Original Research

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

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**Key words: linoleic acid, CYP 2C9, leukotoxin, leukotoxin diol, oxidative stress**

**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, $H_2$DCF-DA = Dichlorodihydrofluorescein diacetate, MnTMPyP = [Mn (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride], AP-1 = activator protein 1, NF-κB = nuclear factor κB.

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 ($H_2$DCF-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 $\mu$g 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 $\mu$M). 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 $\mu$L of cDNA. One $\mu$L of cDNA was used for PCR (total reaction volume of 25 $\mu$L) 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 $\mu$L of the reverse transcriptase product and 10 pmol of primer pairs in a total volume of 25 $\mu$L. 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 $H_2$DCF-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 $H_2$DCF-DA (10 $\mu$M) 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 $\pm$ 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 $\mu$M) 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 $CaCl_2$, 0.5 mM

$MgCl_2$, 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 [$^{32}$P]-ATP (Amersham Pharmacia Biotech, Piscataway, NJ) using $T_4$ 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 $^{32}$P-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 (Micromass 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 $H_2$DCF-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

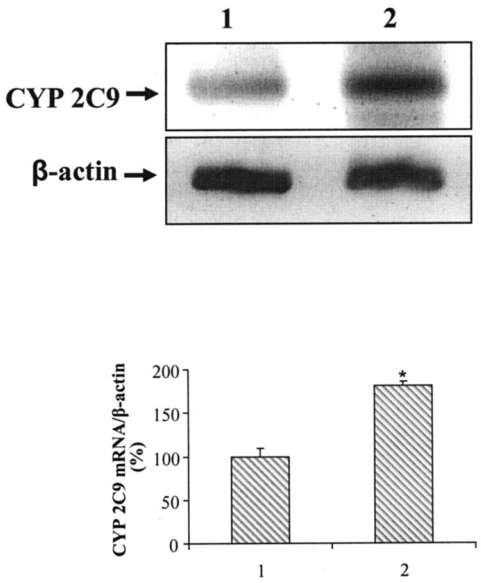


**Fig. 1.** Effect of linoleic acid (LA) on CYP 2C9 messenger RNA levels in human endothelial cells as measured by reverse transcriptase-polymerase chain reaction (RT-PCR). Human umbilical vein endothelial cells were exposed to LA for six hours. The amplified PCR products were visualized using phosphor-imaging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

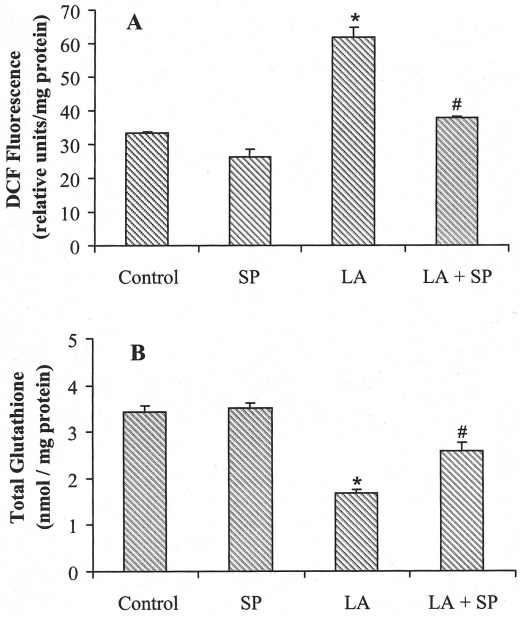


**Fig. 2.** Effect of sulfaphenazole on cellular oxidative stress as measured by DCFH oxidation (A) and cellular glutathione status (B) in endothelial cells exposed to linoleic acid (LA). For the measurement of DCF fluorescence, cells were exposed to 90 $\mu$M LA in the presence or absence of 10 $\mu$M 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 $\mu$M LA and/or SP for six hours. The values are presented as mean ± SEM of three sets of experiments. *Significantly different from control values. #Significantly different from LA group.

