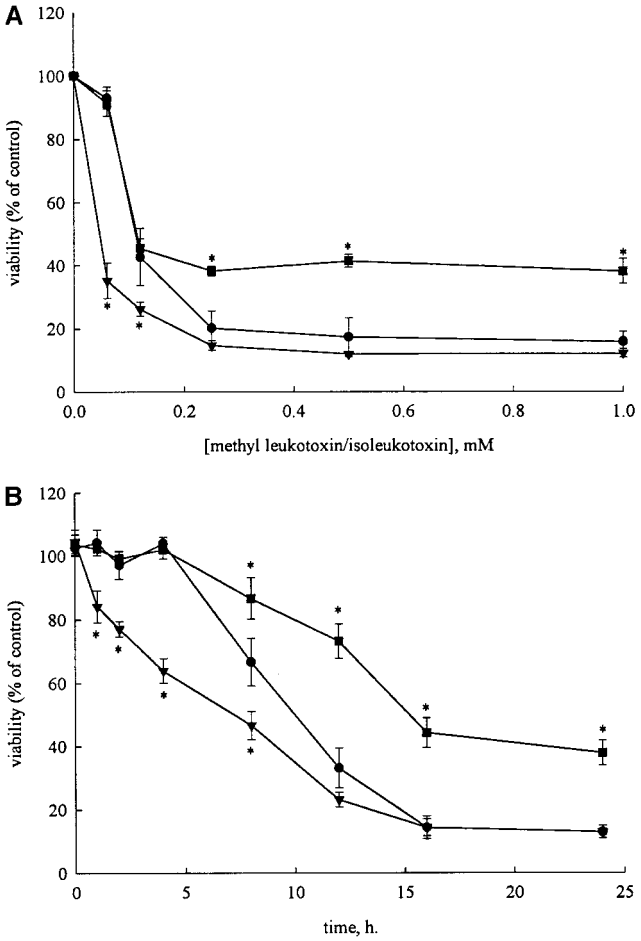

FIG. 6. Protection from ltx/iltx toxicity by EEG. Sf-21 cells expressing hsEH were preincubated with DMSO (●), 0.125 mM DEM (▼), or 10 mM EEG (■). (A) Cells were then treated with various concentrations of ltx/iltx and toxicity assessed by MTT. (B) Alternately, cells were treated with 0.125 mM ltx/iltx and toxicity assayed over time. Values represent mean ± SD from at least three independent experiments. *Significantly different ($P \leq 0.05$) from control cells (treated with DMSO).

icity of ltx/iltx and that decreased free sulfhydryl levels would increase the toxicity of ltx/iltx. Sf-21 cells expressing hsEH were preincubated with DMSO (vehicle control), 0.125 mM DEM, or 10 mM EEG for 2 h, then treated with various concentrations of ltx/iltx (Fig. 6A). DEM decreased the LC_{50} from 0.12 to 0.055 mM. EEG protected the cells from ltx/iltx-induced toxicity at higher concentrations (0.25–1.0 mM), increasing viability 20%. This same pattern held true when time was the independent variable (Fig. 6B). As before, cells were pretreated with DMSO, 0.125 mM DEM, or 10 mM EEG for 2 h, then challenged with 0.125 mM ltx/iltx. DEM reduced viability earlier, decreasing the LT_{50} from 10 to 6 h. EEG, conversely, protected the cells at later time points (12–24 h), increasing the LT_{50} to 15 h. These data, taken in concert, suggest that free sulfhydryls play an important role in moderating the toxicity of ltx/iltx.

