Leukotoxin-Diol
A Putative Toxic Mediator Involved in Acute Respiratory Distress Syndrome
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Leukotoxin is clinically associated with acute respiratory distress syndrome (ARDS). Recently, we found that leukotoxin-diol, the hydrated product of leukotoxin, is more toxic than the parent leukotoxin in vitro (Moghaddam and colleagues, Nature Med. 1997;3:562–566). To test if this difference in the toxicity of leukotoxin and leukotoxin-diol exists in vivo, Swiss Webster mice were administered leukotoxin or leukotoxin-diol. All mice treated with leukotoxin-diol died of ARDS-like respiratory distress, whereas the animals exposed to leukotoxin at the same dose survived. Histopathologic evaluation of the lungs revealed massive alveolar edema and hemorrhage with interstitial edema around blood vessels in the lungs of mice treated with leukotoxin-diol, whereas the lungs of mice treated with identical doses of leukotoxin had perivasculare edema only and little change in alveolar spaces. Immunohistochemistry showed that the soluble epoxide hydrolase responsible for the hydrolysis of leukotoxin to its diol is concentrated in the vascular smooth muscle of small and medium-sized pulmonary vessels. In addition, 4-phenylchalcone oxide, an inhibitor of soluble epoxide hydrolase, was found to decrease the mortality induced by leukotoxin but had no effect on mortality induced by leukotoxin-diol. These studies provide strong in vivo evidence that leukotoxin may act as a protoxicant and that the corresponding diol is a putative toxic mediator involved in the development of ARDS.

Acute respiratory distress syndrome (ARDS) is a descriptive term that has been applied to many acute, diffuse infiltrative lung lesions of diverse etiologies when they are accompanied by severe arterial hypoxemia. An estimated 150,000 cases of ARDS with up to 90% mortality are reported each year in the United States (2). Leukotoxin (9,10-epoxy-12-octadecenoate) (Scheme 1) has been suggested to be a toxic mediator possibly responsible for the development of ARDS. Clinically, leukotoxin has been associated with ARDS based on the evidence that leukotoxin was found in lung lavage fluid obtained from patients with ARDS (3) and that high levels of leukotoxin were reported in the skin and plasma of burn patients suffering from ARDS (4). Epoxylipids are natural dietary components, and some, including leukotoxin and isoleukotoxin (vernolic acid, 12,13-epoxy-9-octadecenoate), are possible targets for expression in transgenic crops (5).

In mammals, leukotoxin is proposed to be produced from linoleic acid by cytochrome P450 (6) (Scheme 1) as well as via reactive oxygen species generated by neutrophil respiratory burst oxidase (7). Leukotoxin is further hydrolyzed to leukotoxin-diol (Scheme 1) by soluble epoxide hydrolase (1). Recently, we reported that leukotoxin-diol is considerably more toxic than its parent epoxide leukotoxin in vitro (1). Spodoptera frugiperda (SF21) cells were killed by leukotoxin only if the human or murine soluble epoxide hydrolase was expressed. In the absence of epoxide hydrolase expression, high concentrations of leukotoxin were required for cytotoxicity (1). In addition, leukotoxin-diol was found to be toxic to renal proximal tubular cells, but no cytotoxicity was observed after the cells were exposed to equivalent levels of leukotoxin (8). These in vitro studies suggest that leukotoxin may act as a protoxicant that is converted to the corresponding diol to produce cytotoxic effects.

Materials and Methods

Animals
Male Swiss Webster (25 to 28 g) mice were purchased from Simonsen (Gilroy, CA) and had free access to water and food. The mice (three in each group) were administered a 1:1 mixture of methyl leukotoxin/isoleukotoxin esters (methyl 9,10-epoxy-12-octadecenoate/methyl 12,13-epoxy-9-octadecenoate) (300 to 1,000 mg/kg, 1.0 ml/kg) or a mixture of the corresponding diols dissolved in ethanol (100 to 400 mg/kg, 1.0 ml/kg) by tail vein injection. Some mice were treated intraperitoneally with 4-phenylchalcone oxide (400 mg/kg, 6.0 ml/kg) suspended in corn oil before exposure to leukotoxin or its diol.

Histopathology
Three different approaches were taken for lung preservation to characterize the extent of injury: inflation with fixation via a tracheal cannula, immersion deflated, or immersion after maintenance of inflation by ligation of the trachea before thoracotomy. All lungs were fixed with 10% neutral-buffered formalin. The same conclusions were drawn from each method. All lobes were sliced 0.2 to 0.3 mm thick and the pieces were embedded in paraffin. Blocks were sectioned at 5 μm and stained with hematoxylin and eosin. Representative areas were recorded by computer imaging with a Sony Caysce Camera mounted on an Olympus Provis microscope.

