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TOXICITY OF LINOLEIC ACID METABOLITES

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INTRODUCTION

Leukotoxin, *cis*-9,10-epoxyoctadeca-12(Z)-enoic acid (LTX), and isoleukotoxin, *cis*-12,13-epoxyoctadeca-9(Z)-enoic acid (iLTX), are the monoepoxides of linoleic acid (*cis*-9,12-octadecadienoic acid). In the systems so far examined two primary metabolites are LTX diol, *threo*-9,10-dihydroxy-octadeca-12(Z)-enoic acid, and iLTX diol, *threo*-12,13-dihydroxyoctadeca-9(Z)-enoic acid. See figure 1. The first mention of LTX, *per se*, in the literature was in 1986 (1), but the two isomers of epoxyoctadecenoic acid have been discussed since before 1971. LTX and iLTX are found endogenously in both animals and plants. While their defensive role in plants seems fairly well established (2), their role in animals is less clear. What is clear is that LTX and iLTX are formed from linoleic acid, usually when the organism is under less than optimal conditions, and that they have a wide range of effects.

It is somewhat surprising that LTX and iLTX have so many effects. While many compounds containing the epoxide functionality are known to be extremely reactive due to the ease with which the highly strained three-membered oxirane ring can be opened, fatty acid epoxides are relatively stable and are poor alkylating agents (3). Epoxides can be degraded by reaction with glutathione to form conjugates, or water to form *vic*-diols (however, it should be noted that hydrolysis of fatty acid epoxides by water alone is extremely slow). See figure 1. These reactions usually represent detoxification pathways and may be catalyzed by glutathione S-transferases or epoxide hydrolases (4).

In this laboratory, we regularly screen a number of epoxide containing compounds for toxicity in an eukaryotic expression system. We generally find that cells transfected with

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epoxide hydrolase are resistant to the cytotoxic and genotoxic effects of most epoxide containing compounds. However, when we screened LTX and iLTX, we found that they weren't cytotoxic unless the cells were expressing soluble epoxide hydrolase (5). This suggests that LTX diol and iLTX diol are the toxic agents, rather than their epoxide precursors. The identification of the final toxic metabolite has provoked a lively debate between our lab and Ishizaki's group in Japan, which has done the majority

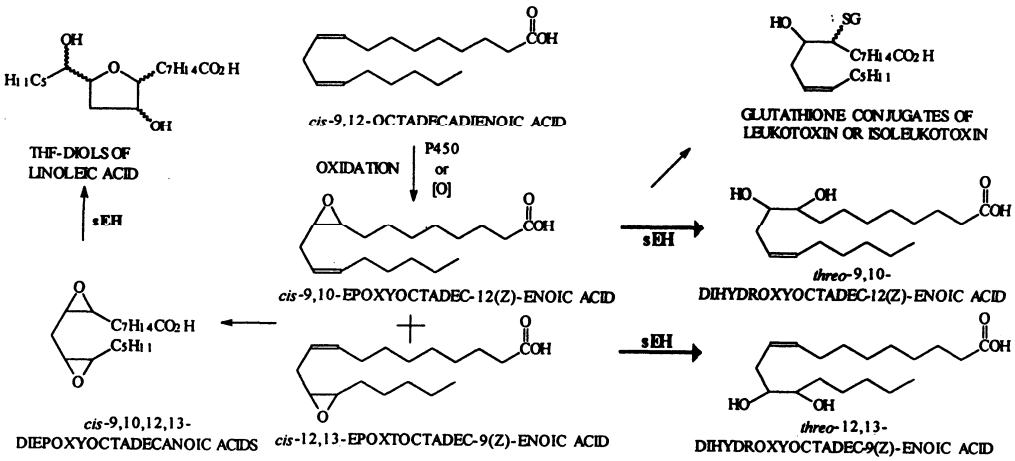

Figure 1. Formation of linoleic acid metabolites. Reactions known to occur enzymatically in cells transfected with soluble epoxide hydrolase are shown in bold. Regio- and optical isomers of thf-diols and gluathione conjugates are not shown; but conjugation could occur in the 9,10,12, or 13 positions. Additionally, the diols could be further metabolized by sulfation or glucuronidation (not shown).

of the work on LTX (6) (7). We hypothesize that LTX diol and iLTX diol account directly for many of the effects observed with LTX.

