TOXICOLOGY AND APPLIED PHARMACOLOGY **146,** 53–59 (1997)
ARTICLE NO. TO978197

Cytotoxicity of Linoleic Acid Diols to Renal Proximal Tubular Cells

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Received January 3, 1997; accepted April 23, 1997

Cytotoxicity of Linoleic Acid Diols to Renal Proximal Tubular Cells. Moran, J. H., Weise, R., Schnellmann, R. G., Freeman, J. P., and Grant, D. F. (1997). *Toxicol. Appl. Pharmacol.* **146, 53–59.**

Monoepoxides of linoleic acid (leukotoxin and isoleukotoxin) have been associated with a variety of pathophysiological diseases in humans including multiple organ failure. They also have been shown to be toxic when injected into experimental animals. Because leukotoxin and isoleukotoxin are excellent substrates for epoxide hydrolases, we tested the hypothesis that the diol metabolites are less toxic than the parent monoepoxides using the rabbit renal proximal tubule (RPT) suspension model. An equimolar mixture of the positional isomers of the methyl esters of leukotoxin and isoleukotoxin did not cause cell death to RPT cells at concentrations up to 1 mM using lactate dehydrogenase release as the endpoint. The corresponding diols, however, caused cell death in a time- and concentration-dependent manner beginning at 4 hr and reaching 42% cell death in 6 hr at 1 mM. Cell death was not due to oxidative stress since malondialdehyde content did not increase and the iron chelator deferoxamine and the antioxidant *N,N′*-diphenyl-1,4-phenylenediamine were not cytoprotective. In contrast, cell death was associated with mitochondrial dysfunction with respiration decreasing 54% prior to the onset of cell death. Secondary to the mitochondrial dysfunction, the diols completely inhibited active Na^+ transport within 30 min of addition. These results suggest that the *in vivo* toxicity and pathophysiology previously attributed to the monoepoxides of linoleic acid may be due to the diol metabolites.

Analogous to arachidonic acid, linoleic acid is metabolized *in vivo* to a variety of potentially important products. Two of these, *cis*-9,10-epoxyoctadec-12(*Z*)-enoic acid (leukotoxin) and *cis*-12,13-epoxyoctadec-9(*Z*)-enoic acid (isoleukotoxin), have been identified in leukocytes and are produced in high levels during acute inflammation (Hayakawa *et al.,* 1986; Ozawa *et al.,* 1986). They cause cardiac failure in dogs (Fukushima *et al.,* 1988; Sugiyama *et al.,* 1987) and have been isolated from the lungs of rats exposed to toxic concentrations of O_2. They are found in the lungs of humans with adult respiratory distress syndrome (Ozawa *et al.,* 1988) and in the serum of severely burned patients with levels ranging from 30 to 100 μM (Kosaka *et al.,* 1994). In the past 8 years numerous studies have shown that leukotoxin and isoleukotoxin cause pathophysiological effects *in vitro* and *in vivo* (Hanaki *et al.,* 1991; Hayakawa *et al.,* 1989, 1990; Ishizaki *et al.,* 1995a,b; Iwase *et al.,* 1995; Ozawa *et al.,* 1991; Ozawa *et al.,* 1989; Sakai *et al.,* 1995; Zhang *et al.,* 1995).

Leukotoxin and isoleukotoxin can be formed during lipid peroxidation (Fig. 1) (Sevanian *et al.,* 1979). They are synthesized in neutrophils (Hayakawa *et al.,* 1986) and can form spontaneously in neutrophil membranes by the reaction of hydroxyl radicals with linoleic acid (Hayakawa *et al.,* 1989). They are produced also via P450-mediated epoxidation of linoleic acid (Laethem *et al.,* 1992, 1996; Moghaddam *et al.,* 1996). The rates of epoxidation obtained with linoleic acid are similar to the rates obtained with arachidonic acid (Laethem *et al.,* 1992).