A possible detoxification pathway for ltx/iltx is through conjugation with cellular sulfhydryls; we would expect this to be a viable pathway if glutathione transferases (GST) were present. Thus, we assayed Sf-21 cell homogenate for glutathione transferase activity but were unable to detect any, using CDNB as a substrate, under conditions where we could easily see conjugation of 0.03 μmol/min/mg protein. To assess the potential for direct conjugation of ltx/iltx by GSH, we attempted to create the conjugate chemically using 0.5 mM ltx/iltx and either 1, 5, or 10 mM GSH in acetonitrile and sodium phosphate buffer vigorously stirring over 24 h. We recovered >98% of the ltx/iltx added and were unable to detect any conjugation (data not shown). We then repeated the experiment with Sf-21 cell homogenate and 0.5 mM ltx/iltx and 1, 5, or 10 mM GSH in sodium phosphate buffer. We again recovered >96% of the ltx/iltx added and were again unable to detect any conjugation (data not shown). However, at 24 h, 75 ± 8% of the ltx/iltx recovered had been converted to the carboxylic acid, indicating that cellular esterases were still active.

DISCUSSION

Collectively, these results indicate an expanded pathway of metabolism from that previously suggested (21) (Fig. 7). Reported investigations of ltx/iltx metabolism have been sparse, primarily resulting from investigations of either epoxide formation and hydrolysis or, conversely, metabolism of mono- or dihydroxy long chain fatty acids. In comparison, the related epoxy

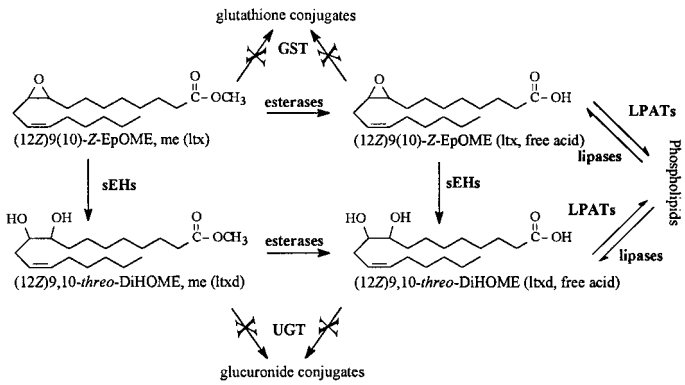

FIG. 7. Proposed pathway for metabolism of ltx/iltx. Only the single regioisomer, ltx, is shown, but this pathway is valid for iltx as well. LPATs are lysophospholipid acyl transferases. UGTs are UDP-glucuronosyl transferases; it is expected that glucuronide conjugation of ltxd will occur in cells containing UGTs, as has been previously shown (70), but that does not occur in this system. Likewise, it is expected that the GSH conjugate will be formed in the presence of GSTs, but that also does not occur in this system. Based on monitoring for $^{14}CO_2$, no evidence of β-oxidation was seen.

eicosanoids have received a great deal of attention. Where relevant, these data have been incorporated into the proposed pathway.

Various studies on epoxidation of polyunsaturated fatty acids indicate that a cytochrome P450 of the 2C, 2B, or 2J class is likely responsible for the enzymatic formation of ltx/iltx in mammalian systems (41–48). Recent work performed in our laboratory has lent further support to this hypothesis by identifying cytochrome P450 2C9 as the primary NADPH-dependent oxidase involved in linoleic acid epoxidation in human liver microsomes (49). In some cases chemical oxidation by active oxygen is thought to be involved (50).

Once formed, aliphatic epoxides can be metabolized by hydrolysis, secondary chain oxidation, β-oxidation, conjugation with cellular nucleophiles (e.g., free sulfhydryls) or esterification with glycerides, cholesterol, or the phospholipids of membranes. Each of these steps was examined during this investigation. Notably, neither secondary oxidation products nor significant incorporation into triglycerides or cholesterol esters were observed in our Sf-21 cell model.

As suggested by Halarnkar (19) who used murine and human liver homogenate and affinity purified sEH, our results with recombinant hsEH support the hypothesis that sEH hydrolyzes ltx/iltx to ltxd/iltxd. Additionally, the kinetic investigations indicate that ltx and iltx are preferred substrates for this enzyme with high catalytic turnover rates. Previous studies using recombinant murine sEH, and the ltx analog, epoxy stearic acid (9,10-Z-EpO) (20), or epoxyeicosanoates and hsEH (44) have also reported high rates of hydration.