It is interesting that in the last two generations we have gone from a predominately steric acid based diet to a predominately linoleic acid based diet (8). Since specific P450s and sEH are

known to be induced by common industrial and environmental compounds, diet could increase the danger of exposure to LTX, iLTX and their metabolites in part of the population (9) (10). It is also important to remember that despite the massive amount of work that has been done on the arachidonic acid metabolites and their role in the body, linoleic acid constitutes the majority of the unsaturated fatty acids found in the body.

In this paper, we will review what is known about the synthesis, effects, metabolism, and mechanisms of action of LTX and iLTX in animals.

SYNTHESIS OF LEUKOTOXIN & ISOLEUKOTOXIN

Ozawa *et al.* found in 1986 (1) that exposure of rats to pure oxygen for 60 hrs resulted in the formation of LTX and iLTX in the lung lavage of these rats. They also found that when linoleic acid was added to leukocytes isolated from the same lung lavage, LTX and isoleuktoxin were formed. Thus, they named the compounds "leukotoxins." While lung lavage from their control rat didn't appear to contain any LTX or iLTX, it is unclear whether the isolated leukocytes were tested for the presence of LTX and iLTX before addition of linoleic acid. It seems reasonable that if the rats are, in fact, biosynthesizing LTX and iLTX, that some amount of these compounds would still be present after isolation of the leukocytes. They also mention, without showing data, that they have seen non-enzymatic conversion of LTX to iLTX *in vitro*. They suggest that iLTX is formed as a rearrangement product from LTX, but this is a very unlikely chemical reaction.

Also in 1986, Hayakawa *et al.* (11) found that LTX and iLTX could be generated by addition of linoleic acid to various cell types, including human and canine blood neutrophils and guinea pig peritoneal neutrophils. They reported that addition of linoleate to lipoxygenase *in vitro* resulted in hydroperoxy linoleate which was changed to LTX on addition of sonicated neutrophils; however, the data were not shown. They also found that addition of calcium and calcium ionophore significantly increased the amount of LTX biosynthesized by guinea pig neutrophils. They again suggested that iLTX is a by-product of LTX based on the fact that they found higher concentrations of LTX than iLTX in the guinea pig neutrophils as well as higher concentrations of 9-hydroxy-linoleate than 12-hydroxy-linoleate. Unequivocal identification of oxylipins by mass spectrometry is quite difficult. Hayakawa et al. (11) report that 9-hydroxylinoleate or 12-hydroxylinoleate are formed from the corresponding epoxide by epoxide hydrolase. Rearrangements of some epoxides under anhydrous acid can lead to allylic alcohol and reduction can lead to alcohols; however it is very unlikely that either of these products were produced by epoxide hydrolase. Exposure of epoxides to strong acid in their extraction procedure can cause some compounds to undergo complex rearrangements.

Iwase *et al.* (12) tested the hypothesis that LTX and iLTX were formed *in vivo* by reactive oxygen species other than hydroxyl radical. They made a monoepoxide generating system by combining cytochrome c with a hydrogen peroxide generating system. When they added linoleic acid to this system they found that monoepoxide formation increased in direct proportion with the amount of H_2O_2 or hypoxanthine added. Addition of catalase or radical scavenger, *para*-nitrosodimethyl-aniline, reduced the formation of the monoepoxides. However, elimination of

superoxide dismutase or addition of mannitol did not reduce monoepoxide formation. They explained these somewhat contradictory results by the possibilities that H_2O_2 was generated even in the absence of superoxide dismutase and that, based on their relative lipophilicities, mannitol is not as effective a hydroxyl radical scavenger as *para*-nitrosodimethylaniline. They concluded that linoleic acid is probably an effective hydroxyl radical scavenger and that in conditions when linoleic acid and cytochrome c are both present, LTX toxicity may occur. These conditions could include ischemia-reperfusion induced cardiac injury and inhibition of mitochondrial respiration.

TOXIC EFFECTS OF LEUKOTOXIN AND ISOLEUKOTOXIN

LTX and iLTX have been reported to have a number of toxic effects, both *in vitro* and *in vivo*. These range from relaxation of smooth muscle to a decrease in mitochondrial respiration to death of the organism.

Ozawa *et al.* (1) and Hayakawa *et al.*(11) both in 1986, found that LTX decreased the rate of state III O_2 consumption in mitochondria at concentrations between 10^{-3} and 10^{-4} M, but consumption at concentrations between 10^{-4} and 10^{-5} M. They also found that iLTX had the same effect but was only half as potent as LTX. Additionally, they saw a dose-dependent relaxation of smooth muscle cells from guinea pig stomach, which stopped quickly after addition of LTX. This suggested to them that the action took place on the membrane surface, rather than within the cell itself.