Soluble and microsomal epoxide hydrolases (EC 3.3.2.3) hydrolyze cytotoxic and mutagenic epoxides to their corresponding diols as part of the general detoxification system of most eukaryotic cells. They hydrolyze mono- and diepoxides of C_{18} fatty acids and esters and these substrates have the highest K_{cat}/K_m ratios for any substrate examined (Fig. 1) (Halarnkar *et al.,* 1989; Nourooz-Zadeh *et al.,* 1992). We therefore hypothesized that the diols of leukotoxin and isoleukotoxin would be much less toxic than the parent epoxides. To test this hypothesis, we synthesized the methyl ester monoepoxides and corresponding diols of linoleic acid and compared their cytotoxic effects in the rabbit renal proximal tubule model. This model is relevant *in vivo* since kidney function is often impaired during multiple organ failure following acute inflammation or severe trauma (Regel *et al.,* 1996). Furthermore, since the mechanism of toxicity of this class of compounds is poorly characterized, we determined whether mitochondrial dysfunction or oxidative stress play a role in their toxicity. Unexpectedly, our results show that the diols are significantly more cytotoxic than the epoxides and suggest that epoxide hydrolases may activate rather than inactivate C_{18} fatty acid epoxides.

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0041-008X/97 $25.00

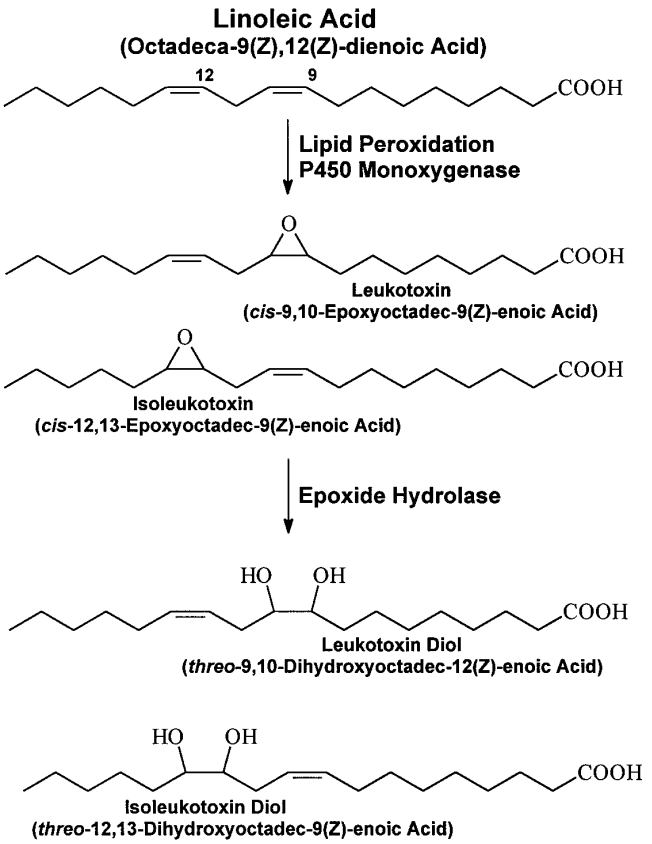

FIG. 1. Synthetic scheme for the *in vivo* production of leukotoxin diols from linoleic acid.

MATERIALS AND METHODS

Synthesis of leukotoxins and isoleukotoxins. Methyl leukotoxin, methyl isoleukotoxin, and their corresponding methylated diols were chemically synthesized from methyl linoleate using *m*-chloroperoxybenzoic acid and perchloric acid, as previously described (Borhan *et al.*, 1993; Moghaddam *et al.*, 1996). Briefly, the mixture of monoepoxides formed by the epoxidation of methyl linoleate by *m*-CPBA was separated from the diepoxides and starting material by silica column chromatography using a mobile phase of 3:7 diethyl ether:hexane. The mixture of monoepoxides was subsequently hydrolyzed to the free fatty acid diols with perchloric acid. The free fatty acid diols were then methylated using trimethylsilyl diazomethane in methanol. The monoepoxides and diol methyl esters were purified by reverse-phase HPLC utilizing a Partisil 5 ODS-3 (Whatman) column, an isocratic mobile phase of 65:35 acetonitrile:H_2O (0.1% phosphoric acid) at a flow rate of 5 ml/min and a detection wavelength of 192 nm. Fractions were collected and extracted with diethyl ether, washed, and concentrated. Structures of the synthesized compounds were confirmed using atmospheric pressure chemical ionization (APCI) with flow injection analysis, fast atom bombardment (FAB), and direct exposure probe (DEP) electron impact (EI) ionization mass spectrometry (MS). The conditions for the analysis were as follows: scan range 50–650 μ, 1.2 sec/scan; APCI temperature, 450°C for the vaporizer, 150°C for the capillary. The corona was set to 4 kV, the current was 5 μA. The mass spectrometer was a Finnigan TSQ-7000. The sources of the remaining chemicals have been reported previously (Schnellmann, 1988; Rodeheaver *et al.* 1990).

