

Original Research

Involvement of CYP 2C9 in Mediating the Proinflammatory Effects of Linoleic Acid in Vascular Endothelial Cells

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Objective: Polyunsaturated fatty acids such as linoleic acid are well known dietary lipids that may be atherogenic by activating vascular endothelial cells. In the liver, fatty acids can be metabolized by cytochrome P450 (CYP) enzymes, but little is known about the role of these enzymes in the vascular endothelium. CYP 2C9 is involved in linoleic acid epoxygenation, and the major product of this reaction is leukotoxin (LTX). We investigated the role of CYP-mediated mechanisms of linoleic acid metabolism in endothelial cell activation by examining the effects of linoleic acid or its oxidized metabolites such as LTX and leukotoxin diol (LTD).

Methods: The effect of linoleic acid on CYP 2C9 gene expression was studied by RT-PCR. Oxidative stress was monitored by measuring DCF fluorescence and intracellular glutathione levels, and electrophoretic mobility shift assay was carried out to study the activation of oxidative stress sensitive transcription factors. Analysis of oxidized lipids was carried out by liquid chromatography/mass spectrometry.

Results: Linoleic acid treatment for six hours increased the expression of CYP 2C9 in endothelial cells. Linoleic acid-mediated increase in oxidative stress and activation of AP-1 were blocked by sulfaphenazole, a specific inhibitor of CYP 2C9. The linoleic acid metabolites LTX and LTD increased oxidative stress and activation of transcription factors only at high concentrations.

Conclusion: Our data show that CYP 2C9 plays a key role in linoleic acid-induced oxidative stress and subsequent proinflammatory events in vascular endothelial cells by possibly causing superoxide generation through uncoupling processes.

INTRODUCTION

Atherosclerosis is believed to be a chronic inflammatory disease, and the earliest event of coronary atherosclerosis is characterized by endothelial activation and dysfunction [1]. Several factors are implicated in the initiation of endothelial dysfunction of which the formation of reactive oxygen species is believed to play a critical role during this process [2,3].

Endothelial cells are continuously exposed to circulating lipids (e.g., dietary fatty acids) and to lipids that have accumulated

in sub-endothelial regions. These biologically active lipids play an important role in the development of atherosclerosis. Polyunsaturated fatty acids and/or their metabolites can have potent biological effects in various cell types by functioning as signaling molecules. Evidence suggests that linoleic acid, a major dietary unsaturated fatty acid in the American diet, has proinflammatory and proatherogenic effects by causing endothelial cell activation [4]. Linoleic acid-induced endothelial activation is considered to be mediated through oxidative stress [4,5]. However, the precise mechanism involved in linoleic acid-induced oxidative stress and

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Abbreviations: CYP = cytochrome P450, ROS = reactive oxygen species, LTX = leukotoxin, LTD = leukotoxin diol, LA = linoleic acid, H₂DCF-DA = Dichlorodihydrofluorescein diacetate, MnTMPyP = [Mn (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride], AP-1 = activator protein 1, NF- κ B = nuclear factor κ B.

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subsequent endothelial cell activation is not known. When considering the mechanism of action of fatty acids, most attention has been focused on the generation of vasoactive fatty acid metabolites [6,7]. However, superoxide anions, hydrogen peroxide, and hydroxyl radicals can also be generated during fatty acid metabolism which can modulate the effect of metabolites that are being formed.

CYP enzymes are considered to be critical in fatty acid metabolism in addition to cyclooxygenase and lipoxygenase pathways. Most CYPs are primarily expressed in the liver. Specific CYPs that are localized in extra-hepatic tissues such as vascular smooth muscle and endothelium can contribute to the regulation of vascular tone and homeostasis [8]. CYP 2C9, an isoform of cytochrome P450, is reported to be a significant source of ROS in coronary arteries [9]. This enzyme is constitutively present in the endothelium, and it can be activated by the rhythmic vessel distension that occurs during the cardiac cycle [10]. It has been reported that CYP 2C9 is the predominant linoleate epoxygenase in human liver microsomes [11] and that the major product of this epoxidation reaction is leukotoxin (LTX). LTX may regulate many physiological and pathological processes. It has been reported that LTX can induce vasodilatation [12], provide defense against infectious diseases [13] and at high concentrations cause multiple organ failure associated with severe burns, acute trauma and adult respiratory distress syndrome [14–16]. Understanding how these biologically active compounds are produced and what role they have in normal physiological and pathological processes could lead to new strategies to prevent endothelial dysfunction, a common denominator of atherosclerosis.

Thus, the aim of the present investigation was to determine whether the pro-inflammatory effect of certain dietary fatty acids such as linoleic acid are due to the generation of reactive oxygen species during the CYP reaction cycle or to CYP-derived metabolites of linoleic acid such as LTX or LTD.