In 1988, Fukshima *et al.* (13) looked at the effect of LTX and other free fatty acids on the cardiovascular health of dogs. They found that 50 mg/kg i.v. LTX depressed the cardiac function of dogs as measured by aortic flow, dP/dt, systolic and diastolic aortic pressure, and change in heart rate. They also found that free unsaturated fatty acids had similar, but weaker effects.

In 1995, Ishizaki *et al.* (14), (15), (16) published three papers detailing the interaction of LTX and iLTX with nitric oxide synthase. Essentially, they treated isolated perfused rat lungs with various concentrations of LTX and N^G-monomethyl-L-arginine, a putative nitric oxide synthase inhibitor, or superoxide dismutase, or oxyhemoglobin. Pretreatment with any of these compounds decreased the toxic effect due to LTX alone. This suggested to them that LTX was either activating nitric oxide synthase, resulting in pulmonary edema, or that nitric oxide synthase was a marker for lung injury.

In 1988 Jia-ning *et al.* (17) saw a significant increase over controls in various parameters indicating lung cell toxicity after injecting rats with 100 μmol/kg LTX and sacrificing them after ten minutes. They also saw a significant increase in these parameters when they injected the rats with 50 μmol/kg LTX and sacrificed after twelve hours. Using a semi-quantitative measure of histological change, they found that for rats sacrificed at ten minutes, both 500 μmol/kg and 200 μmol/kg LTX resulted in histological changes, including intravascular congestion and coagulation and alveolar exudation, edema, hemorrhage and emphysema. For the rats in the subacute group (100 μmol/kg, sacrifice at six hours) they saw essentially the same changes with the addition of

infiltration of neutrophils. These changes are similar to those seen in human patients presenting with adult respiratory distress syndrome.

This is particularly interesting since LTX was associated with toxicity in patients who had symptoms of multiple organ failure, of which adult respiratory distress syndrome is one part. Hayakawa *et al.* (18) and Ozawa *et al.* (19) both published papers in 1990 addressing this. They found that patients with severe burns (>50% body surface area) seemed to recover from the primary shock of the burns, but subsequently became severely ill or died. The patients displayed pulmonary edema, cardiac failure and coagulation abnormalities, all of which are consistent with a diagnosis of multiple organ failure. LTX was present in the plasma of all four patients tested. The concentrations varied between 11.4 nmol and 37 nmol LTX/ml serum. The researchers hypothesized that LTX could be the cause of all these toxic events, having been synthesized by neutrophils in response to the shock of the burn and then spread throughout the body.

In 1994 Kosaka *et al.* (20) correlated high plasma LTX levels in burn patients with death. They saw that patients without extensive burns (<70% body surface area) had a low but fairly steady concentration of plasma LTX over time. The maximum concentration reached was 22.6 ±11.9 nmol/ml at 5 weeks post injury. In contrast, patients with extensive burns (70% body surface area) had two peak plasma LTX levels; one initially and one beginning at week 3. The concentrations varied between 99.6 ±45.1 nmol/ml immediately after injury to 29.8 ±9.6 nmol/ml at day 14 and back up to 98.7 ±24.6 nmol/ml 6 weeks post injury. The control patients had less than 2 nmol/ml plasma LTX. The mortality rate for patients with extensive burns was 66.7%, while it was 22.2% for those without extensive burns. They also found that the mortality rate was significantly higher (61%) for patients with peak plasma LTX concentrations greater than 30 nmol/ml, while the mortality rate was only 8% for patients with peak plasma LTX concentrations less than 30 nmol/ml. They saw a correlation between peak plasma LTX concentrations in the late phase and burn surface area; they also saw a mild correlation between burn surface area and early phase peak plasma LTX levels. They concluded that LTX was either causing the death of these patients or exacerbating their condition.

