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The Role of Methyl-Linoleic Acid Epoxide and Diol Metabolites in the Amplified Toxicity of Linoleic Acid and Polychlorinated Biphenyls to Vascular Endothelial Cells

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Selected dietary lipids may increase the atherogenic effects of environmental chemicals, such as polychlorinated biphenyls (PCBs), by cross-amplifying mechanisms leading to dysfunction of the vascular endothelium. We have shown previously that the ω-6 parent fatty acid, linoleic acid, or 3,3′,4,4′-tetrachlorobiphenyl (PCB 77), an aryl hydrocarbon (Ah) receptor agonist, independently can cause disruption of endothelial barrier function. Furthermore, cellular enrichment with linoleic acid can amplify PCB-induced endothelial cell dysfunction. We hypothesize that the amplified toxicity of linoleic acid and PCBs to endothelial cells could be mediated in part by cytotoxic epoxide metabolites of linoleic acid called leukotoxins (LTX) or their diol derivatives (LTXD). Exposure to LTXD resulted in a dose-dependent increase in albumin transfer across endothelial cell monolayers, whereas this disruption of endothelial barrier function was observed only at a high concentration of LTX. Pretreatment with the cytosolic epoxide hydrolase inhibitor 1-cyclohexyl-3-dodecyl urea partially protected against the observed LTX-induced endothelial dysfunction. Endothelial cell activation mediated by LTX and/or LTXD also enhanced nuclear translocation of the transcription factor NF-κB and gene expression of the inflammatory cytokine IL-6. Inhibiting cytosolic epoxide hydrolase decreased the LTX-mediated induction of both NF-κB and the IL-6 gene, whereas the antioxidant vitamin E did not block LTX-induced endothelial cell activation. Most importantly, inhibition of cytosolic epoxide hydrolase blocked both linoleic acid-induced cytotoxicity, as well as the additive toxicity of linoleic acid plus PCB 77 to endothelial cells. Interestingly, cellular uptake and accumulation of linoleic acid was markedly enhanced in the presence of PCB 77. These data suggest that cytotoxic epoxide metabolites of linoleic acid play a critical role in linoleic acid-induced endothelial cell dysfunction. Furthermore, the severe toxicity of PCBs in the presence of linoleic acid may be due in part to the generation of epoxide and diol metabolites. These findings have implications in understanding interactive mechanisms of how dietary fats can modulate dysfunction of the vascular endothelium mediated by certain environmental contaminants. © 2001 Academic Press

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Activation or dysfunction of the vascular endothelium can result in its reduced effectiveness as a selectively permeable barrier to plasma components, which is a critical event in the etiology of vascular diseases such as atherosclerosis (Flarahan, 1992; Ross, 1993). The endothelium interacts with the blood and underlying tissues, serves as both a pro- and antithrombotic surface, and releases regulatory factors important in modulating vascular tone. Factors implicated in the pathogenesis of atherosclerosis include chronic and cumulative metabolic alterations of the endothelium induced by certain lipids, prooxidants, inflammatory cytokines, and environmental contaminants, such as polyhalogenated aromatic hydrocarbons.

There is ample evidence suggesting that serum cholesterol is a predictor of atherosclerosis and that serum cholesterol concentrations can be modified by varying the composition of dietary fat. Less is known, however, about the role of specific fatty acids in atherosclerosis. The role of saturated fatty acids in atherosclerosis has been questioned (Mensink, 1993; Hegsted *et al.*, 1965). In fact, data from subjects with varying degrees of coronary atherosclerosis support the hypothesis that high serum polyunsaturated fatty acid levels (e.g., linoleic acid or 18:2n−6), when insufficiently protected by antioxidants (e.g., vitamin E), may indicate a higher risk of atherosclerosis (Kok *et al.*, 1991). Recent research with a population from a country with one of the highest dietary polyunsaturated/satu-

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rated fat ratios in the world has concluded that diets rich in ω-6 (or n−6) fatty acids may contribute to an increased incidence in atherosclerosis, hyperinsulinemia, and tumorigenesis (Yam *et al.*, 1996). There appears to be a positive correlation between linoleic acid levels in the phospholipid fractions of human coronary arteries and ischemic heart disease (Loustarinen *et al.*, 1993). In addition, linoleic acid can increase expression of CD36, a scavenger receptor for oxidized low-density lipoprotein (LDL) (Pietsch *et al.*, 1995), and concentrations of linoleic acid in adipose tissue were positively correlated with the degree of coronary artery disease (Hodgson *et al.*, 1993).

Selected fatty acids, and especially ω-6 or n−6 unsaturated fatty acids, derived from the hydrolysis of triglyceride-rich lipoproteins, may be atherogenic by causing endothelial injury or dysfunction and subsequent endothelial barrier dysfunction (reviewed in Hennig *et al.*, 1996b). In support of this hypothesis, we have shown that saturated fatty acids in general had little effect on endothelial barrier function. On the other hand, unsaturated fatty acids, and mostly linoleic acid, can markedly disrupt endothelial barrier function, expressed as an increased transfer of both albumin and LDL across the endothelium (Hennig *et al.*, 1984; Hennig *et al.*, 1985; Hennig *et al.*, 1990). Most interestingly, we found that, when comparing fatty acid extracts derived from different animal fats and plant oils, the fat-induced disruption of endothelial barrier function was related to the amount of linoleic acid present in the fat source (Hennig *et al.*, 1993). The impairment of the endothelial barrier function by linoleic acid or linoleic acid hydroperoxide was greatly reduced by preenrichment of cells with vitamin E (Hennig *et al.*, 1987). In addition to vitamin E, we have shown that endothelial cell exposure to unsaturated fatty acids, and in particular to linoleic acid, can deplete cellular glutathione levels (Toborek and Hennig, 1994). Similar results were observed after cell exposure to the inflammatory cytokine TNF-α (Toborek *et al.*, 1996) or PCB 77 (Slim *et al.*, 2000). Furthermore, our data strongly support the fact that selected unsaturated fatty acids (e.g., linoleic acid) and inflammatory cytokines may cross-amplify vascular endothelial cell activation, an inflammatory response, and atherosclerosis (Toborek *et al.*, 1996).