MATERIALS AND METHODS

Chemicals

Linoleic acid (>99% pure) was obtained from Nu-Chek Prep (Elysian, MN). LTX and LTD were synthesized in the laboratory of Dr. Bruce D. Hammock (University of California, Davis, CA). Dichlorodihydrofluorescein diacetate (H₂DCF-DA) and dihydroethidine (DHE) were obtained from Molecular probes (Eugene, OR) and sulfaphenazole was purchased from Sigma (St. Louis, MO).

Cell Culture and Experimental Media

Endothelial cells were isolated from porcine pulmonary arteries and cultured as previously described [17]. The basic culture medium consisted of M199 (GIBCO Laboratories, Grant Island, NY) containing 10% fetal bovine serum (FBS;

Hyclone Laboratories, Inc., Logan, UT), 100 units per mL of penicillin and 100 µg per mL of streptomycin sulphate (GIBCO). Human umbilical vein endothelial cells were cultured in enriched M-199 medium containing 20% FBS as described previously [18]. The experimental media contained 5% FBS, supplemented with linoleic acid (90 µM). Preparation of experimental media with linoleic acid was performed as described earlier [18].

CYP 2C9 Expression Studies

Total RNA was extracted from endothelial cells using TRI reagent (Sigma, St. Louis, MO) according to the manufacturer's protocol, and it was used to synthesize 20 µL of cDNA. One µL of cDNA was used for PCR (total reaction volume of 25 µL) to examine the induction of CYP 2C9. The following primers were employed in the PCRs: CYP 2C9-forward: 5'-ATT GAC CTT CTC CCC ACC AGC-3', reverse: 5'-GCA AAT CCA TTG ACA ACT GGA GT-3'. The PCR mixture consisted of a Taq PCR Master Mix Kit (Qiagen, Valencia, CA), 1 µL of the reverse transcriptase product and 10 pmol of primer pairs in a total volume of 25 µL. Thermocycling was carried out according to the following profile: 94°C for 1 minute before the first cycle, 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 45 seconds, repeated 30 times and followed by a final extension at 72°C for 7 minutes. PCR products were separated by 2% agarose gel electrophoresis, stained with SYBR® Green I (Molecular Probes, Eugene, OR) and visualized using phosphor-imaging technology (FLA-2000, Fuji, Stamford, CN).

Measurement of Oxidative Stress

Induction of reactive oxygen species was measured using H₂DCF-DA [19]. Endothelial cells were cultured in 24-well plates and treated with linoleic acid and/or sulfaphenazole, a specific CYP 2C9 inhibitor, for three hours. Cells were stained with H₂DCF-DA (10 µM) for the last 30 minutes of the treatment period. In a separate experiment, linoleic acid was treated with or without MnTMPyP, a SOD mimetic to identify the specific reactive oxygen species being generated. After staining, the extracellular dye was washed two times with 10.0 mM HEPES buffer, (pH 7.4) and the fluorescence was measured at an excitation wavelength of 490 nm and an emission wavelength of 525 ± 5 nm, using a multi-well fluorescent plate reader (Molecular Devices, Sunnyvale, CA).

Measurement of Superoxide Anions

Endothelial cells were cultured in 24-well plates and treated with linoleic acid and/or MnTMPyP, a superoxide dismutase mimetic, for three hours. Cells were stained with DHE (10 µM) for the last 30 minutes of the treatment period. After staining, the extracellular dye was washed two times with a buffer containing 145 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 0.5 mM

MgCl₂, 10.0 mM D-glucose and 10.0 mM HEPES (pH 7.4) and the fluorescence was measured at an excitation wavelength of 520 ± 10 nm and an emission wavelength of 610 ± 5 nm, using a multi-well fluorescent plate reader (Molecular Devices).

Glutathione Measurement

Determination of glutathione was carried out by an enzymatic recycling method described by Baker *et al.* [20] using microtiter plate technology. Cellular protein was extracted by adding 100 μL of ice-cold 0.09% sulfosalicylic acid (SSA) to cells, which were collected from P-100 tissue culture plates. Cells were lysed by freezing and thawing, and centrifuged at 10,000 g for five minutes. Each supernatant was collected and used for the glutathione assay. The assay mixture contained 50 μL of the supernatant and 100 μL of the reaction buffer (125 mM phosphate buffer containing 0.225 mM DTNB, 0.302 mM NADPH and glutathione reductase at a concentration of 1.25 U/μL). The blank contained 50 μL of 0.09% 5-SSA instead of supernatant, and the control reaction contained the glutathione standard in place of the supernatant. The mixtures were equilibrated at room temperature for three minutes, and the reaction was started by the addition of 100 μL of the reaction buffer to the cell extract. The absorbance was measured at 405 nm in a 96-well plate reader (Molecular Devices, Sunnyvale, CA).