The ability of the KOH traps to retain $<$1% of the radioactivity in the *in situ* metabolism study suggests that little, if any, of the ltx/iltx or ltxd/iltxd enters the β-oxidation pathway in this insect ovarian cell system. Likewise, the high recovery of total radioactivity and the lack of a significant portion of the radioactivity associated with the proteinaceous fraction suggests that little or no covalent binding to cellular proteins is occurring. Were a GSH-ltx conjugate present, we would expect this molecular species to be found in the aqueous fraction. However, even at 24 h, $<$5% of the radioactivity was assigned to the aqueous fraction even though there is GSH in the cells. This, combined with our inability to form the GSH-ltx conjugate chemically or with cell homogenate over 24 h, illustrates that 1,2-epoxides on aliphatic systems are very stable to attack by nonactivated sulfur nucleophiles. These chemical data combined with data from cell homogenate and cells *in situ* argue that GSH conjugation in the absence of enzymatic catalysis does not occur in this system. Were a GST active on fatty acid oxides present, however, we would expect conjugation to occur.

As for most somatic cell membranes, phosphotidylcholine and phosphotidylethanolamine are the major phospholipids in Sf-9 and Tn cells (51, 52) and it has been shown that infection increases phosphotidylcholine levels (51) in these insect cell lines. Therefore, once it was recognized that the epoxides and diols were entering phospholipids, it was not surprising to find them incorporated into phosphotidylcholine and phosphotidylethanolamine containing residues. Interestingly, Fang *et al.* found that 13-HODE (linoleic acid with a monohydroxy at the C-13 position) was also quickly incorporated into phospholipids, primarily phosphotidylcholine, of bovine aortic endothelial cells, then gradually released (53). Similarly, the incorporation of epoxyeicosanoids (48, 54–58) and dihydroxyeicosanoids (37, 38, 59) into phospholipids has been noted by several research groups. Interestingly, in porcine endothelial cell cultures epoxyeicosanoids are incorporated into phospholipids at 3$\times$ the rate of the dihydroxyeicosanoids (37, 38).

There are suggestions in the literature that epoxy- and dihydroxyeicosanoids phospholipid incorporation is related to their mechanism of action (38, 55, 59). Although we cannot definitively exclude phospholipid incorporation in the enhancement of ltx and ltxd toxicity, the strong correlation of toxicity with the presence of ltxd and ltxd carboxylic acid, the weak correlation with ltx and ltxd incorporation into phospholipid, and the suggestion that ltx incorporation is faster than that of ltxd all argue against phospholipids being directly involved in the toxicity of ltx in our insect cell system. Nonetheless, this compartmentalization represents detoxification by sequestration and, thus, holds some danger. If ltx/iltx and ltxd/iltxd are stored in phospholipids, they can also be released by Ca^{2+}-dependent or independent phospholipases. Therefore, if membrane concentrations of epoxides and/or diols is high enough and their release is rapid, the initiation of a toxic event may result from lipase activation.

By considering the ltx toxicity and metabolic profiling in sEH vs Lac Z expressing cells, a clear association between ltxd acid formation and cytotoxicity emerges. While both Lac Z and sEH expressing cells produce ltx free acids and incorporate these acids into their membranes, only sEH cells show declining epoxy free acid concentrations with a corresponding rise in free acid diols. The strong correlation between toxicity and ltxd/iltxd formation in hsEH expressing cells in conjunction with the lack of any ltxd/iltxd formation or toxicity in the Lac Z-expressing cells suggests that other metabolites are not involved with the observed acute toxicity. These results are consistent with the hypothesis that ltx and iltx are protoxins, which are activated by hydrolysis. Similar findings have been observed in every cell type and animal system recently tested (21, 40, 60). These results may be in contrast to the earlier results

seen by Ozawa *et al.* (7, 10, 11, 14, 61–68) who have described the epoxide as the toxic metabolite. This apparent discrepancy could be explained by the presence of sEH in their cell and animal systems. It is distinctly possible, however, that ltx/iltx may act directly in certain systems.