In addition to generation of free radicals and subsequent oxidative stress, other toxic metabolites of unsaturated lipids such as linoleic acid may contribute significantly to the endothelial cell dysfunction we observed (see Fig. 1). For example, there is evidence that monoepoxides of linoleic acid (or leukotoxins/isoleukotoxins) or their diol derivatives are associated with multiple organ failure and adult respiratory distress syndrome (ARDS) (Hayakwa *et al.*, 1990; Ozawa *et al.*, 1991). In addition, leukotoxin(s) (LTX) has been reported to cause high-permeability pulmonary edema in isolated lungs and to cause dose- and time-dependent damage in cultured pulmonary vascular endothelial cells (Ishizaki *et al.*, 1995). LTX can be formed during lipid peroxidation (Sevanian *et al.*, 1979). Hayakawa *et al.* (1990) have shown that LTX can also be generated spontaneously in neutrophils during interaction of membrane-associated linoleic acid with hydroxyl radicals. In addition, induction of microsomal P450 monooxygenases enhanced autooxidation of polyunsaturated fatty acids to epoxides, suggesting that these intermediates can also be formed by the epoxidation of linoleic acid by catalytic reactions of cytochrome P450 enzymes (Hoebel *et al.*, 1998; Rifkind *et al.*, 1995).

We recently reported an additive or amplified toxicity of linoleic acid and PCBs, such as PCB 77 to endothelial cells (Hennig *et al.*, 1999). However, exact mechanisms of this phenomenon are still unknown. A direct contribution of oxidative stress by selected ω-6 fatty acids (and primarily linoleic acid), as well as an involvement of cytochrome P450 enzymes, in both PCB and fatty acid metabolism appear to be the critical underlying mechanisms in contributing to the observed endothelial dysfunction. Thus, the central hypothesis of this study is that linoleic acid oxidation metabolites, such as leukotoxins and their diol metabolites, are responsible in part for linoleic acid-mediated disruption of vascular endothelial barrier function. We also propose that these toxic metabolites of linoleic acid can contribute to the amplified cytotoxicity of PCB 77 when endothelial cells are enriched with linoleic acid.

MATERIALS AND METHODS

Cell culture and experimental media. Endothelial cells were isolated from porcine pulmonary arteries and cultured as previously described (Hennig *et al.*, 1984). Cultures were verified as endothelial cells by uniform cobblestone morphology and by quantitative determination of angiotensin-converting enzyme activity or by their uptake of fluorescent labeled acetylated (Molecular Probes Inc., Eugene, OR). The basic culture medium consisted of M199 (GIBCO Laboratories, Grant Island, NY) containing 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT). The experimental media were composed of M199 enriched with 5% FBS, selected concentrations of linoleic acid (>99% pure; Nu-Chek Prep, Elysian, MN), methyl leukotoxin (LTX) or methyl leukotoxin diol (LTXD), and/or PCB 77 (3,3′,4,4′-tetrachlorobiphenyl). Cellular accumulation of methyl esters of fatty acids appear to be similar to that of free acids, and, once in cells, methyl esters are converted rapidly to free fatty acids (Greene *et al.*, 2000a,b).

Synthesis of leukotoxins, epoxide hydrolase inhibitors, and PCB. Methyl leukotoxin (methyl leukotoxin and methyl isoleukotoxin) were chemically synthesized from methyl linoleate using *m*-chloroperbenzoic acid, as described previously (Moghaddam *et al.*, 1997). The corresponding diols were obtained by the hydrolysis of these epoxides in a 5% perchloric acid solution in 40% aqueous tetrahydrofuran (Moghaddam *et al.*, 1997). The soluble epoxide hydrolase inhibitors used herein were synthesized as described by Morisseau *et al.* (1999). In the present study, primarily two kinds of epoxide hydrolase inhibitors were used, a soluble or cytosolic (1-cyclohexyl-3-dodecyl urea) and a microsomal (dodecyl-amine) epoxide hydrolase inhibitor. Other soluble inhibitors, e.g., 1,3-dicyclohexyl urea, that are structurally related to 1-cyclohexyl-3-dodecyl urea but were less potent also were tested in selected experimental settings.

PCB 77 was synthesized from 3,3′-dichlorobenzidine, as described by Schramm *et al.* (1985). The PCB was purified by Florisil (Macherey-Nagel, Düren, Germany) and Alumina (Aluminumoxid 90, Merck, Darmstadt, Germany) chromatography and recrystallization from methanol. Structural assignments were confirmed by nuclear magnetic resonance spectrometry and mass spectroscopy.

Endothelial barrier function (albumin transfer studies). Endothelial barrier function was measured as transendothelial albumin transfer using polystyrene chambers with a 0.8-μm pore size polycarbonate membrane (Millipore Corporation, Bedford, MA) according to the method of Hennig *et al.* (1984). After approximate confluence, endothelial monolayers were exposed to control media or media enriched with lipid derivatives and/or PCB 77. Some cultures were first pretreated with an epoxide hydrolase inhibitor and then followed by coexposure to combinations of lipids and PCB for 24 h. Following treatments, chambers with endothelial cells attached to the membranes were washed with M-199 and exposed to 200 μM bovine serum albumin (fatty acid-free, Sigma Chemical Company, St. Louis, MO) in M-199 for 1 h. After incubation with albumin, the albumin transferred across endothelial monolayers was determined using bromcresol green (Sigma Chemical Company) and recorded spectrophotometrically at 630 nm.