Electrophoretic Mobility Shift Assay

Nuclear protein extracts from treated cells were prepared according to the method of Dignam *et al.* [21], and an electrophoretic mobility shift assay was performed using a commercially available kit from Promega Corp. (Madison, WI). Double-stranded oligonucleotides containing the consensus sequence of the binding site for transcription factor AP-1 (5-CGC TTG ATG AGT CAG CCG GAA-3) or NF-κB (5-AGT TGA GGG GAC TTT CCC AGG-3) were purchased from Promega (Madison, WI) and labeled with [³²P]-ATP (Amersham Pharmacia Biotech, Piscataway, NJ) using T₄ polynucleotidyl kinase.

Binding reactions were carried out in a 20 μL volume containing 4 μg of nuclear protein extracts, 10 mM Tris-Cl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, 1 μg of poly[dI-dC] (nonspecific competitor) and 40,000 cpm of ³²P-labeled specific oligonucleotide probe. The mixture (20 μL of total volume) was incubated for 25 minutes at room temperature, and the resulting DNA-protein complexes were resolved on a 5 % non-denaturing polyacrylamide gel.

Analysis of Oxidized Lipids

Linoleic and arachidonic acid epoxide and diol concentrations were quantified as previously described [22]. Briefly, aliquots of cell culture media (6 mL) from triplicate experiments were spiked with analytical surrogates and extracted twice with 2 mL aliquots of ethyl acetate. The organic extracts

were evaporated, redissolved in 100 μL of methanol and analyzed using negative mode electrospray ionization and tandem mass spectrometry on a triple quadrupole instrument (Micro-mass Ultima, Manchester, UK).

Statistical Analysis

Statistical analysis of data was carried out using SYSTAT 7.0 (SPSS, Chicago, IL). One-way ANOVA was used to compare mean responses among the treatments, with *post-hoc* comparisons of the means by Bonferroni least significant difference procedure. Statistical probability of P < 0.05 was considered significant.

RESULTS

Linoleic Acid Induces CYP 2C9 Expression in Endothelial Cells

CYP 2C9 gene expression studies were carried out with HUVEC cells as the porcine gene sequence is not available. RNA samples were extracted from HUVEC cells treated with or without linoleic acid, and CYP 2C9 mRNA was quantified by RT-PCR, as described under materials and methods. A marked increase in CYP 2C9 gene expression was observed upon linoleic acid treatment for six hours, suggesting that this isoform of CYP plays a major role in linoleic acid metabolism in endothelial cells (Fig. 1).

Linoleic Acid Induces Oxidative Stress in Endothelial Cells

In order to understand if CYP 2C9 induction is accompanied by ROS production in endothelial cells, the effect of sulfaphenazole on linoleic acid-induced oxidative stress was monitored using the fluorescent probe H₂DCF-DA. The oxidative stress as observed by DCF fluorescence was increased significantly upon linoleic acid treatment for three hours. The ROS production by linoleic acid was significantly suppressed in the presence of sulfaphenazole, a specific inhibitor of CYP 2C9 (Fig. 2A), suggesting that this isoform of CYP is involved in generating free radicals upon treatment with linoleic acid.

To ascertain linoleic acid-induced oxidative stress in endothelial cells, the cellular glutathione status was also monitored. Glutathione is the most abundant low molecular weight thiol compound in cells and plays an important role in antioxidant defense and cellular detoxification. The cellular glutathione pool was significantly depleted after a six hour exposure to 90 μM linoleic acid. However, co-treatment with sulfaphenazole significantly inhibited the linoleic acid-mediated depletion of intracellular glutathione (Fig. 2B).

MnTMPyP, a superoxide dismutase mimetic, significantly blocked the ROS production by linoleic acid, suggesting that superoxide radicals are a major ROS produced upon linoleic

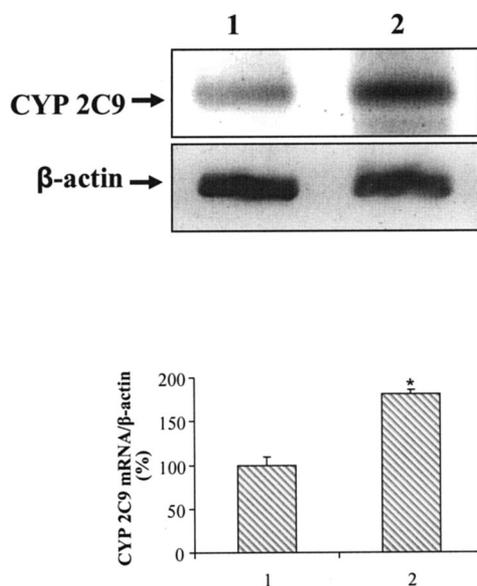


Fig. 1. Effect of linoleic acid (LA) on CYP 2C9 messenger RNA levels in human endothelial cells as measured by reverse transcriptase-polymerase chain reaction (RT-PCR). Human umbilical vein endothelial cells were exposed to LA for six hours. The amplified PCR products were visualized using phosphor-imaging technology. Lane 1, control; lane 2, LA (90 μ M). The values are presented as mean \pm SEM of three sets of experiments. *Significantly different from control values.