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 $\mu$M (data not shown). Both the metabolites caused oxidative stress only at 90 $\mu$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-$\kappa$B and AP-1 was carried out. Figs. 6A and 6B show the effects of LTX and LTD on the activation of NF-$\kappa$B and AP-1, respectively. After a six hour exposure, LTX caused activation of NF-$\kappa$B and AP-1 at 90 $\mu$M concentration, whereas LTD increased the activation of these two transcription factors at both 60 $\mu$M and 90 $\mu$M concentrations. The specificity of NF-$\kappa$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

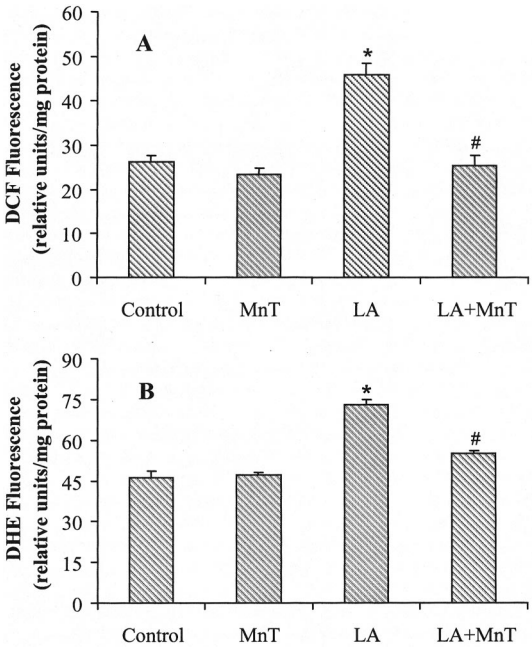


**Fig. 3.** Effect of MnTMPyP on cellular oxidative stress (DCFH oxidation) (Fig. 3A) and superoxide generation (Figure 3B) in endothelial cells exposed to linoleic acid (LA). Cells were exposed to 90 $\mu$M LA in the presence or absence of 10 $\mu$M MnTMPyP for three hours. Cells were loaded with either DCFH-DA or DHE (10 $\mu$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 $\pm$ 5 nm for DCF and at an excitation wavelength of 520 $\pm$ 5 nm and an emission wavelength of 620 $\pm$ 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.

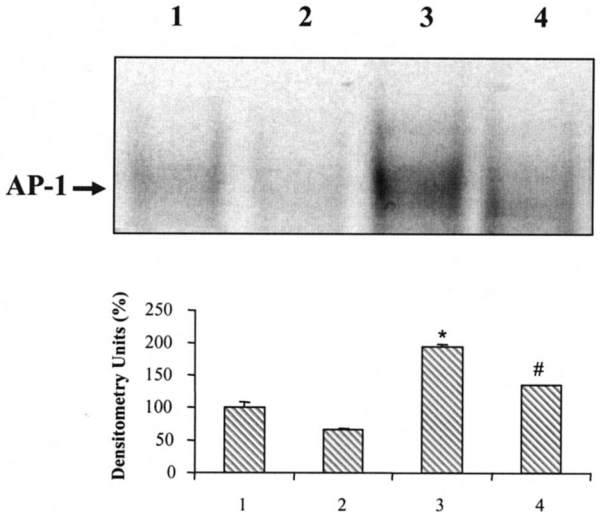


**Fig. 4.** Effect of 10 $\mu$M sulfaphenazole (SP) on linoleic acid (LA)-mediated nuclear translocation of AP-1 in porcine pulmonary artery endothelial cells. Confluent monolayers were treated with 90 $\mu$M LA in the presence or absence of SP for six hours. Lane 1, control; lane 2, SP (10 $\mu$M);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