Renal proximal tubule isolation. Renal proximal tubules (RPT) were isolated and purified by the method of Vinay *et al.* (1981) as modified by Rodeheaver *et al.* (1990) and Groves *et al.* (1996) from 1.5- to 2.0-kg female New Zealand White rabbits (Myrtle's Rabbitry, Thompson Station, TN). RPTs were suspended at a protein concentration of 1 mg/ml in an incubation buffer containing (in mM) alanine, 1; dextrose, 5; heptanoate, 2; lactate, 4; malate, 5; NaCl, 115; $NaHCO_3$, 15; KCl, 5; NaH_2PO_4, 2; $MgSO_4$, 1, and Hepes, 10 (pH 7.4, 295 mOsm/kg). RPT suspensions were incubated under air/CO_2 (95%/5%) at 37°C in a gyrating water bath (180 rpm). After a 15-min preincubation period, RPT suspensions were subjected to 0.2% dimethyl sulfoxide (diluent), methyl leukotoxins (1 mM), or methyl leukotoxin diols (0.25–1 mM) for 0.5, 1, 2, 4, or 6 hr. In some experiments the iron chelator, deferoxamine (DEF, 500 μM) or *N,N′*-diphenyl-1,4-phenylenediamine (DPPD, 2 μM) was added simultaneously with methyl leukotoxin diols to RPT suspensions and the incubation continued for 6 hr.

Oxygen consumption. Oxygen consumption of RPT suspensions was monitored by the method of Schnellmann (1994) using a Clark-type oxygen probe. After basal oxygen consumption was determined, the Na^+/K^+-ATPase inhibitor ouabain (0.1 mM) was added to the RPT suspension. The difference between basal oxygen consumption and ouabain-insensitive oxygen consumption yielded the ouabain-sensitive consumption associated with Na^+/K^+-ATPase activity and active Na^+ transport.

Biochemical assays. Na^+/K^+-ATPase activity in RPT membrane fragments was quantified by the method of Schwartz *et al.* (1969). Soluble epoxide hydrolase activity in homogenized RPT was measured using *trans*-diphenylpropyleneoxide as previously described (Borhan *et al.*, 1995). Glutathione *S*-transferase activity in homogenized RPT was measured using a SpectraMax 96-well plate reader as previously described (Grant *et al.*, 1989) using 1-chloro-2,4-dinitrobenzene as the substrate. Cellular death/lysis was measured by the release of lactate dehydrogenase (LDH) by the method of Moran and Schnellmann (1996) using a CytoFluor measurement system (PerSeptive Biosystems, Bedford, MA). Protein content was determined by the method of Lowry *et al.* (1951). Lipid peroxidation was determined by measuring malondialdehyde content (Rush, *et al.*, 1985; Schnellmann, 1988).

Statistical analysis. The data are presented as means + SE. Data were analyzed by analysis of variance using SigmaStat (Jandel, San Rafael, CA). RPT isolated from one rabbit represents an *n* of 1. Multiple means were tested for significance using Student–Newman–Keuls test and a value of $p < 0.05$.

RESULTS

Mass Spectral Analysis of Leukotoxins and Leukotoxin Diols

Mass spectral analysis of the positional isomers of the diol and monoepoxide methyl esters (Figs. 2A and 2B, respectively) are shown. Prominent ions for the diols (Fig. 2A) include the protonated molecule (*m/z* 329), a dehydration product (*m/z* 311, −18 μ), a double dehydration product (*m/z* 293, −36 μ), loss of methanol (*m/z* 297) and the combined loss of water and methanol (*m/z* 279). A small signal was observed also corresponding to the loss of two waters and methanol (*m/z*261). When the sample was analyzed by FAB/MS and DEP/EI/MS, similar results were obtained. With FAB/MS an additional adduct ion from the matrix (thioglycerol) as well as water loss from the adduct was also observed. With DEP/EI/MS the spectra also showed protonated mole-