acid treatment (Fig. 3A). We also used DHE as a fluorescent probe, which is currently the most accepted probe to measure superoxide radicals in intact cells [23]. DHE is reasonably specific for superoxide [24]. Similar to its effects on DCF fluorescence, linoleic acid showed a remarkable increase in superoxide production in endothelial cells as measured by DHE fluorescence. Similar to its effects on DCF fluorescence, MnTMPyP decreased linoleic acid-induced DHE fluorescence in endothelial cells, suggesting that superoxide radicals are a major source of ROS produced upon linoleic acid treatment (Fig. 3B).

Role of CYP 2C9 in Linoleic Acid-Mediated AP-1 Activation

Electrophoretic mobility shift assay was carried out to investigate the DNA binding activity of the redox-responsive transcription factor AP-1 in endothelial cells exposed to linoleic acid. Nuclear extracts of cells treated with linoleic acid for six hours increased the activation of AP-1 whereas co-treatment with sulfaphenazole suppressed AP-1 activation by linoleic acid (Fig. 4).

The Effects of Epoxide and Diol Metabolites are Concentration Dependent

When examining the effect of LTX and LTD on oxidative stress in endothelial cells, oxidative stress was induced neither by LTX nor by LTD up to a concentration of 30 μ M (data not

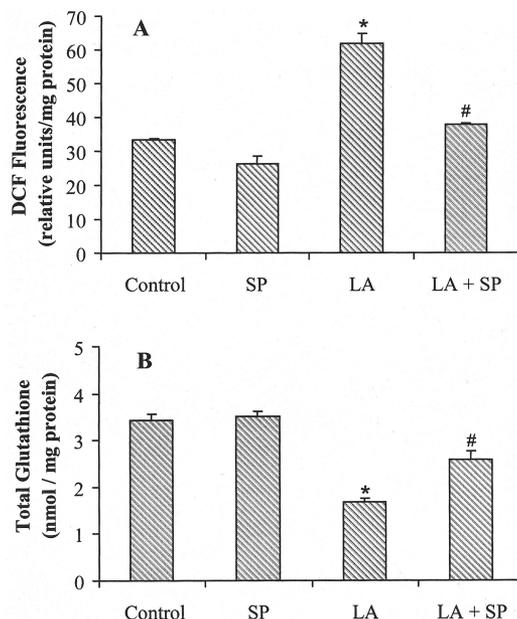


Fig. 2. Effect of sulfaphenazole on cellular oxidative stress as measured by DCFH oxidation (A) and cellular glutathione status (B) in endothelial cells exposed to linoleic acid (LA). For the measurement of DCF fluorescence, cells were exposed to 90 μ M LA in the presence or absence of 10 μ M sulfaphenazole (SP) for three hours. Fluorescence intensity was measured using a fluorescent plate reader at an excitation wavelength of 490 nm and an emission wavelength of 525 ± 5 nm. For the measurement of intracellular glutathione, cells were treated with 90 μ M LA and/or SP for six hours. The values are presented as mean \pm SEM of three sets of experiments. *Significantly different from control values. #Significantly different from LA group.

shown). Both the metabolites caused oxidative stress only at 90 μ M concentration after three hours of exposure as measured by DCF fluorescence (Figs. 5A and 5B).

To further understand the effect of these two linoleic acid-derived metabolites on endothelial cell activation, electrophoretic mobility shift assay of transcription factors such as NF- κ B and AP-1 was carried out. Figs. 6A and 6B show the effects of LTX and LTD on the activation of NF- κ B and AP-1, respectively. After a six hour exposure, LTX caused activation of NF- κ B and AP-1 at 90 μ M concentration, whereas LTD increased the activation of these two transcription factors at both 60 μ M and 90 μ M concentrations. The specificity of NF- κ B and AP-1 binding was determined by supershift analysis with antibodies against p65 and c-Jun, respectively.