METABOLITE TOXICITY: LEUKOTOXIN DIOL & ISOLEUKOTOXIN DIOL

In order to examine possible methods for ameliorating LTX toxicity, Moghaddam *et al.*, in 1997, (5) exposed eukaryotic cells expressing recombinant human soluble epoxide hydrolase to LTX and its isomer, iLTX. Surprisingly, they found that LTX and iLTX were only toxic to those cells expressing soluble epoxide hydrolase (LC_{50}=210 μM). Cells expressing control enzymes were not affected by LTX or iLTX. They had expected LTX diol and iLTX diol (*threo*-9,10-dihydroxyoctadec-12-(Z)enoic acid and *threo*-12,13-dihydroxyoctadec-9-(Z)enoic acid, respectively) to be non-toxic metabolites. The results suggested otherwise, so they challenged the cells with LTX diol and iLTX diol. They found that the diol compounds were equally cytotoxic (LC_{50}=180 μM) regardless of which enzyme the cell was expressing. This is a much higher concentration than the 30 nmol/ml plasma LTX seen by Kosaka *et al.* (20) in burn

patients. However, it is unclear if the plasma LTX concentrations are of the same magnitude as what might be found in the affected areas. In the same paper, Moghaddam *et al.* reported that 300 μM LTX diol and iLTX diol, but not LTX or iLTX, decreased net transepithelial ion transport and increased paracellular permeability in five hours in rat pulmonary alveolar epithelial cells. They also saw mortality in rats within two hours with 35 mg/kg LTX diol but no mortality with up to 100 mg/kg LTX (injection via cardiac puncture). In mice (injection via the tail vein), 30 % mortality occurred within 4 minutes with 200 mg/kg LTX diol. In contrast, it took 18 to 24 hours for them to see 25 % mortality with 400 mg/kg LTX.

When eukaryotic cells expressing soluble epoxide hydrolase were challenged with 200 μM [^{14}C]-labeled LTX and iLTX, the only metabolites which correlated with toxicity were LTX diol and iLTX diol. The diols accounted for 85% of the radioactivity. Toxicity could be lessened by pre-incubation of soluble epoxide hydrolase expressing cells with a soluble epoxide hydrolase inhibitor. Interestingly, endogenous glutathione levels decrease after incubation with LTX; however, the glutathione conjugate did not appear to be formed in an appreciable concentration. This suggests that the diol may be the final toxic metabolite, but some downstream event may be affecting glutathione levels.

Moran *et al.*, also in 1997 (21), argued that since renal failure often occurs in multiple organ failure, renal cells were a good model for testing the toxicity LTX, iLTX and their respective diols. They found that 1 mM LTX diol and iLTX diol caused 42 % death, as measured by lactate dehydrogenase release, whereas, 1 mM LTX and iLTX did not. They also noted, however, that lower concentrations of the diol failed to result in cell death even after 6 hours. They determined that the diols were not causing oxidative stress to the cells by noting that malondialdehyde levels were not significantly different than those in control cells, and that addition of an iron chelator or an antioxidant failed to protect cell death induced by LTX diol and iLTX diol. They mentioned that LTX diol and iLTX diol reduced basal oxygen consumption and completely depressed ouabain-sensitive oxygen consumption. Ouabain-sensitive oxygen consumption is the result of Na^+/K^+-ATPase activity and active Na^+ transport. They did not see direct inhibition of the Na^+/K^+-ATPase by the diols however.

CONCLUSIONS

The work originally done on LTX and iLTX found a number of toxic effects, but failed to look for possible metabolites. It seems likely, because much of the original research was done in whole animals, that soluble epoxide hydrolase was present. The work done by Moghaddam and Moran suggests that LTX diol and iLTX diol are probably the toxic agents. There remains the possibility, however, that the toxic agent is a further metabolite. The epoxide moiety suggests possible conjugation with glutathione. Patients with adult respiratory distress syndrome often have a deficiency of glutathione in their lung lavage (22). This could be due to glutathione conjugation of LTX or iLTX or some downstream event of toxicity initiated by the epoxides.

Whatever the toxic agent, be it the epoxide compound, diol compound or some as yet unknown metabolite, the mechanism of toxicity continues to prove elusive. In all cases, cell

display common symptoms of toxicity: depression of mitochondrial respiration, influx of ions, decrease of membrane potential, release of lactate dehydrogenase and swelling of cells. These are all symptomatic of necrotic cell death. The work by Ishizaki (14) and Iwase (12) suggest that toxicity could be due to creation of various reactive oxygen species, such as hydroxyl radical or nitric oxide, but Moran's (21) work suggests that oxidative stress is not a cause of toxicity.

It seems clear, however, that whether these compounds or their metabolites are toxic, or simply a marker of a toxic event, their study could add a great deal to the field of bioactive lipids. Most previous work on bioactive lipids has concentrated on the eicosanoic acids. This has proved an extremely rich field. It is important to remember, however, that linoleic acid is one of the most abundant fatty acids in the body. It seems quite possible that oxygenated metabolites of linoleic acid could be chemical mediators in a cascade analogous to the arachidonic acid cascade.

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