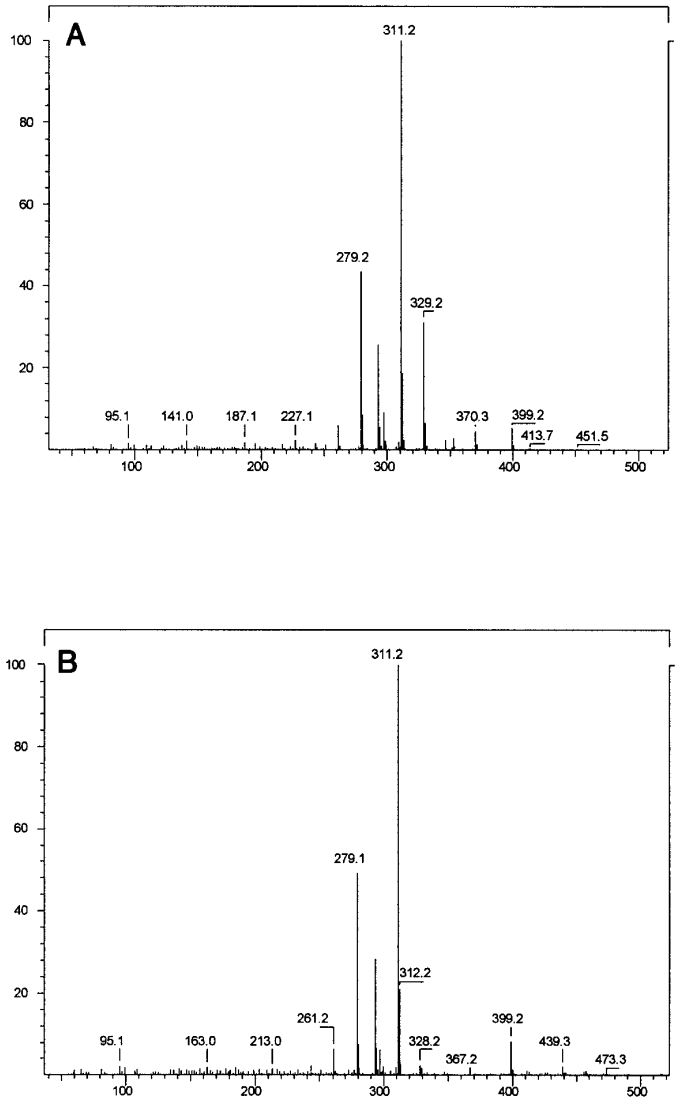

FIG. 2. Mass spectral results of leukotoxin diols (A) and leukotoxins (B). See Materials and Methods for experimental description.

cules and fragments from the protonated molecules. This is attributed to ''auto-chemical ionization'' sometimes observed due to ion–molecule reactions in the mass spectrometer ion source. Characteristic ions for the methylated leukotoxin positional isomers are shown (Fig. 2B) corresponding to the protonated epoxides (*m*/*z* 311), water loss (*m*/*z* 293), loss of methanol (*m*/*z* 279), and loss of water and methanol (*m*/*z* 261). The *m*/*z* 399 is from background in the mass spectrometer (the spectrum has not been background subtracted). As was observed with the diol, the FAB/MS and DEP/EI/MS results were similar to the APCI/MS results. With FAB/MS an additional ion corresponding to an adduct between the protonated molecule and the FAB matrix was observed, whereas with DEP/EI/MS the protonated molecule (associated with auto-chemical ionization) was observed. These results demonstrate the chemical synthesis of the methyl esters of leukotoxin and leukotoxin diols and that these compounds contain their respective positional isomers. Throughout the remainder of this paper we will refer to these compounds as leukotoxins and leukotoxin diols, but in all cases we are referring to the methyl esters of these compounds unless otherwise noted.

Toxicity of Leukotoxins and Leukotoxin Diols to RPT

To examine the cytotoxicity of leukotoxins and leukotoxin diols, RPTs were exposed to various concentrations of leukotoxins or leukotoxin diols for various periods of time, and the degree of cell death/lysis determined. RPT showed no evidence of LDH release compared to controls when exposed to leukotoxins (1 mM) for up to 6 hr (Fig. 3). However, leukotoxin diols produced cell death in a time-dependent manner beginning at 4 hr and reached 42% at 6 hr. Concentration–response studies demonstrated that 0.25 or 0.5 mM leukotoxin diol did not increase LDH release following a 6-hr exposure (Fig. 4). These results show that leukotoxins do not cause RPT cell death at concentrations up to 1 mM, while 1 mM leukotoxin diols produce extensive cell death.