Nuclear extracts and electrophoretic mobility shift assays. Nuclear protein extracts from endothelial cells were prepared according to the method of Dignam *et al.* (1983), and an electrophoretic mobility shift assay (EMSA) was performed using a commercially available kit from Promega Corp. (Madison, WI). Nuclear protein extracts (5 μg) were incubated with the 16 fmol of ^{32}P-endlabeled 22-bp oligonucleotide probe, with the κB enhancer DNA element containing a tandem duplicate of an NF-κB binding site (underlined) (5′-AGTTGA*GGGGACTTTCC*CAGGC-3′). The mixture included 2–3 μg of poly(dI-dC) in a binding buffer, and incubation took place at room temperature for 20 min. The resulting DNA–protein complexes were resolved on a 5% nondenaturing polyacrylamide gel.

Reverse transcriptase–polymerase chain reaction. Interleukin 6 (IL-6) mRNA levels were determined using a semiquantitative RT–PCR method. Treated endothelial cells were lysed and total RNA was extracted using RNA-STAT-60 (Tel TEST, Inc., Friendswood, TX) according to the manufacturer. To each 60-mm dish, 1 mL RNA STAT 60 was added and incubated for 30 min at 4°C. At the end of the incubation period, 200 μL chloroform was added for RNA extraction, and samples were incubated for 5 min and centrifuged for 15 min at 12,000*g*, at 4°C. After collecting the upper phase, 600 μL of isopropanol was added. Tubes were inverted and incubated at −70°C for 20 min. At the end of the incubation period, tubes were further centrifuged at 12,000*g* at 4°C and the supernatant was discarded and each pellet was dissolved in 20 μL DEPEC water and stored at −70°C. Isolated RNA was quantified by determining the absorbance at 260 nm (1 unit absorbance being equivalent to 42 μg/mL RNA). Superscript II reverse transcriptase (Gibco Laboratories) was used for reverse transcription of total RNA to total cDNA. Specific primers were designed using the software package Oligo 5.0 (National Biosciences Inc., Plymouth, MN) and were synthesized by MWG-Biotech Inc. (High Point, NC). The primers sequence used for IL-6 were 5′ (-GCA AGG AGG TAC TGG CAG AAA ACA-) 3′ and 5′ (-GGA CGG CAT CAA TCT CAG GTG-) 3′. Oligonucleotide primers used to amplify the porcine housekeeping gene β-actin were published by Barchowsky *et al.* (1998). The primer sequence for IL-6 resulted in a product size of 365 bp. PCR reaction was performed using *Taq* DNA polymerase (GIBCO Laboratories) using Perkin–Elmer GeneAmp PCR system 9700. The annealing temperature for IL-6 was 62°C. Expression of IL-6 was studied using 41 cycles. At this cycle number the relative concentrations of transcripts for β-actin and IL-6 could be estimated. Cycling times were optimized to ensure that the amplification cycles were below the plateau level for the IL-6 gene. The amplified PCR products were separated on a 2% TBE agarose gel, stained with SYBER Gold (Molecular Probes) and visualized using phosphoimaging technology (FLA-2000, Fuji, Stamford, CT).

Epoxide hydrolase activity. Harvested cells were suspended in 1 mL of chilled sodium phosphate buffer (0.1 M, pH 7.4) containing 1 mM of EDTA, PMSF, and DTT. The cells were disrupted using a Polytron homogenizer (9000 rpm for 30 s). The homogenate was centrifuged at 9000*g* for 10 min at 4°C. The supernatant was then used as enzyme extract. Protein concentration was quantified using the Pierce BCA assay (Pierce, Rockford, IL), using Fraction V bovine serum albumin (BSA) as the calibrating standard. Epoxide hydrolase activity was measured using racemic [^{3}H]*trans*-1,3-diphenylpropene oxide (tDPPO) as substrate (Borhan *et al.*, 1995). Briefly, 1 μL of a 5 mM solution of [^{3}H]tDPPO in DMF was added to 100 μL of enzyme preparation in sodium phosphate buffer (0.1 M, pH 7.4) containing 0.1 mg/mL of BSA ([tDPPO]final = 50 μM). The enzyme was incubated at 30°C for 15 min, and the reaction was quenched by addition of 60 μL of methanol and 200 μL of isooctane, which extracts the remaining epoxide from the aqueous phase. Extractions with 1-hexanol were performed in parallel to assess the possible presence of gluthation transferase activity, which could also transform the substrate (Borhan *et al.*, 1995). The activity was followed by measuring the quantity of radioactive diol formed in the aqueous phase using a scintillation counter (Wallac Model 1409, Gaithersburg, MD). Assays were performed in triplicate.

Fatty acid analysis. Fatty acid determination was performed as reported previously (Hennig and Watkins, 1989; Watkins *et al.*, 2000). Lipids from endothelial cells were extracted with chloroform/methanol (2:1, vol/vol). Methyl esters of fatty acids were prepared using boron trifluoride (14% in methanol) and analyzed using a gas chromatograph (GC HP 5890 series II, autosampler 7673, HP 3365 ChemStation; Hewlett–Packard, Avondale, PA) equipped with a DB 23 column (30 m, 0.53 mm id, 0.5-μm film thickness; J&W Scientific, Folsom, CA). The GC was operated at 140°C for 2 min and the temperature was programmed 1.5°C/min to 198°C and held for 7 min. The injector and flame-ionization detector temperatures were 225 and 250°C, respectively. Fatty acid methyl esters were identified by comparison of retention times with authentic standards (GLC-422, GLC-87, GLC-68A, Nu-Chek-Prep, Elysian, MN). Fatty acid values are presented as μg fatty acid/100 μg DNA.

Statistical analysis. The data were analyzed using SAS (Statistical Analysis System). Comparisons between treatments were made by one-way ANOVA with post-hoc comparisons of the means made by Fischer's least significance difference method (Snedecor and Cochran, 1974). Statistical probability of $p < 0.05$ was considered significant. EMSAs and RT–PCRs (see Figs. 5, 6, and 7–9, respectively) were repeated three times.