Endothelial Cells Generate Epoxide and Diol Metabolites

When the cells were enriched with linoleic acid, other long chain fatty acids (including arachidonic acid) were increased in the media. Linoleic acid treatment increased the production of epoxide and diol metabolites by endothelial cells (Fig. 7). There is also evidence of arachidonic acid-derived epoxides

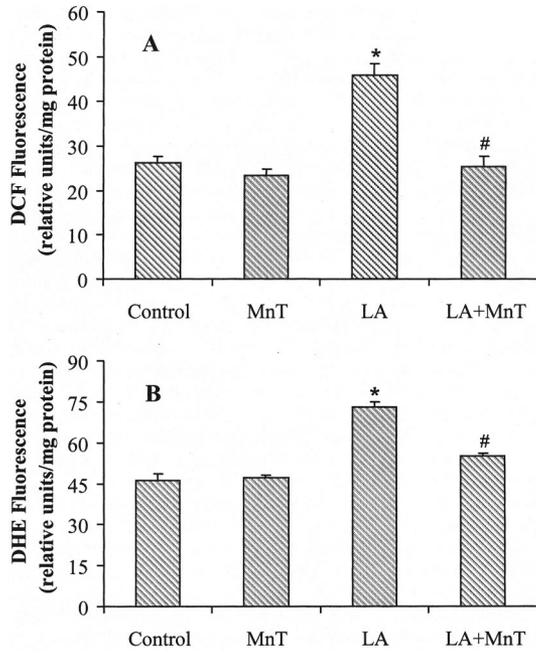


Fig. 3. Effect of MnTMPyP on cellular oxidative stress (DCFH oxidation) (Fig. 3A) and superoxide generation (Figure 3B) in endothelial cells exposed to linoleic acid (LA). Cells were exposed to 90 μ M LA in the presence or absence of 10 μ M MnTMPyP for three hours. Cells were loaded with either DCFH-DA or DHE (10 μ M) for the last 30 minutes of the treatment period. After staining, cells were washed with HEPES buffer (pH 7.4), and the fluorescence intensity was measured using a fluorescent plate reader at an excitation wavelength of 490 nm and an emission wavelength of 525 ± 5 nm for DCF and at an excitation wavelength of 520 ± 5 nm and an emission wavelength of 620 ± 5 nm for DHE. The values are presented as means \pm SEM of three sets of experiments. *Significantly different from control values. #Significantly different from LA group.

and diols but at significantly lower concentrations when compared to those of linoleic acid. The sum of the arachidonic acid epoxides were approximately 1 nm while the linoleic acid epoxides were 3.5 nm. Also, the sum of the measured arachidonic acid diols were 15 nm while those of linoleic acid were 100 nm.

DISCUSSION

The present study demonstrates that linoleic acid can cause oxidative stress and a pro-inflammatory response in endothelial cells that could play a critical role during chronic inflammation in early atherosclerosis. It is well established that fatty acids can be metabolized by cytochrome P450s [25]. Though CYPs are primarily expressed in the liver, some CYPs are also detected in the lung, vasculature, gastrointestinal tract and heart [26,27]. Recent data have suggested that specific CYPs localized in the endothelium contribute to the regulation of vascular tone [28]. CYP 2C9, an isoform of cytochrome P450, is a

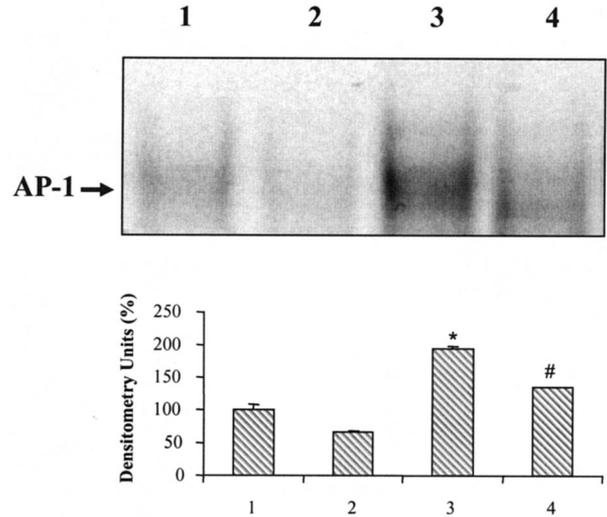


Fig. 4. Effect of 10 μ M sulfaphenazole (SP) on linoleic acid (LA)-mediated nuclear translocation of AP-1 in porcine pulmonary artery endothelial cells. Confluent monolayers were treated with 90 μ M LA in the presence or absence of SP for six hours. Lane 1, control; lane 2, SP (10 μ M); lane 3, LA (90 μ M); and lane 4, LA (90 μ M) + SP (10 μ M). The values are presented as means \pm SEM of three separate experiments. *Significantly different from control values. #Significantly different from LA group.

functionally significant source of ROS in coronary arteries [9]. The present study is the first evidence to show that the expression of CYP 2C9 is increased upon linoleic acid treatment in cultured vascular endothelial cells.