Role of Oxidative Stress in the Toxicity of Leukotoxin Diols

To determine whether oxidative stress plays a significant role in the toxicity of leukotoxin diols, the degree of lipid

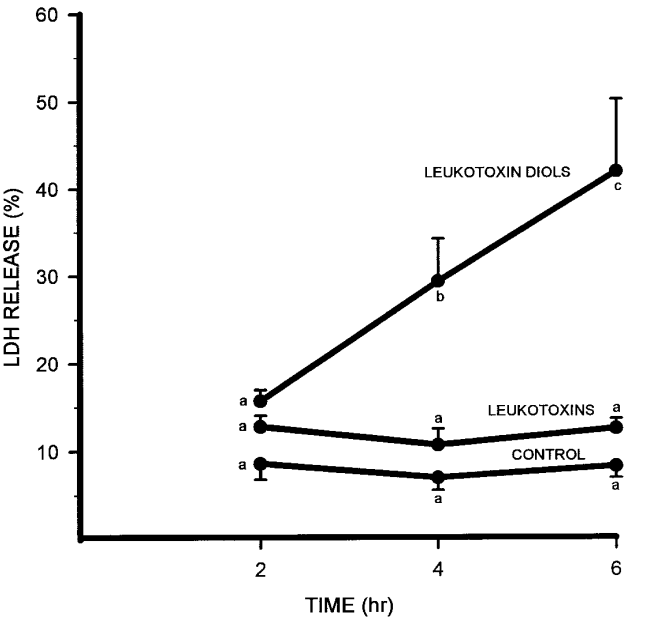

FIG. 3. Time-dependent effects of leukotoxin (1 mM) and leukotoxin diol (1 mM) on rabbit RPT LDH release. Data are represented as means ± SE ($n = 3–8$). Means with different superscripts are significantly different from one another ($p < 0.05$).

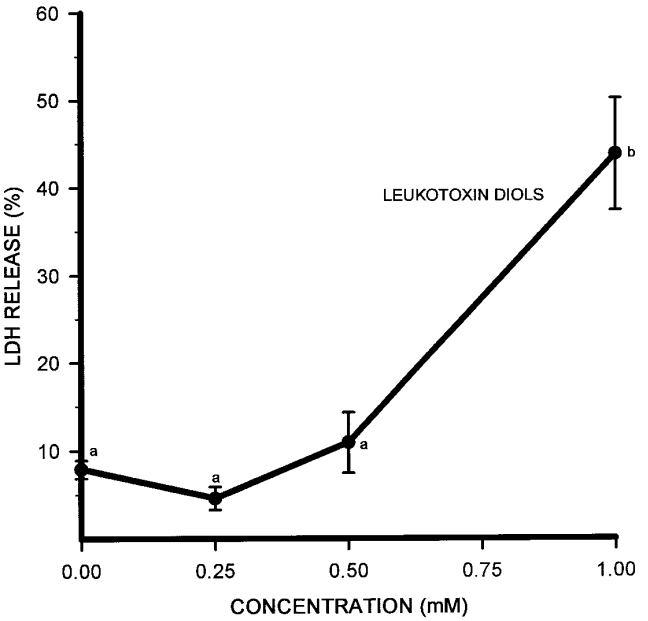

FIG. 4. Concentration-dependent effects of leukotoxin diols on RPT LDH release after 6 hr of exposure. Data are represented as means ± SE (n = 3–8). Means with different superscripts are significantly different from one another ($p < 0.05$).

peroxidation in RPT exposed to 1 mM leukotoxin diols was determined. Malondialdehyde content in RPT treated with leukotoxin diols was 0.03 ± 0.02 nmol/mg cellular protein after a 2-hr exposure and was not significantly different from controls (0.05 ± 0.02 nmol/mg protein). The role of oxidative stress in the toxicity of leukotoxin diols was examined also by determining whether an iron chelator (DEF) or an antioxidant (DPPD) was cytoprotective. Neither DEF nor DPPD had any effect on cell death induced by leukotoxin diols (Fig. 5). Previous studies from this laboratory have demonstrated that these concentrations of DEF and DPPD completely prevent oxidant-induced oxidative stress and/or cell death in this model (Schnellmann, 1988, 1991; Groves *et al.*, 1991). These results show that oxidative stress does not play a significant role in the toxicity of leukotoxin diols.