Synthesis
Methyl leukotoxin/isoleukotoxin esters and the corresponding diols were synthesized as described previously (9). Briefly, methyl linoleate was allowed to react with a 1:1 molar ratio of m-chloroperbenzoic acid in methylene chloride at room temperature for 5 h. A mixture of methyl leukotoxin/isoleukotoxin esters was purified by flash chromatography on silica gel eluted with 5% ethyl acetate in hexane. Methyl leukotoxin/isoleukotoxin ester diols were prepared by hydrolysis of methyl leukotoxin/isoleukotoxin esters using aqueous 1% perchloric acid in the presence of tetrahydrofuran (vol/vol, 50/50) as cosolvent at room temperature for 3 h.

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Abbreviation: acute respiratory distress syndrome, ARDS.
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followed by purification through flash chromatography on silica gel eluted with 10% ethyl acetate in hexane. These synthetic compounds were identified by $^3$H-nuclear magnetic resonance and mass spectrometry. The spectra of those compounds were the same as reported previously (9) and indicated a high degree of purity. A 1:1 ratio of regio isomers of both leukotoxin/isoleukotoxin and their diols was observed by gas liquid chromatography. 4-Phenylchalcone oxide was prepared as described previously (10). Aldol condensation of 4-phenylbenzaldehyde and acetophenone gave 4-phenylchalcone, and epoxidation of the resulting chalcone by hydrogen peroxide produced 4-phenylchalcone oxide. The purity of the previously mentioned synthetic compounds was > 95% as determined by high performance liquid chromatography analysis.

Antibody Preparation and Evaluation

Soluble epoxide hydrolase was affinity purified (11) from the pooled liver cytosol of 25 male Swiss Webster mice (Bantam-Kingman, Fremont, CA) that had been fed 0.5% wt/wt clofibrate ad libitum in ground rodent chow for 14 d ad libitum. Antiserum was subsequently prepared by five immunizing boosts of multiple intradermal injections of pure mouse liver sEH (100 μg each) into a 2.5-kg female New Zealand white rabbit (12) spaced over a 5-mo period. Antibodies were affinity purified from rabbit sera by sodium dodecyl sulfate polyacrylamide gel electrophoresis Western blots of 1 to 25 μg mouse liver homogenate, which provided a very strong band at the endogenous peroxidases present in tissues. Nonspecific protein interactions were blocked by incubating the tissues with bovine serum albumin for 30 min. Antigenic proteins were identified by the avidin-biotin horseradish peroxidase method as outlined by Plopper and coworkers (13). Standard controls were (1) substitution of the primary antibody with phosphate-buffered saline; (2) substitution of secondary antibody for phosphate-buffered saline; or (3) serial culture medium dilutions from 1:50 to 1:50,000 for anticytotoxic epoxide hydrolase. Antibody binding was identified using the Hsu modification of the diaminobenzidine tetrahydrochloride-chromogen method for immunohistochemistry (14).

Results

Although the evidence obtained from our previous in vitro studies supported our theory that leukotoxin is a precursor of leukotoxin-diol as a toxic mediator responsible for the development of ARDS, in vivo confirmation was needed. To compare the toxicity of leukotoxin or leukotoxin-diol in vivo, we administered leukotoxin or leukotoxin-diol methyl esters by tail vein injection in mice and examined the mortality of the animals. As shown in Table 1, all mice given 300 mg/kg leukotoxin-diol died, whereas the animals administered the same dose of leukotoxin survived. One hundred percent mortality was observed for leukotoxin only after intravenous administration with 600 mg/kg or more. Additionally, the leukotoxin dose resulting in 50% mortality was approximately 400 mg/kg, whereas that of the diols of leukotoxin was around 100 mg/kg. This indicates that leukotoxin-diol is more toxic than leukotoxin in vivo, which is consistent with the observation from earlier in vitro studies.

In addition, all the mice treated with either leukotoxin-diol or high doses of leukotoxin died of respiratory distress. The symptoms leading ultimately to death were characterized by rapid shallow breathing followed by gasping and discharge of bloody exudate from the nasal and oral cavities. After the appearance of severe respiratory symptoms, the animals given either leukotoxin-diol or leukotoxin (high doses) died in less than 2 min. However, administration of a higher dose of leukotoxin (1,000 mg/kg) caused acute death (in 2 min post-treatment), but none of the previously mentioned respiratory distress reactions were observed.

To evaluate lung injury induced by leukotoxin and leukotoxin-diol, we examined the histopathologic changes of the lungs of the mice. Massive bronchiolar and alveolar edema were observed in the lungs of mice 5 min after treatment with 400 mg/kg leukotoxin-diol (Figure 1D and Table 2). Furthermore, the majority of the lung was consolidated with some hemorrhage and interstitial edema around blood vessels. However, mice given similar doses of leukotoxin exhibited little change in the lung tissue morphology 5 min after treatment (Table 2). Lung tissues of mice given carrier (ethanol) were unchanged 5 min after treatment (Figure 1A and Table 2). Lungs of mice treated with 100 mg/kg leukotoxin-diol had little pulmonary injury.
5 min after exposure (Table 2) but still showed peribronchiolar and perivascular edema along with alveolar edema in peripheral portions of acini 30 min after treatment (Figure 1C and Table 2). These time- and dose-dependent results supply further evidence for the toxic role of leukotoxin-diol in the development of ARDS.