With respect to the fate of the experimentally delivered epoxy and dihydroxy lipid methyl esters, our analysis of the *in situ* metabolism of ltx/iltx supports the hypothesis that cellular esterases metabolize both of these oxylipids to their carboxylic acid forms. Additionally, the results suggest that ester hydrolysis proceeds at a faster rate than epoxide hydrolase forms diols. These data suggest that cellular esterases are functional in Sf-21 cells and clouds are ability to determine the reliance of toxicity on the presence of the free carboxylate. To address this question, esterase inhibition in Sf-21 cells was explored. Of the inhibitors tested only PMSF both inhibited the Sf-21 cell esterases and was not toxic. Pretreatment with this inhibitor increased the viability of cells treated with the longer epoxy stearate esters, as we would expect if the free acid form were the most toxic agent. However, PMSF also slightly increased the viability of cells treated with the free acid form of epoxy stearate. It is not clear why this occurred, and a simple explanation for this result, e.g., associated protease inhibition, is not consistent with our findings. One interpretation of the data would suggest that toxicity may progress in stages which are differentially effected by ester length and the presence of PMSF.

While the mode of ltx induced toxicity was not the focus of this investigation, limited examination of cellular thiol concentration was performed during the pursuit of GSH conjugation as a potential metabolic process. While conjugation with GSH was not observed, it was observed that depleting or elevating cellular GSH either potentiated or attenuated toxicity, respectively. Interestingly, both ltx and ltxd exposures in Lac Z-expressing cells reduced cellular free sulfhydryl concentrations, suggesting that both ltx/iltx and ltxd/iltxd disrupt the redox status of the cells. This has been previously noted with ltx in rat lung (67).

While Sf-21 cells do not contain glucuronosyl transferases (69), another possible detoxification route *in vivo* in mammals is through glucuronidation. It appears that ltxd/iltxd may be conjugated with glucuronic acid by a UDP-glucuronosyltransferase (D. F. Grant, personal communication, 1999) and that the glucuronide of ltxd/iltxd has appeared in certain disease states (70). Due to the increase in hydrophilicity, and thus excretion rate, it seems likely that glucuronidation would constitute a detoxification pathway. However, ltxd/iltxd was initially thought to be a detoxification product as well, based on the same criteria. To our knowledge, the synthesis and toxicity testing of the glucuronic acid conjugates of ltx/iltx have not been performed.

While this study alone is not sufficient to conclude that a linoleic acid cascade exists similar in scope to the arachidonic acid cascade, it nevertheless opens the field to significant research in this area. The correspondence is striking. The high levels of linoleate in mammalian systems, the dependence on cytochrome P450s and sEH for metabolism, and the sequestration in phospholipids are all significant. Moreover, the changes in membrane ion permeability and flux associated with ltxd (21) and the vasoactive effects of ltx (63, 64, 71) are similar to those seen in the arachidonic acid cascade (46). Additionally, it has been hypothesized that ltx plays a role in inflammation (72) and suggested that linoleic acid can exert proinflammatory effects in endothelial cells (73).

In conclusion, we have demonstrated that ltx/iltx are toxic in a time-dependent manner to hsEH expressing cells and are metabolized by hsEH with a K_M and V_{max} comparable to the best known exogenous substrate and that metabolism can be inhibited both *in situ* and *in vitro.* Our data suggest that the final toxic metabolite of ltx/iltx is the free carboxylic acid of the corresponding diol, suggesting the involvement of cellular esterases in experimental epoxy fatty acid ester exposures. We have shown that both ltx/iltx and ltxd/iltxd are incorporated into phospholipids, eliminating them from the toxicant pool and thereby modulating toxic potency. Finally, we have demonstrated that while free sulfhydryls ameliorate ltx/iltx induced toxicity, conjugation with GSH is not involved in our system. With these data, we have constructed a revised metabolic pathway for ltx/iltx in Sf-21 cells.

