Bioactivation of leukotoxins to their toxic diols by epoxide hydrolase

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Leukotoxin is a linoleic acic 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[1]. 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)[2–4]. This often fatal condition is commonly attributed to the toxicity of leukotoxin produced by leukocytes, which are recruited to the burned skin of victims in order to control infection. 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[5].

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[6]. 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[7,8].

Epoxide hydrolases are members of the α/β-hydrolase fold enzyme family that convert epoxides to the corresponding diols. Two diverse epoxide hydrolases, known as the microsomal and the 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[9,10]. Along with the microsomal epoxide hydrolase, the soluble epoxide hydrolase is thought to provide cellular protection from exogenous and endogenous epoxides by detoxifying them to nonreactive, more water soluble diols. The soluble epoxide hydrolase turns over a variety of fatty acid epoxides at high rates[11,12]. Recently, we have succeeded in cloning murine[13] and human[14] 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[15]. 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. 2*a*). Methyl leukotoxin was not toxic to control cells expressing β-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. 2, *b* 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[12]. Thus, it is not surprising that cells transfected with the virus cod-

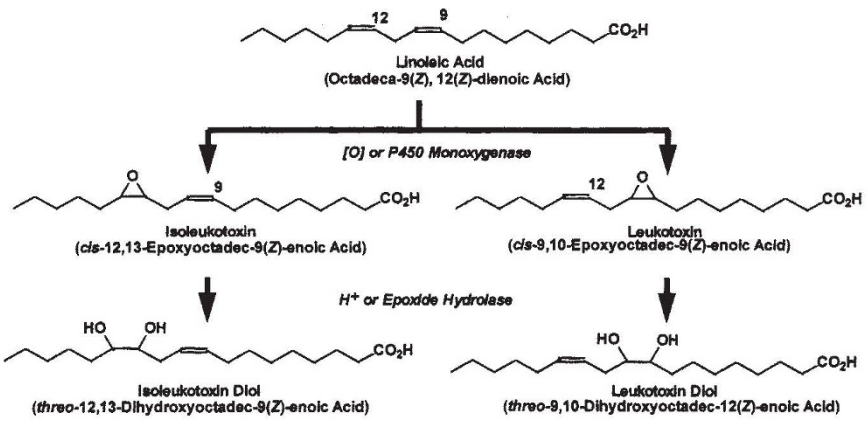

Fig. 1 Linoleic acid is an 18:2 polyunsaturated lipid, which is a very abundant fatty acid in the average American diet. It can be converted by autooxidation or P-450 action to two epoxides commonly known as leukotoxin and isoleukotoxin. Either acid catalyzed hydrolysis or exposure to epoxide hydrolase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

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. 2*b*) as was its free acid[15]. 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 protoxins. In the systems studied here, they are only toxic following activation to the corresponding diols. The low toxicity of the linoleate diepoxides 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[16].

Further support for the above hypothesis was obtained by following the distribution and metabolism of methyl [1-^{14}C]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-