At 400 mg/kg all mice treated with leukotoxin-diol died in less than 10 min, whereas half the mice given leukotoxin survived and the other half died 9 to 12 h later. Additionally, exposure to 400 mg/kg leukotoxin-diol produced massive alveolar, peribronchiolar, and perivascular edema 5 min after administration (Figure 1D and Table 2). No significant lung damage was observed 5 min after treatment with 400 mg/kg leukotoxin (Table 2). However, 30 min after treatment, perivascular edema, but little evidence of edema in alveolar spaces, was observed (Figure 1B and Table 2).

Apparently, leukotoxin does cause lung edema, but it takes a much longer time for leukotoxin to produce edema than it does for leukotoxin-diol at the same doses. It is likely that gradual accumulation of leukotoxin-diol, which is generated from enzymatic hydrolysis of leukotoxin, produces the lung injury.

Soluble epoxide hydrolase is hypothesized to be the principle enzyme that hydrolyzes leukotoxin to leukotoxin-diol (1). We reasoned that tissues and cells of the lung expressing high levels of epoxide hydrolase would be more susceptible to leukotoxin than those expressing low levels of the enzyme if leukotoxin-diol, rather than leukotoxin, was responsible for pulmonary toxicity. Antibodies prepared

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**Figure 1.** Histologic comparison of the acinar changes in lungs of mice exposed to carrier (A), leukotoxin (B), and leukotoxin-diol (C and D). (B) In mice receiving leukotoxin (400 mg/kg), there was exudate lining terminal bronchioles (TB) and edema in perivascular spaces (arrow) of pulmonary arteries and veins, but alveolar ducts (AD) and alveoli were free of material. (C) In mice receiving a low dose of leukotoxin-diol (100 mg/kg), terminal bronchioles were free of exudate, but both perivascular spaces (arrows) and distal alveoli (arrowhead) were filled with eosinophilic exudate. (D) In mice receiving a high dose of leukotoxin-diol (400 mg/kg), all compartments, bronchiolar, perivascular, and alveolar, were nearly filled with exudate and red blood cells (original magnification: × 100).

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**TABLE 2**

<table>
<thead>
<tr>
<th>Compound Treated</th>
<th>Dose (mg/kg)</th>
<th>Time (min after treatment)</th>
<th>Histopathologic Changes in the Lungs</th>
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<tr>
<td>Leukotoxin-diol</td>
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<td>Massive bronchiolar edema</td>
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<td></td>
<td></td>
<td>Massive alveolar edema</td>
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<td></td>
<td></td>
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<td>Interstitial edema</td>
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<td></td>
<td></td>
<td></td>
<td>Hemorrhage</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total lung consolidation</td>
</tr>
<tr>
<td>Leukotoxin-diol</td>
<td>100</td>
<td>5</td>
<td>Little change</td>
</tr>
<tr>
<td>Leukotoxin-diol</td>
<td>100</td>
<td>30</td>
<td>Peribronchiolar edema</td>
</tr>
<tr>
<td>Leukotoxin</td>
<td>400</td>
<td>5</td>
<td>Little change</td>
</tr>
<tr>
<td>Leukotoxin</td>
<td>400</td>
<td>30</td>
<td>Perivascular edema</td>
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</table>

Male Swiss Webster mice were administered leukotoxin or leukotoxin-diol (in ethanol) by tail vein injection. Lungs of the animals were fixed with 10% neutral-buffered formalin and sliced. The pieces were embedded in paraffin and stained with hematoxylin and eosin. Histologic observations were made in a blind fashion.
against mouse-soluble epoxide hydrolase were employed to characterize the enzyme distribution in the lungs of mice. As seen in Figure 2, immunoreactivity with these antibodies was found in the vascular smooth muscle of the small- and medium-sized vessels in the pulmonary parenchyma. In addition, there did appear to be immunoreactivity with cells lining small muscular arteries in some sections. As consistently observed in histopathologic studies, the lungs of mice given leukotoxin at 400 mg/kg 30 min postexposure had severe perivascular edema but little alveolar edema (Figure 1B and Table 2). Edema was present only in the interstitial areas of blood vessels where epoxide hydrolase is heavily distributed. It appears that high epoxide hydrolase concentrations in the pulmonary vascular smooth muscle generates high local concentrations of leukotoxin-diol, which increases permeability of vascular walls resulting in perivascular edema. Systemically, the majority of leukotoxin is certainly hydrolyzed in the liver where abundant epoxide hydrolase is present and the resulting diol metabolites are likely rapidly biotransformed in situ to conjugates and excreted in the urine. High concentrations of leukotoxin-diol as glucuronides have been found in the urine of patients with peroxisomal disorders (9).