RESULTS

The present article is focused on effects of LTX and LTXD on metabolism of cultured vascular endothelial cells. LTX is formed by several cytochrome P450 isozymes (Fig. 1), with cytochrome P450 2C9 being the single most important one in hepatic tissue (Draper and Hammock, 2000). Several recombinant cytochrome P450s have been shown to produce LTX (Moran *et al.*, 2000; Bylund *et al.* 1998), and which cytochrome P450 primarily is responsible for LTX production in endothelial cells is under investigation. In our previous papers we demonstrated cytochrome P450 in our endothelial cell cultures (Hennig *et al.*, 1999; Toborek *et al.,* 1995a). LTXD is formed from LTX by soluble epoxide hydrolase(s) (Fig. 1). This enzyme is active in our endothelial cell cultures, and specific activity of normal endothelial cells was found to be 130 ± 10 pmol of diol formed per min per mg protein.

LTX and LTXD Decrease Endothelial Barrier Function

Figure 2 shows the effect of LTX and LTXD on endothelial barrier function, expressed as albumin flux across endothelial monolayers. The rationale for the chosen experimental treatment times and concentrations were based on results from previous studies. We have shown that exposing porcine vascular endothelial cells to 90 μM linoleic acid for 24 h was

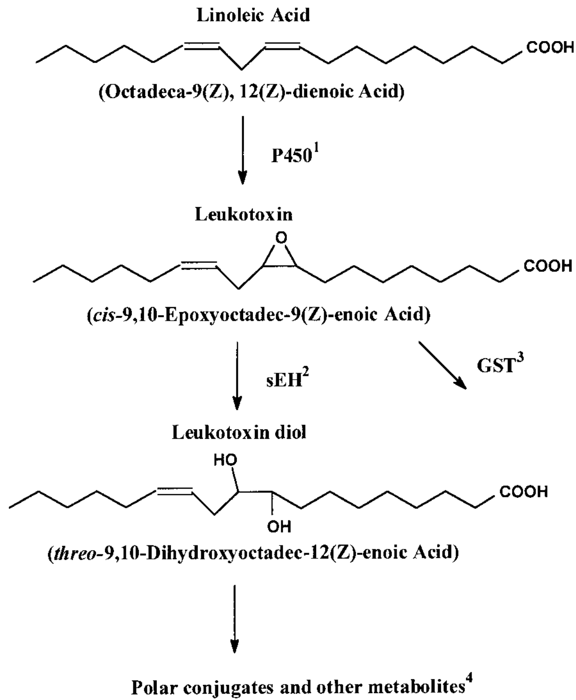

FIG. 1. Structures and proposed metabolic pathway of the formation of leukotoxin and leukotoxin diol from linoleic acid. Cytochrome P450 isozymes (1) are thought to convert the fatty acid linoleate to leukotoxin (shown) or the corresponding 12,13 regioisomer (isoleukotoxin), which are relatively stable 1,2-disubstituted epoxides (Draper and Hammock, 2000). The leukotoxins are converted to their corresponding diols predominantly by the soluble epoxide hydrolase or sEH (2), which can be inhibited by compounds such as dicyclohexyl urea (Greene *et al.*, 2000a). Spontaneous reaction with glutathione is very slow (3), but the leukotoxins can be converted to glutathione conjugates enzymatically (Greene *et al.*, 2000b). The leukotoxin diols, which are thought to be the major biologically reactive metabolite in the endothelial cell system, can be metabolized further by a variety of pathways, including conjugation to the corresponding glucuronic acids, cytochrome P450 based oxidation, or β-oxidation (4).

sufficient to alter endothelial barrier function. By using 20–60 μM LTX or LTXD, we assumed that linoleic acid is partially oxidized to its corresponding epoxide or diol metabolite. Indeed, it was shown that linoleic acid can be metabolized to approximately 41% epoxide/diol metabolites in renal proximal tubular cells (Moran *et al.*, 2000). Treating endothelial cells with LTXD for 24 h resulted in a concentration-dependent increase in albumin transfer across the endothelium. Enriching the media with LTX, on the other hand, only increased albumin flux across the endothelium at 60 μM with no effect observed at 20 or 40 μM.

Figure 3 shows that pretreating endothelial cells with 3 μM of the soluble (cytosolic) epoxide hydrolase inhibitor 1-cyclohexyl-3-dodecyl urea for 2 h prior to treatment with 60 μM LTX decreased albumin flux across endothelial cell monolayers compared to cultures treated only with LTX. Figure 4 shows that 1-cyclohexyl-3-dodecyl urea partially protected against linoleic acid as well as linoleic acid plus PCB 77-

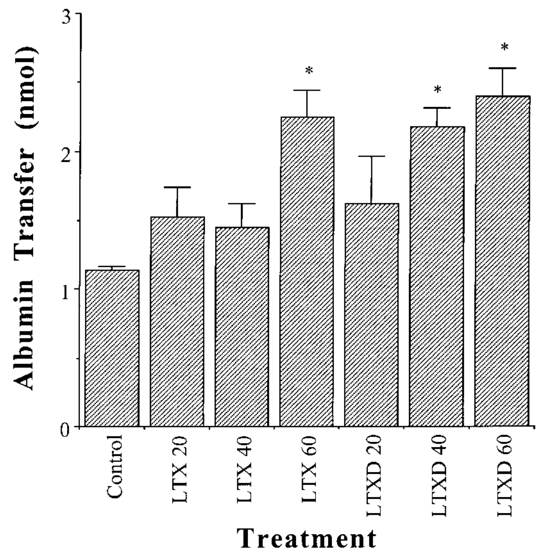

FIG. 2. Effect of 20, 40, or 60 μM methyl leukotoxin (LTX 20, LTX 40, LTX 60) or methyl leukotoxin diol (LTXD 20, LTXD 40, LTXD 60) on albumin transfer across cultured endothelial cell monolayers. Porcine vascular endothelial cells were treated with methyl leukotoxin or methyl leukotoxin diol for 24 h. Values are means ± SEM, $n = 6$. *Significantly different from control cultures.

mediated endothelial barrier dysfunction. Albumin transfer across endothelial monolayers was not affected by 1-cyclohexyl-3-dodecyl urea (1.39 ± 0.36 nmol) alone compared to control cultures (1.14 ± 0.33 nmol).