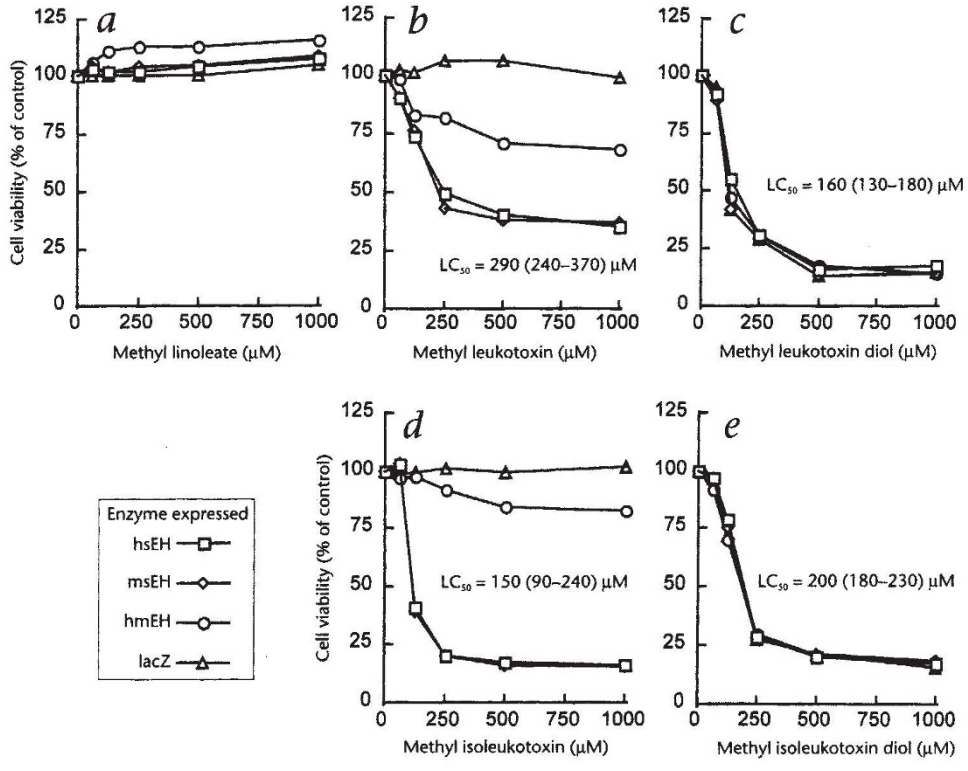

Fig. 2 Effects of lipids on viability of cultured cells of *Spodoptera frugiperda* (Sf-21 cells). *a*, Methyl linoleate produced no change in viability. *b*, Methyl leukotoxin was not toxic to lacZ-expressing cells, mildly toxic to hmEH-expressing cells but was toxic (LC_{50} ≈290 μM) to cells expressing either human or mouse sEH. *c*, Methyl leukotoxin diol was toxic (LC_{50} ≈160 μM) to all cell types. *d*, Methyl isoleukotoxin was not toxic to lacZ-expressing cells, mildly toxic to hmEH-expressing cells and toxic (LC_{50} ≈150 μM) to those cells expressing mouse or human sEH. *e*, Methyl isoleukotoxin diol was toxic (LC_{50} ≈200 μM) to all cells, showing that the hydrolysis of the epoxides of methyl leukotoxin and methyl isoleukotoxin to their diols causes the toxicity attributed to these compounds. On the basis of studies of uptake and metabolism of ^{14}C leukotoxin, the lethal concentration of leukotoxin diol or isoleukotoxin diol produced by cells expressing hsEH is slightly lower than the lethal concentration of exogenous diol (data not shown). All experiments were replicated three to seven times on different days. All of the replicates were used in the calculation of LC_{50} values with the upper and lower fiducial limits calculated with the statistical program Polo. Results similar to the ones reported here for methyl esters were obtained with free fatty acids (data not shown). The diepoxides were much less toxic (LC_{50} ≈600 μM) and their hydration products (data not shown) caused no toxicity to any cell type at concentrations up to 1 mM.

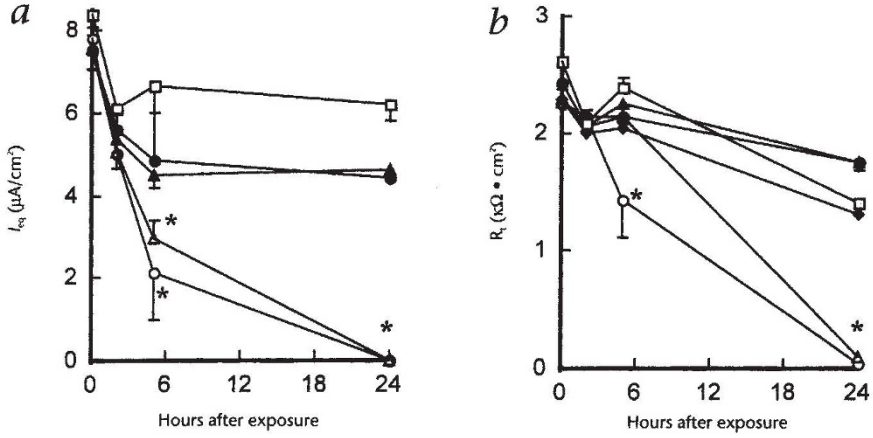

Fig. 3 Effects of lipids on bioelectric properties of primary cultured monolayers of rat pulmonary alveolar epithelial cells. Data are presented as means ± s.d.; n = 5–10 monolayers. * Mean significantly less than that of the parent lipid (methyl linoleate) at the same time after exposure as determined by one-way ANOVA/Scheffé contrast. Monolayers were treated with 300 μM methyl linoleate (□), 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_t), short-circuit current (I_{eq})] were recorded using a voltohmmeter as previously described[20]. *a*, Treatment of monolayers with 300 μM methyl leukotoxin diol or methyl isoleukotoxin diol resulted in decreased I_{eq} by 5 h following exposure, suggestive of reduced net transepithelial ion transport. Complete absence of I_{eq} 24 h following exposure indicated the loss of epithelial cell viability. *b*, R_t values of same monolayers shown in *a*. Decreased R_t 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_t 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).

toxin diol and methyl isoleukotoxin diol, and log P of 4.18 for leukotoxin diol and isoleukotoxin diol. Both of these arguments support the concept that the epoxides are protoxins for the diol or subsequent metabolite, but they do not directly address subtle differences in potency between leukotoxin, isoleukotoxin and their diols.