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REFERENCES

1. Stephen, A., and Wald, N. (1990) *Am. J. Clin. Nutr.* **52,** 457–469.
2. Kang, J. X., and Leaf, A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9886–9890.
3. Spindler, S. A., Clark, K. S., Callewaert, D. M., and Reddy, R. G. (1996) *Biochem. Biophys. Res. Commun.* **218,** 187–191.
4. Vaughan, J. G., and Geissler, C. A. (1997) The New Oxford Book of Food Plants, Oxford Univ. Press, Oxford.
5. Slim, R. M., Toborek, M., Watkins, B. A., Boissonneault, G. A., and Hennig, B. (1996) *J. Am. Coll. Nutr.* **15,** 289–294.
6. Krajcovicova-Kudlackova, M., Simoncic, R., Bederova, A., Klvanova, J., Brtkova, A., and Grancicova, E. (1996) *Nahrung* **40,** 17–20.

7. Kosaka, K., Suzuki, K., Hayakawa, M., Sugiyama, S., and Ozawa, T. (1994) *Mol. Cell. Biochem.* **139,** 141–148.
8. Ozawa, T., Hayakawa, M., Takamura, T., Sugiyama, S., Suzuki, K., Iwata, M., Taki, F., and Tomita, T. (1986) *Biochem. Biophys. Res. Commun.* **134,** 1071–1078.
9. Yamada, Y., Uemura, H., Nakaya, H., Sakata, K., Takatori, T., Nagao, M., Iwase, H., and Iwadate, K. (1996) *Biochem. Biophys. Res. Commun.* **226,** 391–395.
10. Hayakwa, M., Kosaka, K., Sugiyama, S., Yokoo, K., Aoyama, H., Izawa, Y., and Ozawa, T. (1990) *Biochem. Int.* **21,** 573–579.
11. Ozawa, T., Hayakawa, M., Kosaka, K., Sugiyama, S., Ogawa, T., Yokoo, K., Aoyama, H., and Izawa, Y. (1990) *Adv. Prost. Throm. Leuk. Res.* **21,** 569–572.
12. Jia-Ning, H., Taki, F., Sugiyama, S., Asai, J., Izawa, Y., Satake, T., and Ozawa, T. (1988) *Lung* **166,** 327–337.
13. Demling, R. H. (1995) *Ann. Rev. Med.* **46,** 193–202.
14. Fukushima, A., Hayakawa, M., Sugiyama, S., Ajioka, M., Ito, T., Satake, T., and Ozawa, T. (1988) *Cardio. Res.* **22,** 213–218.
15. Hammock, B. D., Storms, D. H., and Grant, D. F. (1996) *in* Biotransformation (Guengerich, F. P., Ed.), Vol. 3, pp. 284–305, Pergamon.
16. Ota, K., and Hammock, B. D. (1980) *Science* **207,** 1479–1481.
17. Wixtrom, R. N., and Hammock, B. D. (1985) *in* Biochemical Pharmacology and Toxicology (Zakim, D., and Vessey, D. A., Eds.), Vol. 1, pp. 1–93, Wiley, New York.
18. Wixtrom, R. N. (1987) Ph.D. dissertation, Davis, CA., p. 183.
19. Halarnkar, P. P., Wixtrom, R. N., Silva, M. H., and Hammock, B. D. (1989) *Arch. Biochem. Biophys.* **272,** 226–236.
20. Borhan, B., Mebrahtu, T., Nazarian, S., Kurth, M. J., and Hammock, B. D. (1995) *Anal. Biochem.* **231,** 188–200.
21. Moghaddam, M. F., Grant, D. F., Cheek, J., Greene, J. F., Williamson, K. C., and Hammock, B. D. (1997) *Nat. Med.* **3,** 562–566.
22. Grant, D. F., Greene, J. F., Pinot, F., Borhan, B., Moghaddam, M. F., Hammock, B. D., McCutchen, B., Ohkawa, H., Luo, G., and Guenthner, T. M. (1996) *Biochem. Pharmacol.* **51,** 503–515.
23. Murray, R. W., and Jeyaraman, R. (1985) *J. Org. Chem.* **50,** 2847–2853.
24. Murray, R. W., and Singh, M. (1997) *in* Organic Synthesis (Shinkai, I., Ed.), Vol. 74, pp. 91–100, Wiley, New York.
25. Gunstone, F. D., and Schuler, H. R. (1975) *Chem. Phys. Lipids* **15,** 174–188.
26. Blee, E., and Shuber, F. (1990) *J. Biol. Chem.* **265,** 12887–12894.
27. Gill, S. S., and Hammock, B. D. (1979) *Biochem. Biophys. Res. Comm.* **89,** 965–971.
28. Beetham, J. K., Tian, T., and Hammock, B. D. (1993) *Arch. Biochem. Biophys.* **305,** 197–201.
29. Wixtrom, R. N., Silva, M. H., and Hammock, B. D. (1988) *Anal. Biochem.* **169,** 71–80.
30. Wilkinson, G. N. (1961) *J. Biochem.* **80,** 324–332.
31. Morisseau, C., Goodrow, M. H., Dowdy, D., Zheng, J., Greene, J. F., Sanborn, J. R., and Hammock, B. D. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 8849–8854.
32. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Phys.* **37,** 911.
