Leukotoxin is a linoleic acid oxide produced by leukocytes and has been associated with the multiple organ failure and adult respiratory distress syndrome seen in some severe burn patients. Leukotoxin has been reported to be toxic when injected into animals intravenously. Herein, we report that this lipid is not directly cytotoxic in at least two in vitro systems. Using a baculovirus expression system we demonstrate that leukotoxin is only cytotoxic in the presence of epoxide hydrolases. In addition, it is the diol metabolite that proves toxic to pulmonary alveolar epithelial cells, suggesting a critical role for the diol in leukotoxin-associated respiratory disease. In vivo data also support the toxicity of leukotoxin diol. For the first time we demonstrate that soluble epoxide hydrolase can bioactivate epoxides to diols that are apparently cytotoxic. Thus leukotoxin should be regarded as a protoxin corresponding to the more toxic diol. This clearly has implications for designing new clinical interventions.

One of the linoleic acid oxides formed by cytochrome P-450 monooxygenase is cis-9,10-epoxyoctadec-12(Z)-enoic acid, commonly known as leukotoxin (Fig. 1). Leukocytes can biosynthesize this compound, which has been shown to be toxic when administered to animals by intravenous injection, hence, the term leukotoxin. The biological importance of leukotoxin is most evident in severe burn patients. After initial stabilization, a number of these patients have been reported to suffer from multiple organ failure, including adult respiratory distress syndrome (ARDS). This often fatal condition is commonly attributed to toxic dialls by epoxide hydrolase. Many other stress conditions can lead to ARDS; more than 150,000 cases per year were reported in the United States alone with over 50 percent mortality.

Other factors may enhance biosynthesis of leukotoxin. It has been demonstrated that exposure of rats to nitrogen dioxide and other oxidants initiates significant epoxidation of pulmonary polyunsaturated fatty acids, resulting in the production of leukotoxin and isoleukotoxin (Fig. 1) as auto-oxidation products in the lung. Moreover, we have demonstrated elevated biosynthesis and metabolism of linoleate epoxides in mice by induction of microsomal enzymes such as P-450 monooxygenases and epoxide hydrolases using clofibrate, a hypolipidemic and peroxisome-proliferating agent.

Epoxide hydrolases are members of the omega- and zygos family that convert epoxides to the corresponding diols. Two diverse epoxide hydrolases, known as the microsomal and soluble epoxide hydrolases, are present in animal tissues at high levels. Epoxide hydrolases are found in all vertebrate tissues examined, although at vastly different levels. Along with the microsomal epoxide hydrolase, the soluble epoxide hydrolase is thought to provide cellular protection from xenogenous and endogenous epoxides by detoxifying them to nonreactive, water soluble diols. The soluble epoxide hydrolase turns over a variety of fatty acid epoxides at high rates. Recently, we have succeeded in cloning murine and human soluble epoxide hydrolases and expressing them in the baculovirus expression system. These and other recombinant viruses were used to transfect cells of Spodoptera frugiperda (SF-21), which are naturally low in endogenous epoxide hydrolase activity, in order to study the roles of epoxide hydrolases in cellular metabolism. We have found that epoxide hydrolases rapidly hydrolyze a variety of mutagenic and carcinogenic epoxides as well as fatty acid epoxides when expressed in the baculovirus/SF-21 system. As expected, cells transfected with the virus containing cDNA coding for epoxide hydrolase were resistant to the cytotoxic and genotoxic effects of most epoxide-containing compounds.