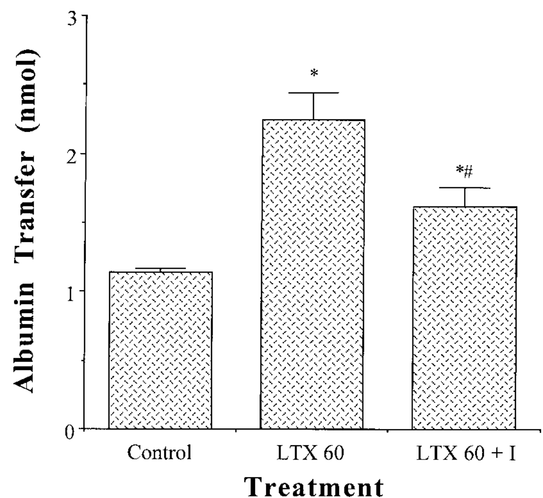

FIG. 3. Effect of 1-cyclohexyl-3-dodecyl urea on leukotoxin-induced increase in albumin transfer across cultured endothelial cell monolayers. Porcine vascular endothelial cells were either left untreated (Control), treated with 60 μM LTX for 24 h (LTX 60), or pretreated with 3 μM 1-cyclohexyl-3-dodecyl urea (I) for 2 h prior to cotreatment with 60 μM LTX for another 24 h (LTX 60 + I). Values are means ± SEM, $n = 6$. *Significantly different from control cultures. #Significantly different from cultures treated with 60 μM LTX.

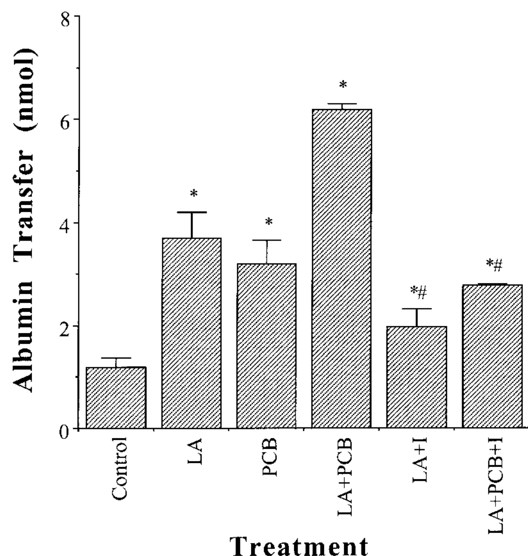

FIG. 4. Effect of 1-cyclohexyl-3-dodecyl urea on linoleic acid or linoleic acid + PCB 77-induced increase in albumin transfer across cultured endothelial cell monolayers. Porcine vascular endothelial cells were either left untreated (Control), treated with 90 μM linoleic acid for 30 h (LA), treated with 3.4 μM PCB 77 for 24 h (PCB), preexposed to 90 μM linoleic acid for 6 h followed by coexposure to PCB 77 for another 24 h (LA + PCB), pretreated with 3 μM 1-cyclohexyl-3-dodecyl urea (I) for 2 h prior to treatment with 90 μM linoleic acid for another 30 h (LA + I), or pretreated with 3 μM 1-cyclohexyl-3-dodecyl urea for 2 h prior to treatment with LA + PCB (LA + PCB + I). Values are means ± SEM, n = 6. *Significantly different from control cultures. #Significantly different than the respected cultures treated without the inhibitor.

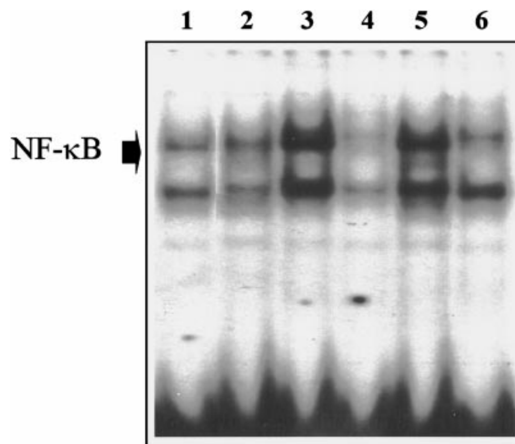

FIG. 5. Effect of LTX, LTXD, or 1-cyclohexyl-3-dodecyl urea on LTX-induced activation of NF-κB. Lane 1, porcine vascular endothelial cells left untreated (control); lanes 2 and 3, treated with 30 or 60 μM LTX for 3 h; lanes 4 and 5, treated with 30 or 60 μM LTXD for 3 h; lane 6, pretreated with 3 μM 1-cyclohexyl-3-dodecyl urea for 2 h prior to treatment with 60 μM LTX for another 3 h.

LTX and LTXD Activate NF-κB

Similar to the endothelial permeability experiments, NF-κB activation, as analyzed by EMSA, also was affected by treatment with LTX or LTXD. NF-κB is the critical transcription factor that regulates the inflammatory cytokine network. For example, NF-κB is involved in regulation of gene expression coding for inflammatory cytokines (e.g., IL-6) and adhesion molecules, such as vascular cell adhesion molecule (VCAM-1) and endothelial-leukocyte adhesion molecule-1. Figure 5 shows that LTX or LTXD can activate NF-κB. Both 60 μM LTX or LTXD caused translocation of NF-κB to the nucleus. This activation of NF-κB was inhibited by pretreating endothelial cells with 1-cyclohexyl-3-dodecyl urea prior to treatment with 60 μM LTX.