Role of Mitochondrial Dysfunction in the Toxicity of Leukotoxin Diols

To determine whether mitochondria are a target of leukotoxin diols, oxygen consumption was measured in RPT exposed to 1 mM leukotoxin diols. After a 0.5-hr exposure to the toxin, basal oxygen consumption was not different from control values (Fig. 6). After 1 and 2 hr of exposure to leukotoxin diols there was an approximate 33 and 54% reduction, respectively, in basal oxygen consumption from control values. These changes in basal oxygen consumption preceded cell death (Fig. 3).

Basal oxygen consumption is composed of ouabain-sensitive and ouabain-insensitive components. Ouabain-sensitive oxygen consumption is the result of Na^+/K^+-ATPase activity and active Na^+ transport, whereas ouabain-insensitive oxygen consumption is the result of all other cellular oxygen-consuming reactions (Schnellmann, 1994). Leukotoxin diols (1 mM) completely inhibited ouabain-sensitive oxygen consumption after 0.5, 1, and 2 hr exposures (Figs. 6 and 7). Ouabain-sensitive oxygen consumption values in controls did not change over time. Ouabain-insensitive oxygen consumption in RPT treated with 1 mM leukotoxin diols increased numerically, but not statistically, after a 30-min exposure (Fig. 6). However, normalizing the data to percentage of control revealed a 27% increase in ouabain-insensitive oxygen consumption (Fig. 7). Ouabain-insensitive oxygen consumption was not different from controls after 1 and 2 hr.

To determine whether leukotoxin diols directly inhibited the Na^+/K^+-ATPase, Na^+/K^+-ATPase activity was determined in RPT membrane fragments following a 30-min exposure to 1 mM leukotoxin diols. There was no significant difference in Na^+/K^+-ATPase activity between controls and leukotoxin diol-treated RPT (225 + 55 mU/mg cellular proteins vs 189 + 49 mU/mg cellular proteins, $p > 0.05$).

The soluble expoxide hydrolase activity in RPT cells was

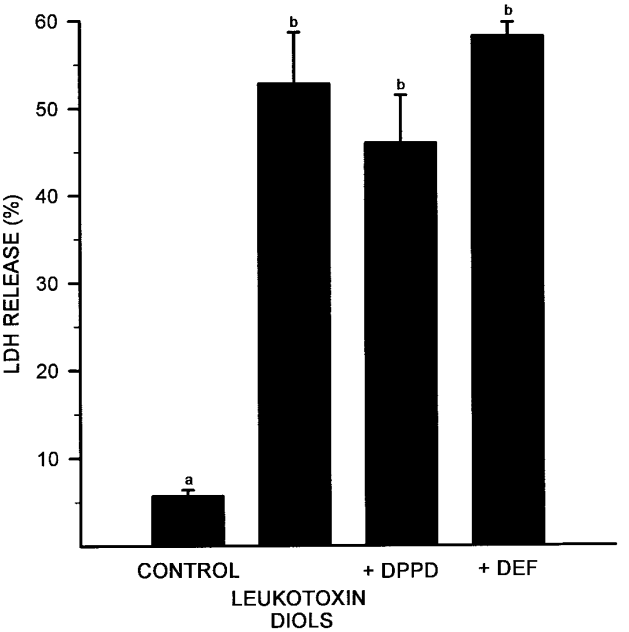

FIG. 5. Effects of *N,N′*-diphenyl-1,4-phenylenediamine (DPPD) and deferoxamine (DEF) on leukotoxin diol-induced RPT LDH release. DEF (0.5 mM) or DPPD (2 μM) was added immediately prior to the leukotoxin diols (1 mM) and the percentage LDH release was determined 6 hr later. Data are represented as means ± SE (n = 3–5). Means with different superscripts are significantly different from one another ($p < 0.05$).