As mentioned above, it generally is accepted that epoxides are toxic, and their hydration to diols usually constitutes a detoxification process. Our surprising discovery is that the diols of leukotoxin and isoleukotoxin (Fig. 1) are much more toxic than their progenitor epoxides (Fig. 2). Methyl linoleate was not toxic to any of the transfected SF-21 cells (Fig. 2a). Methyl leukotoxin was not toxic to control cells expressing beta-galactosidase (lacZ). This epoxy fatty acid exhibited toxicity only when incubated with cells expressing human soluble epoxide hydrolase (hsEH), mouse soluble epoxide hydrolase (msEH), or human microsomal epoxide hydrolase (hmEH). This links epoxide hydrolysis to cytotoxicity (Fig. 2a and c). As with other plants and animals studied, the human soluble epoxide hydrolase hydrolyzes most fatty acid epoxides at more than 1000 times the rate of the microsomal epoxide hydrolase. Thus, it is not surprising that cells transfected with the virus cod-
ing the soluble enzyme are more sensitive to the leukotoxins than those transfected with virus coding the microsomal enzyme. Methyl leukotoxin diol, however, was toxic to all the cells mentioned above (Fig. 2b) as was its free acid. We observed similar results for isoleukotoxin and its corresponding diol with all the above-mentioned transfected cells (Fig. 2, d and e). The diols were of similar toxicity, and the epoxides were nontoxic in Hela cells. These data suggest that leukotoxin and isoleukotoxin are prototoxins. In the systems studied here, they are only toxic following activation to the corresponding diols. The low toxicity of the linoleate epoxides and lack of toxicity of their hydration products (data not shown) suggests that further epoxidation of the leukotoxin diols is not needed for cytotoxicity, unlike the case with the diol epoxides of polycyclic aromatic hydrocarbons.

**Fig. 1.** Linoleic acid is an 18:2 polyunsaturated lipid, which is a very abundant fatty acid in the average American diet. It can be converted by autooxidation or P-450 action to two epoxides commonly known as leukotoxin and isoleukotoxin. Either acid catalyzed hydrolysis or exposure to epoxide hydrolase leads to the corresponding three diols, which we hypothesize to be responsible for at least some of the toxic effects previously attributed to the epoxides.

Further support for the above hypothesis was obtained by following the distribution and metabolism of methyl [1-14C]leukotoxin in aliquots of Sf cells taken over a 24-hour period. No diol was detected in cells expressing lacZ, but approximately 60% of the radioactivity in the cells expressing hsEH was attributable to the diol 16 to 24 hours after dosing. The diols and epoxides clearly have differences in polarity and thus differences in their anticipated rate of crossing biological membranes. Calculation of log P values (octanol/water partition coefficients) for these compounds indicates that all of the compounds will partition preferentially into cells; however, one can anticipate that the epoxides will partition more rapidly than the diols with log P of 6.048 for methyl leukotoxin and methyl isoleukotoxin, 5.632 for leukotoxin and isoleukotoxin, log P of 4.601 for methyl leuko-
After 24 hours, monolayer permeability was relatively unaltered, whereas the other compounds were inactive at doses up to 300 μM (data not shown).

Measurements of leukotoxin concentration in the serum of burn patients revealed a fluctuating pattern for this epoxy fatty acid with the concentrations of leukotoxin always much higher than the parent leukotoxin diols proved to be more toxic and to cause much more pronounced symptoms at a lower concentration than the parent leukotoxins. The leukotoxin diol concentrations showing biological activity in vivo are consistent with in vivo levels of leukotoxin observed in burn and ARDS patients.41,42

Fig. 3 Effects of lipids on bioelectric properties of primary cultured monolayers of rat pulmonary alveolar epithelial cells. Data are presented as means ± s.d.; n = 5–10 monolayers. * Mean significantly less than that of the parent lipid (methyl linolate) at the same time after exposure as determined by one-way ANOVA/Scheffé contrast. Monolayers were treated with 300 μM methyl linolate (○), methyl leukotoxin (▲), methyl isoleukotoxin (●), methyl leukotoxin diol (□) or methyl isoleukotoxin diol (△), delivered in less than 2% methanol (vol/vol). Immediately before exposure (0 h) and at various times after administration of lipid, monolayer bioelectric properties [transepithelial resistance (R), short-circuit current (Isc)] were recorded using a voltmeter as previously described41. a, Treatment of monolayers with 300 μM methyl leukotoxin diol or methyl isoleukotoxin diol resulted in decreased Isc by 5 h following exposure, suggestive of reduced net transepithelial ion transport. Complete absence of Lp, 24 h following exposure indicated the loss of epithelial cell viability. b, R, values of same monolayers shown in a. Decreased R, was noted at 5 h following exposure to 300 μM methyl isoleukotoxin, indicating that paracellular permeability increased as a result of lipid administration. By 24 h after exposure to either methyl leukotoxin diol or methyl isoleukotoxin diol, R, values were negligible, confirming a decrease in cell viability and monolayer integrity as a result of treatment with either diol. No biological activity was observed in this system with the parent compound, monoepoxides(s), or diol(s) in the methyl oleate series. In separate experiments only methyl leukotoxin diol and methyl isoleukotoxin diol were effective at increasing intracellular calcium monitored by Fura-2 with 50 μM the lowest effective dose in alveolar epithelial cells, whereas the other compounds were inactive at doses up to 300 μM (data not shown).