33. Krisch, K. (1966) *Biochim. Biophys. Acta* **122,** 265–280.
34. Grant, D. F., Bender, D. M., and Hammock, B. D. (1989) *Insect Biochem.* **19,** 741–751.
35. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82,** 70–77.
36. Anderson, M. E., Powrie, F., Puri, R. N., and Meister, A. (1985) *Arch. Biochem. Biophys.* **239,** 538–548.
37. VanRollins, M., Kaduce, T. L., Fang, X., Knapp, H. R., and Spector, A. A. (1996) *J. Biol. Chem.* **271,** 14001–14009.
38. Weintraub, N. L., Fang, X., Kaduce, T. L., VanRollins, M., Chatterjee, P., and Spector, A. A. (1997) *Circ. Res.* **81,** 258–267.
39. Charles, J.-P., Wojtasek, H., Lentz, A. J., Thomas, B. A., Bonning, B. C., Palli, S. R., Parker, A. G., Dorman, G., Hammock, B. D., Prestwich, G. D., and Riddiford, L. M. (1996) *Arch. Insect Biochem. Physiol.* **31,** 371–393.
40. Greene, J. F., Newman, J. W., Williamson, K. C., and Hammock, B. D. *Chem. Res. Tox.*, in press.
41. Rifkind, A. B., Lee, C., Chang, T. K., and Waxman, D. J. (1995) *Arch. Biochem. Biophys.* **320,** 380–389.
42. Zeldin, D. C., DuBois, R. N., Falck, J. R., and Capdevila, J. H. (1995) *Arch. Biochem. Biophys.* **322,** 76–86.
43. Zeldin, D. C., Plitman, J. D., Kobayashi, J., Miller, R. F., Snapper, J. R., Falck, J. R., Szarek, J. L., Philpot, R. M., and Capdevila, J. H. (1995) *J. Clin. Invest.* **95,** 2150–2160.
44. Zeldin, D. C., Moomaw, C. R., Jesse, N., Tomer, K. B., Beetham, J., Hammock, B. D., and Wu, S. (1996) *Arch. Biochem. Biophys.* **330,** 87–96.
45. Bafor, M., Smith, M. A., Jonsson, L., Stobart, K., and Stymne, S. (1993) *Arch. Biochem. Biophys.* **303,** 145–151.
46. Rahman, M., Wright, J. T., Jr., and Douglas, J. G. (1997) *Am. J. Hypertens.* **10,** 356–365.
47. Laethem, R. M., Balazy, M., and Koop, D. R. (1996) *Drug Metab. Disp.* **24,** 664–668.
48. Wu, S., Moomaw, C. R., Tomer, K. B., Falck, J. R., and Zeldin, D. C. (1996) *J. Biol. Chem.* **271,** 3460–3468.
49. Draper, A. J., and Hammock, B. D. *Arch. Biochem. Biophys.*, submitted.
50. Iwase, H., Takatori, T., Aono, K., Iwadate, K., Takahashi, M., Nakajima, M., and Nagao, M. (1995) *Biochem. Biophys. Res. Commun.* **216,** 483–488.
51. Marheineke, K., Grunewald, S., Christie, W., and Reilander, H. (1998) *FEBS Lett.* **441,** 49–52.
52. Yeh, L. H., Bajpai, R. K., and Sun, G. Y. (1997) *Lipids* **32,** 481–487.
53. Fang, X., Kaduce, T. L., and Spector, A. A. (1999) *J. Lipid Res.* **40,** 699–707.
54. Shivachar, A. C., Willoughby, K. A., and Ellis, E. F. (1995) *J. Neurochem.* **65,** 338–346.
55. Fang, X., Kaduce, T. L., Weintraub, N. L., and Spector, A. A. (1997) *Prosta. Leuko. Essent. Fatty Acids* **57,** 367–371.
56. Capdevila, J., Pramanik, B., Napoli, J. L., Manna, S., and Falck, J. R. (1984) *Arch. Biochem. Biophys.* **231,** 511–517.
57. Karara, A., Dishman, E., Jacobson, H., Falck, J. R., and Capdevila, J. H. (1990) *FEBS Lett.* **268,** 227–230.
58. Zhu, Y., Brand-Schieber, E., McGiff, J. C., and Balazy, M. (1995) *Hypertens.* **25,** 854–859.
59. Fang, X., VanRollins, M., Kaduce, T. L., and Spector, A. A. (1995) *J. Lipid Res.* **36,** 1236–1246.
60. Moran, J. H., Weise, R., Schnellmann, R. G., Freeman, J. P., and Grant, D. F. (1997) *Toxicol. Appl. Pharmacol.* **146,** 53–59.
61. Ishizaki, T., Shigemori, K., Nakai, T., Miyabo, S., Hayakawa, M., Ozawa, T., Voelkel, N. F., and Chang, S. W. (1995) *J. Appl. Physiol.* **79,** 1106–1111.
62. Sakai, T., Ishizaki, T., Ohnishi, T., Sasaki, F., Ameshima, S., Nakai, T., Miyabo, S., Matsukawa, S., Hayakawa, M., and Ozawa, T. (1995) *Am. J. Physiol.* **269,** L326–331.
63. Ishizaki, T., Shigemori, K., Nakai, T., Miyabo, S., Ozawa, T., Chang, S. W., and Voelkel, N. F. (1995) *Am. J. Physiol.* **269,** L65–70.