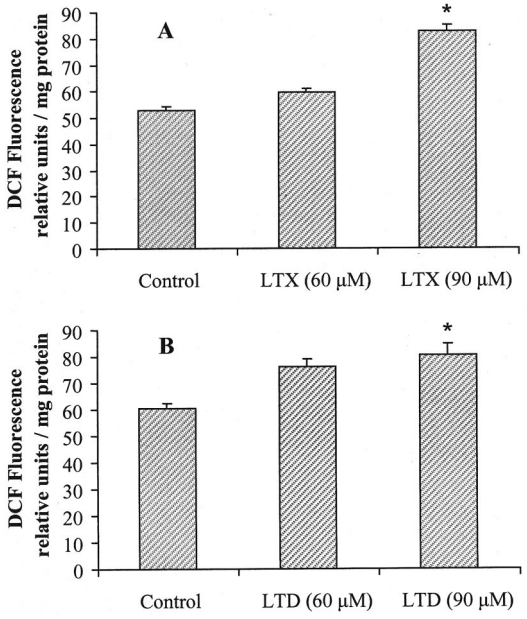


**Fig. 5.** Effect of leukotoxin (LTX) and leukotoxin diol (LTD) on cellular oxidative stress (DCFH oxidation) in endothelial cells. Cells were exposed to LTX and LTD (60 and 90 $\mu$M) for three hours. Fluorescence intensity was measured at an excitation wavelength of 490 nm and an emission wavelength of 525 $\pm$ 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-$\kappa$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

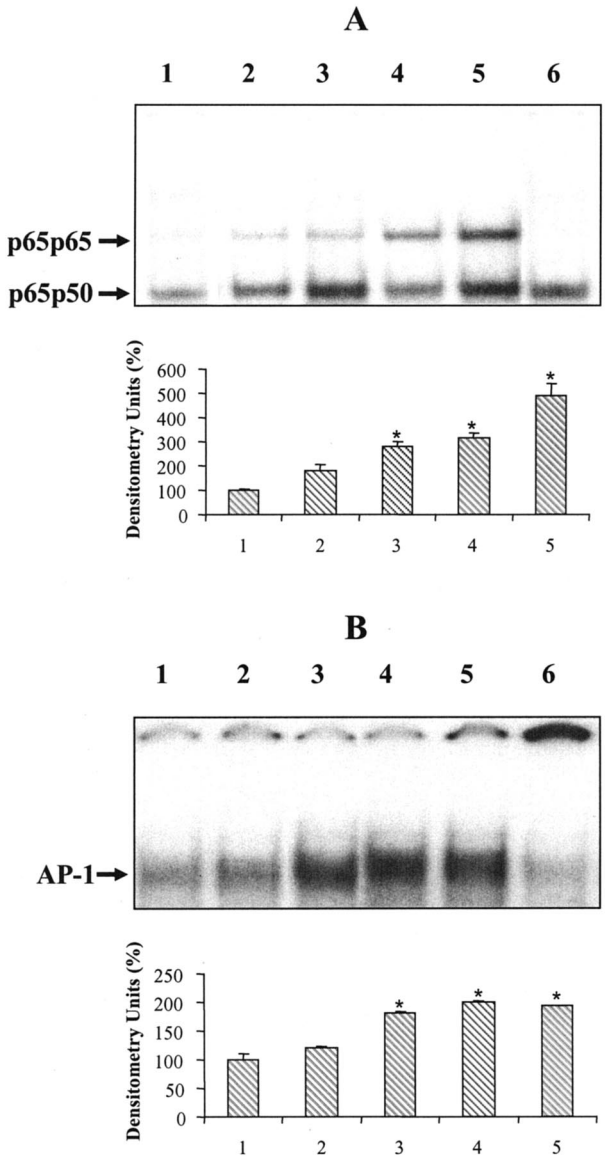


**Fig. 6.** Effect of leukotoxin (LTX) and leukotoxin diol (LTD) on the activation of NF-$\kappa$B (A) and AP-1 (B) in porcine pulmonary artery endothelial cells. Confluent monolayers were treated with LTX (60 and 90 $\mu$M) and LTD (60 and 90 $\mu$M) for six hours. Fig. 5A: lane 1, control; lane 2, LTX (60 $\mu$M); lane 3, LTX (90 $\mu$M); lane 4, LTD (60 $\mu$M); lane 5, LTD (90 $\mu$M) and lane 6, LTX (90 $\mu$M) supershift (p65). Fig. 5B: lane 1, control; lane 2, LTX (60 $\mu$M); lane 3, LTX (90 $\mu$M); lane 4, LTD (60 $\mu$M); lane 5, LTD (90 $\mu$M) and lane 6, LTX (90 $\mu$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-$\kappa$B in endothelial cells. Interestingly, we found that this compound did not induce NF-$\kappa$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