To further probe the role of soluble epoxide hydrolase in ARDS induced by leukotoxin, we compared the toxicity of leukotoxin and its diol in mice that had been pretreated with 4-phenylchalcone oxide, a potent inhibitor of soluble epoxide hydrolase (10). As expected, pretreatment with the chalcone oxide was found to decrease the mortality in mice after exposure to leukotoxin ($P < 0.01$) (Table 3), whereas 4-phenylchalcone oxide demonstrated no protective effects on toxicity induced by leukotoxin-diol.

The in vivo work described previously, together with our previous in vitro studies, provides strong evidence that leukotoxin may act as a precursor that is converted to the corresponding diol, producing cytotoxic effects.

**TABLE 3**

Mortality of leukotoxin and leukotoxin-diol in mice pretreated with vehicle or 4-phenylchalcone oxide

<table>
<thead>
<tr>
<th></th>
<th>Leukotoxin</th>
<th>Leukotoxin-diol</th>
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<tr>
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<td>PCO pretreatment</td>
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<td>1/4</td>
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</table>

**Definition of abbreviation:** 4-phenylchalcone oxide, PCO. Male Swiss Webster mice were administered corn oil or PCO (400 mg/kg) suspended in corn oil intraperitoneally. After 1 h, the animals were injected with leukotoxin (500 mg/kg) or leukotoxin-diol (100 mg/kg, in ethanol) by tail vein injection followed by examination of mortality.

**Discussion**

Earlier studies demonstrated that leukotoxin appears to be a protoxicant of leukotoxin-diol, which is possibly responsible for cytotoxicity in varieties of cell lines (1). We extended the in vitro investigation to examine the association of lung injury with leukotoxin and leukotoxin-diol in vivo. These in vivo studies showed that a higher dose of leukotoxin than of leukotoxin-diol was needed to produce mortality in mice, indicating leukotoxin-diol is more toxic than leukotoxin not only in vitro but also in vivo. Although the mechanism of toxic action has not been identified, leukotoxin-diol is known to be a hydrolytic metabolite of leukotoxin metabolized by soluble epoxide hydrolase (1). Unlike leukotoxin-diol, exposure of mice to the same dose of leukotoxin did not cause acute lung injury based on mortality and histopathologic evaluation. An extremely high dose (> 1,000 mg/kg) of leukotoxin did cause acute death in mice (in 2 min post-treatment), but no respiratory distress reactions were observed. It is likely that gradual accumulation of leukotoxin-diol, which is generated from the enzymatic hydrolysis of leukotoxin given in the mice, produces the lung injury.

Leukotoxin and isoleukotoxin have been found in the lung lavage fluid of patients suffering from ARDS (3) and are suggested to be associated with ARDS. However, our previous in vitro studies showed that leukotoxin is not toxic to SF21 cells unless these cells are transfected with the gene for the soluble epoxide hydrolase that biotransforms leukotoxin to its diol. It was also found that leukotoxin-diol is much more toxic than leukotoxin to a selection of cell lines (8). These in vitro studies indicate that leukotoxin needs to be hydrolyzed to the corresponding diol before it produces toxic effects. As a matter of fact, leukotoxin- and isoleukotoxin-diols as glucuronides have been found in the urine of patients with peroxisomal lung disorders (15). As described previously, massive injury was observed in the lungs of mice given 400 mg/kg leukotoxin-diol in 5 min. However, little change in the lungs of mice exposed to 400 mg/kg leukotoxin in 5 min was found, but appreciable edema was observed in 30 min. This observation supplies strong evidence that leukotoxin may act as a precursor that is converted to the corresponding diol, pro-
ducing cytotoxic effects. Early work by Ozawa and coworkers (3) and Hu and colleagues (16) demonstrated that intravenous administration of leukotoxin in rats at a dose of 160 mg/kg produced massive histopathologic changes within 10 min. This indicates rats may be more susceptible to leukotoxin than are mice, as would be expected based on scaling for a compound that is rapidly metabolized. The pathology in rats was more severe 24 h after injection of leukotoxin than immediately. This parallels our observation in mice where severity of lung injury is proportional to length of leukotoxin administration.

The in vivo toxicity of leukotoxin and its diol raises caution that plans to engineer crop plants for production of industrial oils such as vernolic acid (isoleukotoxin) (5) should be coupled with systems to insure that such oils are not consumed by humans or livestock. In addition, this work provides insight into the role of linoleate oxylipins in the development of ARDS and opens new avenues for development of effective therapies.

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