Selective Inhibition of Cytochrome P450 2C9 Reduces Linoleic Acid-Induced Activation of NF-κB

To provide evidence of LTX as being mediators of endothelial cell activation, cells were coexposed to the cytochrome P450 2C9 inhibitor sulfaphenazole (10 μM) and linoleic acid (90 μM). Sulfaphenazole can inhibit cytochrome P450 2C9, known to contribute to LTX formation (Draper and Hammock, 2000). Compared with control cultures, linoleic acid markedly activated NF-κB, and this activation was inhibited by cotreatment with sulfaphenazole (Fig. 6).

LTX and LTXD Increase IL-6 Gene Expression

Enrichment of the experimental media with 20, 40, or 60 μM LTX or LTXD caused an increase in mRNA levels of IL-6 (Fig. 7). mRNA levels of IL-6 were higher in LTX- or LTXD-treated compared to untreated cultures. However, cells treated with 40 and 60 μM LTXD showed higher levels of IL-6

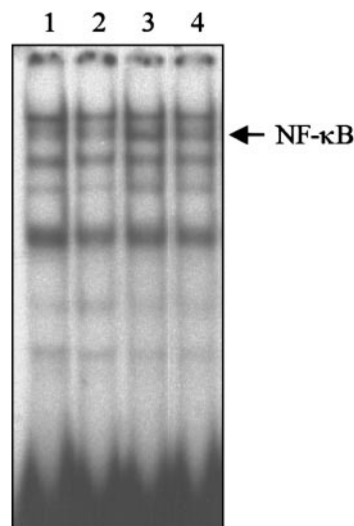

FIG. 6. Effect of linoleic acid or sulfaphenazole on linoleic acid-induced activation of NF-κB. Lane 1, cells left untreated (control); lane 2, treated with 10 μM sulfaphenazole for 6 h; lane 3, treated with 90 μM linoleic acid for 6 h; lane 4, cotreatment with sulfaphenazole and linoleic acid for 6 h. The NF-κB band was confirmed by supershift assay to be the transcriptionally active p65/p65 homodimer.

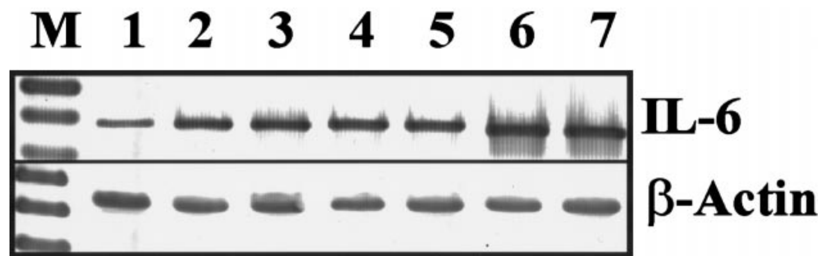

FIG. 7. Effect of LTX and LTXD on the IL-6 gene expression as analyzed by RT–PCR. Porcine vascular endothelial cells were treated with LTX or LTXD for 6 h. M, molecular weight marker; lane 1, control cultures; lanes 2–4, cultures treated with 20, 40, and 60 μM LTX, respectively; lanes 5–7, cultures treated with 20, 40, and 60 μM LTXD, respectively.

mRNA compared with the corresponding concentrations in LTX-treated cells. Similar to IL-6, LTX induced adhesion molecule expression (VCAM-1) at levels as low as 20 μM (data not shown).

Inhibition of Epoxide Hydrolase Prevents IL-6 Gene Expression Mediated by LTX or Linoleic Acid plus PCB 77

The LTX-induced expression of the IL-6 gene was blocked by the cytosolic epoxide hydrolase inhibitor 1-cyclohexyl-3-dodecyl urea (Fig. 8). In contrast, prior enrichment of endothelial cells with 25 μM α-tocopherol (vitamin E) did not prevent LTX-induced IL-6 gene expression (data not shown). Furthermore, 1-cyclohexyl-3-dodecyl urea (a potent soluble epoxide hydrolase inhibitor) and, to a lesser extent, 1,3-dicyclohexyl urea (a moderately potent soluble epoxide hydrolase inhibitor) protected against linoleic acid plus PCB 77-mediated induction of IL-6 mRNA as seen in Fig. 9. In contrast, cellular pretreatment with the microsomal epoxide hydrolase inhibitor dodecyl-amine did not protect against the linoleic acid or PCB 77-mediated increase in mRNA levels of IL-6.

PCB 77 Increases Cellular Uptake and Accumulation of Linoleic Acid

Endothelial cell exposure to culture media supplemented with 90 μM linoleic acid for 30 h resulted in a 7-fold cellular enrichment with this fatty acid compared to control cultures (26.1 ± 1.8 vs 3.7 ± 0.9 μg fatty acid/100 μg DNA; $n = 6$). Exposure to PCB 77 alone had no effect on cellular linoleic acid levels. In contrast, exposure to linoleic acid for 6 h followed by cotreatment with PCB 77 for an additional 24 h resulted in a 11.3-fold cellular enrichment with linoleic acid compared to control cultures (42.4 ± 4.7 vs 3.7 ± 0.9 μg fatty acid/100 μg DNA; $n = 6$).

DISCUSSION

The vascular endothelium is a susceptible target for injury mediated by free fatty acids and environmental toxins. There is ample evidence that free fatty acids and, in particular, linoleic acid may cause activation and dysfunction of endothelial cells (Hennig *et al.*, 1996b, 2000). The observation that linoleic acid can amplify the toxicity of certain PCBs, such as coplanar PCBs, raises questions about mechanisms involved in this phenomenon. It has been proposed that linoleic acid can induce oxidative stress (Hennig *et al.*, 1996a; Toborek *et al.*, 1996), interfere with nitric oxide synthase activity (Davda *et al.*, 1995), affect cell–cell adhesion and tight junction proteins (Jiang *et al.*, 1998), and amplify a cytokine-mediated inflammatory response (Toborek *et al.*, 1996).