indicating an increase in intercellular junction permeability and/or loss of epithelial integrity. Both of the diols apparently reduced net ion flux in alveolar epithelial monolayers, suggesting that the mechanism of toxicity differed from that following oxidant gas exposure43. Both of these effects (decreased active ion transport, elevated paracellular permeability) would contribute to the development of leukotoxin-induced alveolar edema previously reported following in vivo exposures44. As shown in Fig. 3, there is no statistically significant difference between methyl linolate, its monoepoxides leukotoxin and isoleukotoxin, and a variety of other oxidized lipids in this system. Although not significant by simple statistics, the leukotoxins do seem to show a trend toward toxicity in Fig. 3a. If real, these effects could be due to traces of diol formed from the epoxide, alternate metabolites formed from the epoxide, or from direct action of the epoxide itself. Our preliminary results show that the diols, but not the methyl linolate or its monoepoxides, also increase intracellular levels of calcium. The in vitro observations on the relative toxicity of the leukotoxins and their diols were borne out in vivo. In both Sprague-Dawley rats and Swiss-Webster mice the leukotoxin diols proved to be more toxic and to cause more pronounced symptoms at a lower concentration than the parent leukotoxins. The leukotoxin diol concentrations showing biological activity in vitro are consistent with in vivo levels of leukotoxin observed in burn and ARDS patients.
mice support the hypothesis that leukotoxin is a protoxin in both rats and in mice. In our assay system, it is clear that the leukotoxin diols are more potent than the corresponding leukotoxins. However, we cannot exclude the possibility that the precursor epoxides act directly, or through metabolites other than the diol. Doses of only 0.35 mg/kg leukotoxin diol delivered by cardiac puncture in rats caused immediate respiratory distress with 100% death in less than 2 hours following injection, whereas no clear symptoms or mortality were observed with doses of the parent leukotoxin up to 100 mg/kg. Mice received tail vein injections of the free fatty acids. When 200 mg/kg of the leukotoxin diol was administered, 30% mortality occurred within 4 minutes. All of the mice treated were lethargic and exhibited difficulty breathing for at least 2 hours. In contrast, at 400 mg/kg of the parent leukotoxins, only 25% mortality was observed with death between 18 and 24 hours. In no case were clear symptoms of pulmonary distress observed for more than 10 minutes.

Our data indicate that the numerous pathologies attributed to leukotoxin and isoleukotoxin result from enzymatic activation mediated largely by the soluble epoxide hydrolase. If this is the case, there are several obvious pathways for clinical intervention. For example, in the cell system illustrated in Fig. 2, the toxicity of the leukotoxins in cells expressing mEH could be reversed by administration of the potent sEH inhibitor 4-fluorochalcone oxide. In preliminary studies toxicity of leukotoxin to mice seems to be reduced by prior administration of an sEH inhibitor 4-phenylchalcone oxide. One should also try to avoid drugs such as clofibrate, which is known to induce the cytochrome P-450 and epoxide hydrolase, which form leukotoxin diol(s). It may also be of benefit to stimulate other enzymes involved in leukotoxin degradation such as the glutathione transferases and to avoid drugs such as acetaminophen that deplete glutathione.