We subsequently examined the effects of these linoleate–derived compounds in an *in vitro* model of mammalian lung: monolayers of primary cultured rat alveolar epithelial cells[17]. Within the gas exchange region of adult mammalian lung, alveolar epithelium provides the principal resistance to the movement of water and solutes across the air–blood barrier into air spaces[18]. Etiology of adult respiratory distress syndrome likely involves damage to this tissue[19]. Exposing alveolar epithelial cells to oxidant gases induces increased permeability as a result of altered intercellular junctions but spares membrane ion transport function[20]. As part of the present study, we examined the toxicity of methyl linoleate, methyl leukotoxin and methyl isoleukotoxin, and their corresponding diols to alveolar epithelial barrier function *in vitro*. However, as assessed by bioelectric measurements[20], we estimated that monolayer short-circuit current was reduced within 5 hours following exposure to the diols (Fig. 3*a*). After 24 hours, monolayer permeability was relatively unchanged in the linoleate and monoepoxide treatment groups but significantly elevated (as evinced by decreased values of transepithelial resistance) in monolayers exposed to either diol, 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 exposure[20]. 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* exposures[21]. As shown in Fig. 3, there is no statistically significant difference between methyl linoleate, 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 linoleate 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 much more pronounced symptoms at a lower concentration than the parent leukotoxins. The leukotoxin diol concentrations showing biological activity *in vitro* are also consistent with *in vivo* levels of leukotoxin observed in burn and ARDS patients[3,4,22]. 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 in control patients[22]. We suggest that the numerous effects attributed to leukotoxin including pulmonary edema, vasodilatation, capillary damage, coagulation problems, cardiac arrest, mitochondrial dysfunction, and nitric oxide elevation[3,23–26] be reexamined using the diol metabolites.

Both the transfected Sf cells and the alveolar epithelial cells are model systems in which end points of cell death and large bioelectric changes were monitored. One can anticipate that physiological effects will occur much more rapidly *in vivo* when more subtle changes can influence the physiology of an organism. Neither the Sf cells nor the alveolar epithelial cells have significant levels of sEH, thus little bioactivation occurs. However, when leukotoxins are given *in vivo*, there can be metabolism by EHs in target tissues or the diols can be formed in hepatic and renal tissues with high sEH and then released into general circulation. Lungs contain low levels of both sEH and mEH, which could explain the action of leukotoxin in isolated pulmonary tissue. Conversely, leukotoxins may exert their toxicities via activation of other enzyme systems; for example, nitric oxide production[26]. It is clear that sophisticated physiological end points should be used in a comparison of leukotoxins and their diols[3,23–26]; however, our preliminary data using mortality and *in vivo* symptomatology in both rats and

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 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 msEH could be reversed by administration of the potent sEH inhibitor 4-fluorochalcone oxide[27]. 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)[28,29]. 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[30].

Methods

Leukotoxin synthesis. Methyl leukotoxin and methyl isoleukotoxin, and their diols were chemically synthesized from methyl linoleate using *m*-chloroperbenzoic acid, as described previously[7,31]. 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/LREI/MS, and ^{1}H and ^{13}C NMR and comparisons of these data to those found in the literature[32]. 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[14]. Sf-21 cells were infected with recombinant baculoviruses producing either human soluble epoxide hydrolase (hsEH), mouse soluble epoxide hydrolase (msEH), human microsomal epoxide hydrolase (hmEH), 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)[15]. For tracer studies leukotoxin was prepared as described above from 1-^{14}C-labeled methyl linoleate (supplied by NEN Life Science Products, 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[20]. 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. Blood-borne 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% CO_2/air, the initial medium was removed from the cultures and replaced with serum-free F-12 + 6 nutrient medium as previously described[20]. 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 zyphoid 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-methoxyethanol after they were anesthetized by inhaling methoxyflurane.

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