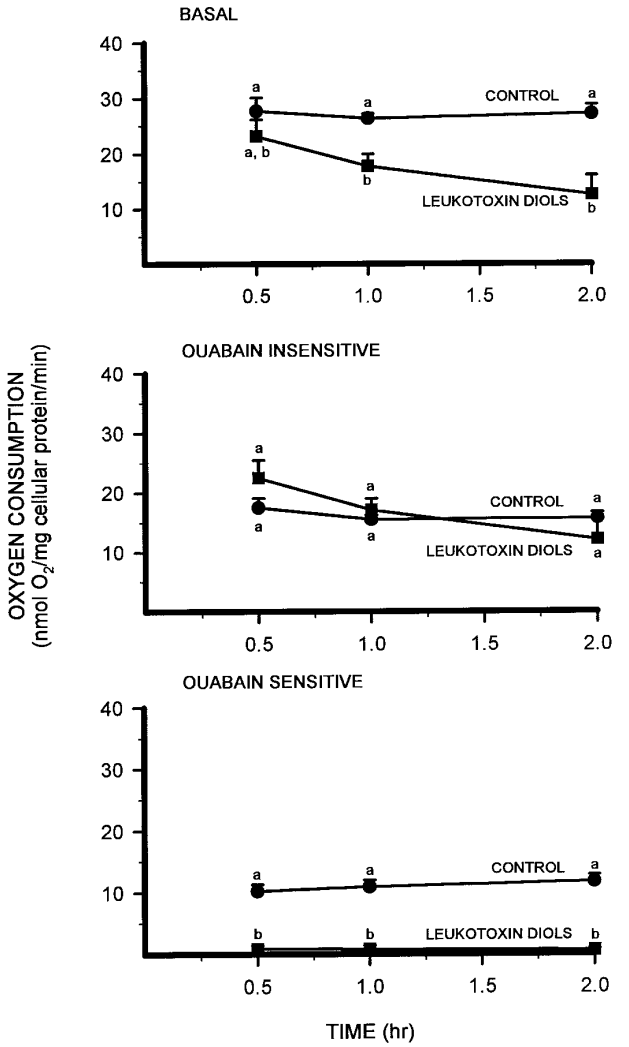

FIG. 6. Time-dependent effects of leukotoxin diols (1 mM) on RPT basal, ouabain-insensitive, and ouabain-sensitive oxygen consumption. Data are represented as means ± SE ($n = 5–6$). Means with different superscripts are significantly different from one another ($p < 0.05$).

17 + 1 nmol/mg/min and the glutathione *S*-transferase activity was 1.8 + 0.1 μmol/mg/min.

DISCUSSION

Because it is generally thought that diols are less toxic than the parent epoxides (Glatt and Oesch 1987; Manson 1980), we initially hypothesized that the diols of leukotoxin and isoleukotoxin would be nontoxic to RPT. Furthermore, since the mechanism of toxicity of this class of compounds is poorly characterized, we proposed to examine whether mitochondrial dysfunction or oxidative stress play a role in their toxicity. Unexpectedly, the leukotoxins did not produce RPT cell death at concentrations up to 1 mM, while the leukotoxin diols produced time-dependent cell death. Since RPTs contain high levels of SEH, it is not clear why the leukotoxins were not cytotoxic especially in light of our finding that the synthesized diols were cytotoxic. The reason for the lack of toxicity of leukotoxin and isoleukotoxin in RPT may result from the ability of these cells to incorporate the leukotoxin into membrane phospholipids and thereby prevent its actions, and/or detoxification through a nonepoxide hydrolase pathway such as glutathione *S*-transferase conjugation. Although this study was not designed to elucidate the metabolism of leukotoxin and isoleukotoxin in RPT cells, we did find that RPT cells have high levels of glutathione *S*-transferase activity. Whether these enzymes conjugate glutathione to leukotoxin and/or isoleukotoxin, analogous to the conjugation of glutathione to leukotriene A_4 by leukotriene C_4 synthase, remains to be determined; however, our results suggest that this is a distinct possibility.

Our finding that the diol methyl esters of linoleic acid are cytotoxic is consistent with the recent results reported by Grant *et al.* (1996) who showed that the methyl esters and the free acid diols of epoxystearic acid were more toxic than the parent epoxide. In addition, we have shown that the diol methyl esters have the same potency as do the diol free acids in an *Sf*-21 cell system (Moghaddam *et al.*, 1997). These results suggest that the methyl ester is rapidly hydrolyzed to form the free acid within cells and that the epoxide group of monoepoxidized C_{18} fatty acids is relatively nonreactive. Previous studies which have shown that leukotoxin and iso-