64. Ishizaki, T., Takahashi, H., Ozawa, T., Chang, S. W., and Voelkel, N. F. (1995) *Am. J. Physiol.* **268,** L123–128.
65. Ishizaki, T., Shigemori, K., Ameshima, S., Nakai, T., Miyabo, S., Hayakawa, M., Ozawa, T., and Voelkel, N. F. (1996) *Am. J. Physiol.* **271,** L459–463.
66. Hanaki, Y., Kamiya, H., Ohno, M., Hayakawa, M., Sugiyama, S., and Ozawa, T. (1991) *Japan J. Med.* **30,** 224–228.
67. Akai, M., Ishizaki, T., Matsukawa, S., Shigemori, K., and Miyamori, I. (1998) *Free Rad. Biol. Med.* **25,** 596–604.
68. Ishizaki, T., Ozawa, T., and Voelkel, N. F. (1999) *Pulmon. Pharmacol. Ther.* **12,** 145–155.
69. Nguyen, N., and Tukey, R. (1997) *Drug Metab. Disp.* **25,** 745–749.
70. Street, J. M., Evans, J. E., and Natowicz, M. R. (1996) *J. Biol. Chem.* **271,** 3507–3516.
71. Takahashi, H., Ishizaki, T., Ameshima, S., Kishi, Y., Sasaki, F., Nakai, T., Miyabo, S., and Ozawa, T. (1992) *Nippon Kyobu Shikkan Gakkai Zasshi* **30,** 418–424.
72. Draper, A. J., and Hammock, B. D. (1999) *Toxicol. Sci.* **50,** 30–35.
73. Young, V. M., Toborek, M., Yang, F., McClain, C. J., and Hennig, B. (1998) *Metabol.* **47,** 566–572.