Different CYPs have been reported to be involved in the metabolism of different dietary fatty acids. The epoxidation of arachidonic acid and linoleic acid by CYP is well established and is associated with the CYP 2C gene family [29–33]. It also has been reported that different isozymes of the CYP 2C gene family are capable of metabolizing linoleic acid and of forming similar products [29,30]. However, the most convincing evidence obtained in support of our hypothesis that CYP 2C9 is involved in linoleic acid metabolism and is a physiologically relevant source of ROS was provided using a specific inhibitor of CYP 2C9. In the present study, we showed that linoleic acid can significantly increase the oxidative stress in endothelial cells. However, co-treatment with sulfaphenazole was found to inhibit linoleic acid-mediated oxidative stress as observed by DCF fluorescence. Also, the intracellular glutathione level was significantly depleted by linoleic acid which was partially prevented by sulfaphenazole. These results suggest that CYP 2C9 plays a major role in generating ROS upon treatment with linoleic acid in endothelial cells. This is in agreement with previous findings suggesting that CYPs can generate varying amounts of oxygen-derived free radicals and are associated with markers of oxidative damage in cultured cells [34–36]. The mechanism by which CYP enzymes generate ROS may be through uncoupling processes which occurs during the CYP

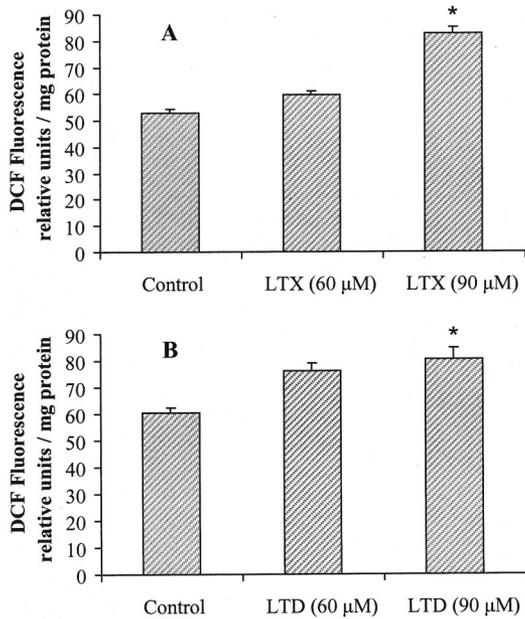


Fig. 5. Effect of leukotoxin (LTX) and leukotoxin diol (LTD) on cellular oxidative stress (DCFH oxidation) in endothelial cells. Cells were exposed to LTX and LTD (60 and 90 μ M) for three hours. Fluorescence intensity was measured at an excitation wavelength of 490 nm and an emission wavelength of 525 ± 5 nm. Values are mean \pm SEM of three separate experiments. *Significantly different from control values.

reaction cycle resulting in the formation of superoxide anion radicals and hydrogen peroxide [37–39].

Several lines of evidence suggest that an imbalance between cellular oxidant/antioxidant levels can lead to the activation of certain redox sensitive transcription factors and the expression of pro-inflammatory genes in endothelial cells [40,41]. Fatty acids, and in particular linoleic acid, have been reported to increase nuclear translocation of NF- κ B and AP-1 in vascular endothelial cells [42–44]. In the present study, co-treatment with sulfaphenazole suppressed the activation of AP-1 by linoleic acid. AP-1 is a critical oxidative stress-sensitive transcription factor in the regulation of vascular inflammation [45,46]. As sulfaphenazole also decreases the oxidative stress caused by linoleic acid, its observed inhibitory effect on the activation of transcription factors can be attributed to the suppression of reactive oxygen species formed via the CYP 2C9 pathway. The consequences of superoxide anion or hydrogen peroxide production by CYP 2C9 may include a chronic elevation in the activity of the redox-sensitive transcription factors and the expression of inflammatory cytokines and adhesion molecules, leading to endothelial cell dysfunction. Thus, scavenging ROS during CYP reaction cycle may protect endothelial cells from the deleterious effects of these oxidizing species.