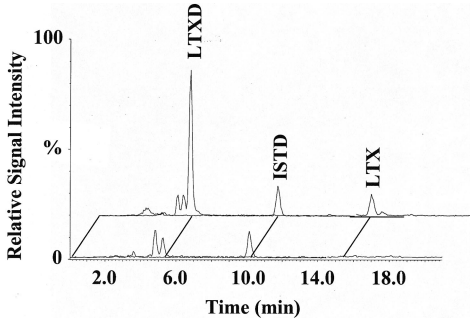


**Fig. 7.** Endothelial cell-derived linoleic acid metabolites. Cells were cultured in the presence of linoleic acid (90 $\mu$M; upper trace) for 24 hours, and media concentrations of linoleate-derived epoxides and diols were 3.2 $\pm$ 0.5 nM and 90 $\pm$ 15 nM, respectively. These metabolites were present at $<$0.5 and 10 $\pm$ 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 $\mu$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 $\mu$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].

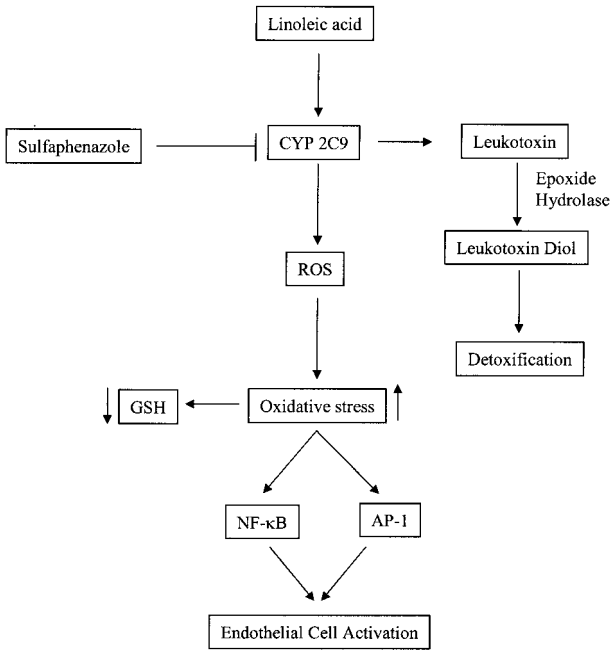


**Fig. 8.** Proposed model for the mechanism of linoleic acid (LA)-mediated endothelial cell activation. LA treatment results in CYP 2C9 activation and production of superoxide radicals as well as depletion of glutathione in endothelial cells. The increased oxidative stress results in the activation of oxidative stress sensitive transcription factors such as NF-$\kappa$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 $\mu$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-$\kappa$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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## REFERENCES