It is not clear if linoleic acid itself or its oxidative derivatives mediate these metabolic and structural changes in endothelial cells. Linoleic acid hydroperoxide, for example, has been reported to be associated with an increase in lipid peroxidation of

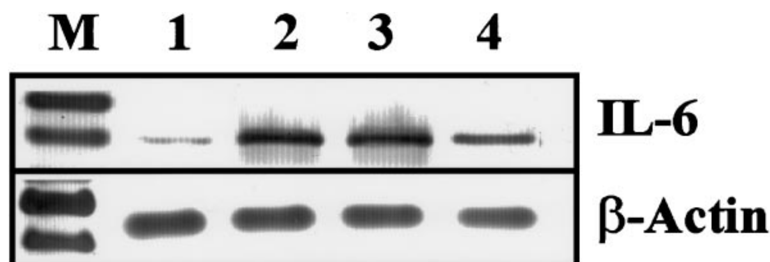

FIG. 8. Effect of epoxide hydrolase inhibitors on LTX-induced IL-6 gene expression as analyzed by RT–PCR. Porcine vascular endothelial cells were treated with 60 μM LTX for 6 h. In experiments that involved epoxide hydrolase inhibitors, cultures were pretreated with 3 μM for 2 h prior to treatment with 60 μM LTX. In cotreatment experiments the inhibitors were maintained for a total of 8 h. M, molecular weight marker; lane 1, control cultures; lane 2, 60 μM LTX for 6 h; lane 3, 3 μM dodecyl-amine + 60 μM LTX; lane 4, 3 μM 1-cyclohexyl-3-dodecyl urea + 60 μM LTX.

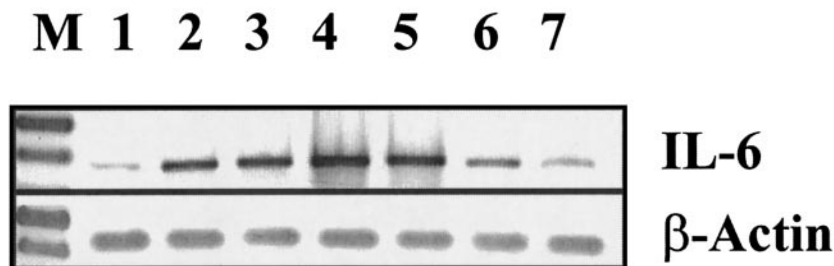

FIG. 9. Effect of linoleic acid, PCB, and/or epoxide hydrolase inhibitors on the IL-6 gene expression as analyzed by RT–PCR. Porcine vascular endothelial cells were pretreated with 90 μM linoleic acid for 3 h before exposure to 3.4 μM PCB for another 3 h. In experiments that involved epoxide hydrolase inhibitors, cultures were pretreated with 3 μM for 2 h prior to treatment with fatty acids and/or PCB. In cotreatment experiments, linoleic acid was maintained for a total of 6 h and the epoxide hydrolase inhibitors were maintained for a total of 8 h. M, molecular weight marker; lane 1, control cultures; lane 2, linoleic acid for 6 h; lane 3, PCB 77 for 3 h; lane 4, linoleic acid + PCB 77; lane 5, dodecyl-amine + linoleic acid + PCB 77; lane 6, 1,3-dicyclohexyl urea + linoleic acid + PCB 77; lane 7, 1-cyclohexyl-3-dodecyl urea + linoleic acid + PCB 77.

endothelial cell phospholipids (Pacifici *et al.*, 1994), an increase in intracellular calcium levels (Sweetman *et al.*, 1995), and disruption of the selective barrier function of vascular endothelial cells (Hennig *et al.*, 1986). Another candidate of oxidative derivatives of polyunsaturated fatty acids that might mediate endothelial cell injury are 15-hydroperoxyeicosatetraenoic acid (15-HPETE) or 13-hydroperoxyoctadecadienoic acid (13-HPODE), which are the peroxidative derivatives of arachidonic acid and linoleic acid, respectively. 15-HPETE and 13-HPODE have been shown to cause cytotoxicity in endothelial cells (Friedrichs *et al.*, 1999).

There is evidence that cytochrome P450 enzymes are involved in the production of leukotoxins (Rifkind *et al.*, 1995; Laethem *et al.*, 1996). It also has been reported that the monoepoxides of linoleic acid, *cis*-9,10-epoxyoctadec-12(z)-enoic acid/*cis*-12,13-epoxyoctadec-9(z)-enoic acid (leukotoxin or LTX) or their diol derivatives (LTXD) are associated with multiple organ failure and a fatal disorder called ARDS (Hayakwa *et al.*, 1990; Ozawa *et al.*, 1991). In addition, LTX can cause high-permeability pulmonary edema in isolated lungs and a dose- and time-dependent damage of cultured pulmonary vascular endothelial cells (Ishizaki *et al.*, 1995). It is possible that LTX or LTXD mediate the activation of endothelial cells after exposure to linoleic acid. In fact, our data show that both LTX and LTXD disrupt endothelial barrier function as measured by increased albumin transfer across endothelial monolayers. Furthermore, LTXD caused endothelial barrier dysfunction at lower concentrations compared with LTX, suggesting that the diol metabolites are the most cytotoxic in this system. Others have reported that the critical toxic lipid species are the result of the bioactivation of leukotoxins to their diol metabolites by epoxide hydrolases (Moghaddam *et al.*, 1997). Symptoms reminiscent of ARDS and related symptoms can be caused in mice by injection of leukotoxin or its diol, but only the diol is active if the animals are pretreated with a potent epoxide hydrolase inhibitor (Moghaddam *et al.*, 1997, Morisseau *et al.*, 1999). Perivascular edema appears to be the first lesion in the lung, suggesting the vascular endothelium as a target for the cytotoxicity of leukotoxin diols (J. Zheng, personal communication). We demonstrated that preexposure to the highly potent soluble epoxide hydrolase inhibitor 1-cyclohexyl-3-dodecyl urea, but not the microsomal epoxide hydrolase inhibitor dodecyl-amine, can prevent the LTX-mediated increase in albumin transfer across the endothelium. Moreover, inhibition of the epoxide hydrolase markedly reduced the amplified cytotoxicity of linoleic acid plus PCB 77.