Although liver is the most critical tissue in drug metabolism, it is imperative to examine the effect of a pharmacological agent in endothelial cells as considerable amount of CYPs are expressed in these cells [8]. In addition to linoleic acid, we used

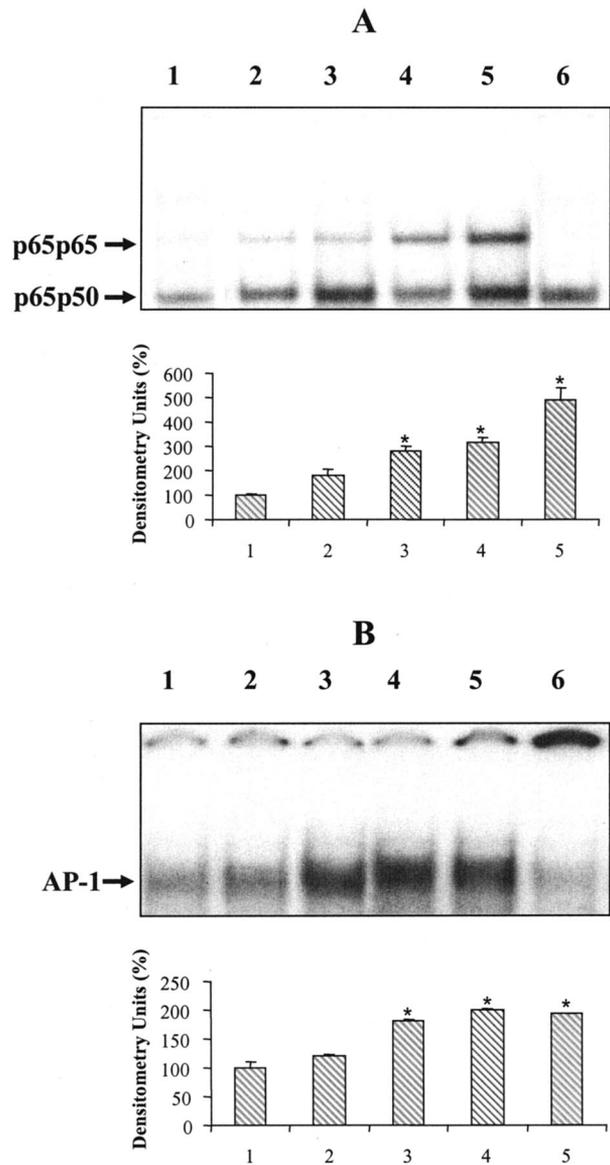


Fig. 6. Effect of leukotoxin (LTX) and leukotoxin diol (LTD) on the activation of NF- κ B (A) and AP-1 (B) in porcine pulmonary artery endothelial cells. Confluent monolayers were treated with LTX (60 and 90 μ M) and LTD (60 and 90 μ M) for six hours. Fig. 5A: lane 1, control; lane 2, LTX (60 μ M); lane 3, LTX (90 μ M); lane 4, LTD (60 μ M); lane 5, LTD (90 μ M) and lane 6, LTX (90 μ M) supershift (p65). Fig. 5B: lane 1, control; lane 2, LTX (60 μ M); lane 3, LTX (90 μ M); lane 4, LTD (60 μ M); lane 5, LTD (90 μ M) and lane 6, LTX (90 μ M) supershift (c-jun). Values are mean \pm SEM of three separate experiments. *Significantly different from control values.

nifedipine, a known CYP2C9 inducer [9], to examine if this compound can also activate NF- κ B in endothelial cells. Interestingly, we found that this compound did not induce NF- κ B activation in endothelial cells (unpublished data), suggesting that the observed proinflammatory events may be more specific for fatty acids than general pharmacological agents.

Fatty acids and their metabolites, in particular CYP-derived

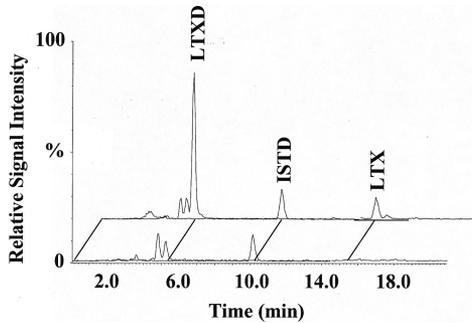


Fig. 7. Endothelial cell-derived linoleic acid metabolites. Cells were cultured in the presence of linoleic acid (90 μ M; upper trace) for 24 hours, and media concentrations of linoleate-derived epoxides and diols were 3.2 ± 0.5 nM and 90 ± 15 nM, respectively. These metabolites were present at <0.5 and 10 ± 5 nM, respectively without supplemental linoleic acid. Traces of epoxy and dihydroxy arachidonates were also observed in linoleic acid-treated cultures (data not shown). Results are from triplicate analyses of 6 mL culture media aliquots analyzed by LC/MS/MS.

metabolites, from a spectrum of unsaturated fatty acids may be potential modulators of vascular function. To examine if the linoleic acid metabolites can contribute to oxidative stress, studies were carried out with epoxide and diol metabolites of linoleic acid such as LTX and LTD. Although LTX is reported to be involved in conditions such as acute respiratory distress syndrome and acute lung injury [16,47], reports also suggest that LTX causes endothelium dependent pulmonary vasodilatation in isolated rat lungs and in isolated pulmonary artery ring structures [12].