Methods

Leukotoxin synthesis. Methyl leukotoxin and methyl isoleukotoxin, and their diols were chemically synthesized from methyl linoleate using m-chloroperbenzoic acid, as described previously. These two monoepoxides and the diastereomers of the 9,10,12,13-diepoxide (not shown) were separated from each other and interfering compounds using normal-phase silica column chromatography. Each of these monoepoxides then was hydrolyzed to its diol using perchloric acid and purified. The purity and identity of all compounds were confirmed using a combination of at least TLC, GC, GC/MS/MS, and 'H and 13C NMR and comparisons of these data to those found in the literature. Free fatty acids were purified using the same solvent system containing 1% acetic acid.

In vitro toxicity. The effects of lipids on the viability of cells expressing transgenic enzymes was evaluated using the baculovirus expression system. SF-21 cells were infected with recombinant baculoviruses producing either human soluble epoxide hydrolase (hSEH), mouse soluble epoxide hydrolase (mEH), human microsomal epoxide hydrolase (hmmEH), or β-galactosidase (lacZ) as a control virus. Two days post infection varying concentrations of lipids were added dissolved in 2% final concentration of DMSO (vol/vol). Three days post infection a viability test was performed with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). For tracer studies leukotoxin was prepared as described above from 1-[^14]C-labeled methyl linoleate (supplied by NEN Life Science, Du Pont). The compound was added as described above to give a final concentration of 220 μM. Aliquots from various times were extracted with ethyl ether, mixed with authentic standards, concentrated, and spotted on 250-μm silica TLC plates. Location of radioactive spots was by positron scanning and quantification by liquid scintillation counting. Log P values were calculated with MacLogP 2.0 (BioByte Corp., Claremont, CA).

Cell cultures. Alveolar type II cells were isolated from adult rats using discontinuous gradients and differential adherence techniques as reported previously. Briefly, lungs from male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were filled with an emulsion of Fluorinert FC-75 (3M, St. Paul, MN) and bovine serum albumin in balanced salt solution for 20 min at 37°C. After displacement of the emulsion by lavage, a solution of elastase (Worthington Biochemical, Freehold, NJ) was infused via the airways and allowed to incubate for 20 min at 37°C. Elastase-digested lungs were minced, filtered and pooled to yield a single-cell suspension, which was centrifuged on a discontinuous Percoll (Pharmacia) gradient. Bloodborne leukocytes were subsequently removed from this enriched cell suspension using IgG-coated plates, resulting in a final yield of ~85% type II cells. Isolated type II cells were plated onto tissue culture-treated Transwell inserts (Costar, Van Nuys, CA) in Ham's F-12 medium supplemented with 10% newborn bovine serum and 0.1 μM dexamethasone. After 48 h in culture at 37°C in 5% CO2/air, the initial medium was removed from the cultures and replaced with serum-free F-12 + 1% nutrient medium as previously described. This serum-free medium consisted of Ham's F-12 supplemented with sodium bicarbonate (14 mM), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, 10 mM) and l-cystine (0.15 mM) and contained the following growth factors: insulin (5 μg/ml), transferrin (5 μg/ml), epidermal growth factor (25 ng/ml), hydrocortisone (1 μM), bovine hypothalamus extract (7.5 μg/ml) and retinol (0.1 μM). Monolayers were then incubated in serum-free medium for an additional 24 to 48 h until removed from the incubator for experiments.

In vivo toxicity. The toxicity of the compounds was evaluated in Sprague-Dawley rats from Simonson Laboratories, Gilroy, CA. Rats received free fatty acids dissolved in PBS containing 5% DMSO by cardiac puncture after they were anesthetized by intraperitoneal injections of pentobarbital. Rats were anesthetized and laid on their backs. Their abdomens were punctured vertically after placing the tip of the syringe needle on the right side of the xyphoid process and below the last rib. Next, the syringe was rotated downwards 45 degrees and rotated clockwise 45 degrees. At this point, the syringe was pushed forward to puncture the diaphragm and the heart. This would place the tip of the needle into the right side of the heart. To ensure dispensing of the test material into the circulation, blood was withdrawn into the syringe before releasing the test material. Male Swiss-Webster mice (18-20 g) from Bantin-Kingman (Fremont, CA) received tail vein injections of free fatty acids dissolved in 2-methoxethanol after they were anesthetized by inhalation methoxyflurane.

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