The amplified toxicity of linoleic acid and PCBs to endothelial cells could also be mediated by cellular accumulation of this fatty acid and thus its availability as a substrate for the formation of cytotoxic epoxide metabolites. Due to the very low basal activity of endothelial cell delta 6-desaturase, arachidonic acid is not produced from linoleic acid significantly in this type of cell (Spector *et al.*, 1981; Debry and Pelletier, 1991), which can result in linoleic acid accumulation within endothelial cells (Spector *et al.*, 1981; Hennig and Watkins, 1989). Furthermore, Matsusue *et al.* (1999) demonstrated that coplanar PCBs can suppress delta 5 and delta 6 desaturase activities. Our data show that linoleic acid uptake and cellular accumulation of this fatty acid is markedly increased in the presence of PCB 77, further supporting our hypothesis that PCB-induced endothelial cell dysfunction can be modulated by the cellular lipid milieu. These findings suggest that the amplified toxicity observed when endothelial cells were exposed to both linoleic acid and PCB 77 could be due to the accumulation of linoleic acid, which then could act as a substrate for the formation of LTX or LTXD.

It is also known that endothelial cells play a key role in the inflammatory response, both by the production of proinflammatory cytokines and by their interaction with leukocytes. Linoleic acid has been shown to be a potent activator of the transcription factor NF-κB in vascular endothelial cells (Hennig *et al.*, 1996a, 2000). Here we show that LTX and LTXD can increase mRNA levels of interleukin-6, which was associated with NF-κB activation. Coexposure of endothelial cells with the cytochrome P450 2C9 inhibitor sulfaphenazole decreased

linoleic acid-induced activation of NF-κB. Furthermore, both IL-6 mRNA and NF-κB were downregulated upon preexposure to the cytoplasmic epoxide hydrolase inhibitor. This suggests that LTX and LTXD are critical mediators in linoleic acid-induced endothelial cell activation and dysfunction. Further support of the cytotoxic properties of these linoleic acid metabolites was recently demonstrated by Moran *et al.* (2000) in renal proximal tubular cells, which metabolized linoleic acid approximately to 41% epoxide/diol metabolites. Functional NF-κB sites are involved in the transcription of IL-6 and IL-8 genes in response to inflammatory mediators. These cytokines can themselves exert a proinflammatory action (Mantovani *et al.*, 1992; Gerritsen and Bloor, 1993). Activated endothelial cells also can express various adhesion molecules, including VCAM-1 and ICAM-1, which facilitate the adhesion of hematopoietic cells to the endothelium (Yamaguchi *et al.*, 1998). In fact, Young *et al.* (1998) have shown that treating vascular endothelial cells with linoleic acid resulted in increased production of IL-8 and expression of ICAM-1. Our data suggest that LTX may be in part responsible for the observed linoleic acid-mediated inflammatory response. Furthermore, by inhibiting cytoplasmic epoxide hydrolase with 1-cyclohexyl-3-dodecyl urea, partial protection was observed against linoleic acid-mediated increase in albumin transfer as well as the upregulation of IL-6 mRNA. These data support our hypothesis that LTXD is more cytotoxic than LTX and that linoleic acid-mediated activation of endothelial cells is partly mediated via cytoplasmic epoxide hydrolase, i.e., formation of LTXD from LTX.

The induction of enzymes involved in the epoxidation of linoleic acid or hydration of leukotoxin to its corresponding diol also may be important in the regulation of the observed cytotoxicity. Fisslthaler *et al.* (2000) have shown that a cytochrome P450 2C arachidonic acid epoxygenase, which is homologous to cytochrome P450 2C8/9, is expressed in cultured human endothelial cells and native porcine coronary artery endothelial cells. Interestingly, the induction of cytochrome P450 2C in native porcine coronary artery endothelial cells by β-naphthoflavone enhanced the formation of 11,12-epoxyeicosatrienoic acid (Fisslthaler *et al.*, 1999). Knowing that both β-naphthoflavone and coplanar PCB 77 activate genes under the control of aryl hydrocarbon receptors (AhR), one could speculate that AhR agonists may have the capability of inducing the same battery of genes, including the cytochrome P450 2C family, which in turn could convert linoleic acid to its epoxide(s). Based on relative polarity and experiments with other cell types, we anticipate that LTX and LTXD will enter cells both as their free acids and the methyl esters used in this study.

In summary, our data support the concept that specific dietary fatty acids (e.g., linoleic acid) can activate vascular endothelial cells. We provide evidence that linoleic acid metabolites, such as leukotoxins and their diol derivatives, are critical in endothelial cell activation. Furthermore, our data show that ω-6 fatty acids (e.g., linoleic acid) and environmental contaminants (e.g., PCB 77) can additively exacerbate endothelial cell dysfunction. Mechanisms of this amplification of response may include oxidative stress, enhanced cellular uptake and accumulation of linoleic acid, epoxide and diol formation, and induction of epoxide hydrolase and cytochrome P450 enzymes. Our data may have implications in understanding mechanisms of atherosclerosis mediated by dietary lipids and environmental contaminants.

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