We used 10–90 μ M concentrations of LTX or LTD in the present study. The epoxide and diol metabolites are produced in exceptionally high amounts *in vivo* during specific pathological events. For example, Kosaka *et al.* [15] showed that LTX can reach high concentrations (>100 μ M) in the serum of patients with significant burns. Also, Ozawa *et al.* [48] reported considerable amounts of LTX in bronchoalveolar lavage fluid (diluted specimens) in patients with acute respiratory distress syndrome. Furthermore, studies suggest that renal proximal tubules can metabolize linoleic acid approximately to 41% LTX and LTD [49]. Therefore, the concentrations of LTX and LTD used in the present study range from physiological to pathological levels.

It is evident from our data that the epoxide/diol metabolites can be formed by the endothelial cells. In addition to the epoxide/diol metabolites of linoleic acid, there is also evidence that these metabolites can be derived from arachidonic acid but at significantly lower concentrations. Especially after exposure to linoleic acid, our data provide evidence that linoleic acid is a major substrate for CYP 2C9 in endothelial cells. However, we cannot exclude the possibility that linoleic acid may also have an indirect effect on CYP 2C9 activity possibly via increased release of arachidonic acid from the phospholipid pool [50].

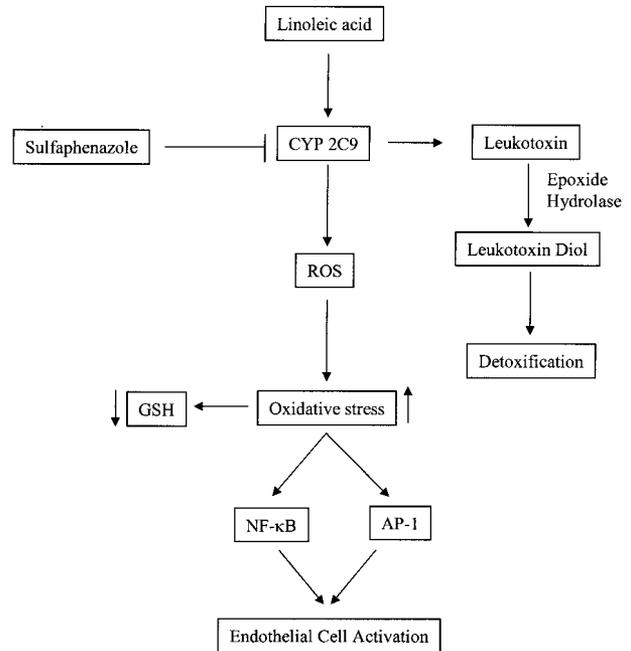


Fig. 8. Proposed model for the mechanism of linoleic acid (LA)-mediated endothelial cell activation. LA treatment results in CYP 2C9 activation and production of superoxide radicals as well as depletion of glutathione in endothelial cells. The increased oxidative stress results in the activation of oxidative stress sensitive transcription factors such as NF- κ B and AP-1, leading to endothelial cell activation. Sulfaphenazole, a specific inhibitor of CYP 2C9 suppresses the oxidative stress caused by LA treatment. The formation of leukotoxin and leukotoxin diol under physiological condition may help in the fatty acid detoxification process.

In the present study, the deleterious effects of the epoxide and diol metabolites were seen only at very high concentrations. For example, neither LTX nor LTD induced oxidative stress up to 30 μ M concentration as measured by DCF fluorescence. On the other hand, a profound increase in oxidative stress was observed at high concentrations of LTX or LTD. The fact that both LTX and LTD induced oxidative stress only at high concentrations suggests the possibility that the metabolites may not be as toxic as the parent fatty acid in endothelial cells. Furthermore, these metabolites and especially the diol metabolites, which are produced endogenously, may help to detoxify specific fatty acids and thus be beneficial for the maintenance of vascular homeostasis [51]. For example, epoxyeicosatrienoic acid (EET), an extensively studied epoxide metabolite of arachidonic acid, has been reported to exert an anti-inflammatory effect in endothelial cells by inhibiting the activation of NF- κ B and by decreasing the cytokine-induced adhesion molecule expression [52].

In conclusion, our results indicate that linoleic acid can induce CYP 2C9 expression in vascular endothelial cells (Fig. 8). Due to possible uncoupling processes and generation of ROS, CYP 2C9 induction appears to be responsible for increased cellular oxidation and activation of redox-responsive

transcription factors in linoleic acid-treated cells. On the other hand, linoleic acid metabolites produced via the CYP 2C9 pathways, such as LTX or LTD, may contribute to vascular pathology only at very high concentrations.

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