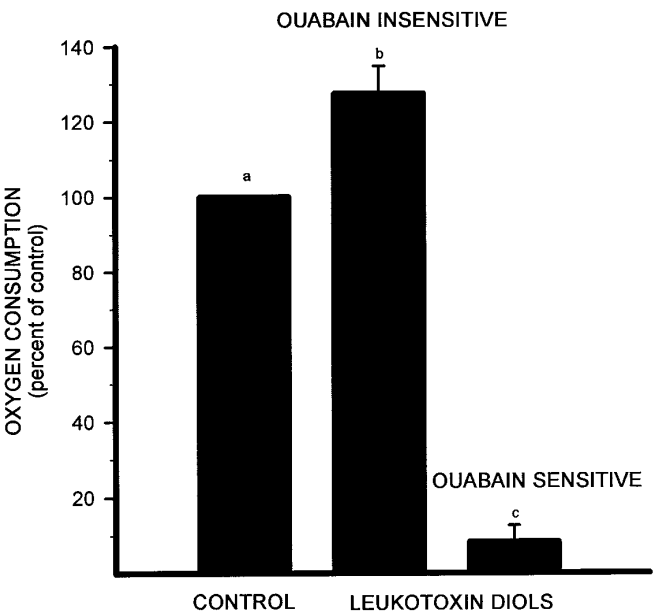

FIG. 7. The effect of a 30-min exposure to leukotoxin diols (1 mM) on RPT ouabain-insensitive and ouabain-sensitive oxygen consumption. Data are represented as means ± SE ($n = 5–6$). Means with different superscripts are significantly different from one another ($p < 0.05$).

leukotoxin are toxic *in vitro* or *in vivo* will require further evaluation based on these results.

We investigated two possible pathways of cell injury and death produced by leukotoxin diols. Oxidative stress is known to cause cell injury and death produced by a variety of oxidants and chemicals. For example, in the RPT model we have demonstrated that oxidants (hydroperoxide, *t*-butyl hydroperoxide) and halocarbons (dichlorovinyl-L-cysteine, tetrafluoroethyl-L-cysteine) cause oxidative stress and that oxidative stress plays a role in the cell death produced by these compounds (Schnellmann, 1988, 1991; Groves *et al.*, 1991). In both cases, lipid peroxidation was a sensitive marker of oxidative stress and the iron chelator DEF and the antioxidant DPPD blocked the lipid peroxidation and diminished or blocked cell death. Leukotoxin diols did not produce lipid peroxidation nor did DEF or DPPD have any effect on leukotoxin diol-induced cell death. In comparison, a concentration of *t*-butylhydroperoxide (0.5 mM) that produces a similar degree of cell death increased malondialdehyde content to greater than 1 nmol/mg cellular protein (Schnellmann, 1988). These results suggest that oxidative stress is not a significant factor in RPT cell injury and death produced by leukotoxin diols.

Another pathway by which leukotoxin diols may cause cell injury and death is through mitochondrial dysfunction. Leukotoxin diols inhibited basal respiration in RPT within 1 hr of addition and significantly prior to the onset of cell death. Probing of basal RPT respiration revealed that leukotoxin diols completely inhibited ouabain-sensitive respiration and active Na^+ transport within 30 min and remained inhibited over time. To determine whether the inhibition of ouabain-sensitive respiration by leukotoxin diols was the result of direct inhibition of Na^+/K^+-ATPase activity, Na^+/K^+-ATPase activity was measured in RPT membrane fragments. Leukotoxin diols had no direct effect on Na^+/K^+-ATPase activity. These results show that an early event in the toxicity of leukotoxin diols is mitochondrial dysfunction which leads to the inhibition of Na^+/K^+-ATPase activity and active Na^+ transport.

Ouabain-insensitive respiration, respiration associated with all other cellular processes excluding active Na^+ transport, increased 27% following a 30-min exposure to leukotoxin diol. An increase in ouabain-insensitive respiration is suggestive of uncoupling of oxidative phosphorylation (Schnellmann, 1994). Previous studies have not examined the mitochondrial effects of lekotoxin diols and studies that have examined the uncoupling effects of leukotoxin have been contradictory. Leukotoxin was shown to uncouple oxidative respiration in rat liver mitochondria (Ozawa *et al.*, 1986), but did not uncouple oxidative respiration in mitochondria isolated from rat lungs. This difference may be explained by differences in the metabolism of leukotoxin in liver, lungs, and kidneys.

In summary, we have found that leukotoxin diols are cytotoxic compounds that may be involved in a variety of pathophysiological consequences that have been previously attributed to the corresponding epoxides. The cytoxotoxicity of leukotoxin diols in RPT is mediated in part through mitochondrial dysfunction and by an indirect inhibition of the Na^+/K^+-ATPase. We found no evidence that oxidative stress plays a significant role in the toxicity of leukotoxin diols.

ACKNOWLEDGMENT

The authors thank Andrew Stanley for his technical help.

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