1. Behrendt D, Ganz P: Endothelial function. From vascular biology to clinical applications. Am J Cardiol 90:40L–48L, 2002.
2. Sydow K, Schwedhelm E, Arakawa N, Bode-Boger SM, Tsikas D, Hornig B, Frolich JC, Boger RH: ADMA and oxidative stress are responsible for endothelial dysfunction in hyperhomocyst(e)inemia: effects of L-arginine and B vitamins. Cardiovasc Res 57:244–252, 2003.
3. Landmesser U, Spiekermann S, Dikalov S, Tatge H, Wilke R, Kohler C, Harrison DG, Hornig B, Drexler H: Vascular oxidative stress and endothelial dysfunction in patients with chronic heart failure: role of xanthine-oxidase and extracellular superoxide dismutase. Circulation 106:3073–3078, 2002.
4. Hennig B, Toborek M: Nutrition and endothelial cell function: Implications in Atherosclerosis. Nutr Res 21:279–293, 2001.
5. Toborek M, Lee YW, Garrido R, Kaiser S, Hennig B: Unsaturated fatty acids selectively induce an inflammatory environment in human endothelial cells. Am J Clin Nutr 75:119–125, 2002.
6. Hoebel BG, Steyrer E, Graier WF: Origin and function of epoxyeicosatrienoic acids in vascular endothelial cells: more than just endothelium-derived hyperpolarizing factor? Clin Exp Pharmacol Physiol 25:826–830, 1998.
7. Roman RJ: P-450 metabolites of arachidonic acid in the control of cardiovascular function. Physiol Rev 82:131–185, 2002.
8. Fleming I: Cytochrome p450 and vascular homeostasis. Circ Res 89:753–762, 2001.
9. Fleming I, Michaelis UR, Bredenkotter D, Fisslthaler B, Dehghani F, Brandes RP, Busse R: Endothelium-derived hyperpolarizing factor synthase (Cytochrome P450 2C9) is a functionally significant source of reactive oxygen species in coronary arteries. Circ Res 88:44–51, 2001.
10. Popp R, Fleming I, Busse R: Pulsatile stretch in coronary arteries elicits release of endothelium-derived hyperpolarizing factor: a modulator of arterial compliance. Circ Res 82:696–703, 1998.
11. Draper AJ, Hammock BD: Identification of CYP2C9 as a human liver microsomal linoleic acid epoxygenase. Arch Biochem Biophys 376:199–205, 2000.
12. Ishizaki T, Takahashi H, Ozawa T, Chang SW, Voelkel NF: Leukotoxin, 9,10-epoxy-12-octadecenoate causes pulmonary vasodilation in rats. Am J Physiol 268:L123–128, 1995.
13. Reddy PS, Kumar TC, Reddy MN, Sarada C, Reddanna P: Differential formation of octadecadienoic acid and octadecatrienoic acid products in control and injured/infected potato tubers. Biochim Biophys Acta 1483:294–300, 2000.
14. Fukushima A, Hayakawa M, Sugiyama S, Ajioka M, Ito T, Satake T, Ozawa T: Cardiovascular effects of leukotoxin (9, 10-epoxy-12-octadecenoate) and free fatty acids in dogs. Cardiovasc Res 22:213–218, 1988.
15. Kosaka K, Suzuki K, Hayakawa M, Sugiyama S, Ozawa T: Leukotoxin, a linoleate epoxide: its implication in the late death of patients with extensive burns. Mol Cell Biochem 139:141–148, 1994.
16. Ozawa T, Hayakawa M, Kosaka K, Sugiyama S, Ogawa T, Yokoo K, Aoyama H, Izawa Y: Leukotoxin, 9,10-epoxy-12-octadecenoate, as a burn toxin causing adult respiratory distress syndrome. Adv Prostaglandin Thromboxane Leukot Res 21B:569–572, 1991.
17. Hennig B, Shasby DM, Fulton AB, Spector AA: Exposure to free fatty acid increases the transfer of albumin across cultured endothelial monolayers. Arteriosclerosis 4:489–497, 1984.
18. Toborek M, Lee YW, Kaiser S, Hennig B: Measurement of inflammatory properties of fatty acids in human endothelial cells. Methods Enzymol 352:198–219, 2002.
19. Yang HW, Hwang KJ, Kwon HC, Kim HS, Choi KW, Oh KS: Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. Hum Reprod 13:998–1002, 1998.
20. Baker MA, Cerniglia GJ, Zaman A: Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Anal Biochem 190:360–365, 1990.
21. Dignam JD, Lebovitz RM, Roeder RG: Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1489, 1983.
22. Newman JW, Watanabe T, Hammock BD: The simultaneous quantification of cytochrome P450 dependent linoleate and arachidonate metabolites in urine by HPLC-MS/MS. J Lipid Res 43:1563–1578, 2002.
23. Munzel T, Afanas'ev IB, Kleschyov AL, Harrison DG: Detection of superoxide in vascular tissue. Arterioscler Thromb Vasc Biol 22:1761–1768, 2002.
24. Benov L, Sztejnberg L, Fridovich I: Critical evaluation of the use of hydroethidine as a measure of superoxide anion radical. Free Radic Biol Med 25:826–831, 1998.
25. Oyekan AO, McAward K, Conetta J, Rosenfeld L, McGiff JC: Endothelin-1 and CYP450 arachidonate metabolites interact to promote tissue injury in DOCA-salt hypertension. Am J Physiol 276:R766–775, 1999.
26. Scarborough PE, Ma J, Qu W, Zeldin DC: P450 subfamily CYP2J and their role in the bioactivation of arachidonic acid in extrahepatic tissues. Drug Metab Rev 31:205–234, 1999.
27. Wu S, Moomaw CR, Tomer KB, Falck JR, Zeldin DC: Molecular cloning and expression of CYP2J2, a human cytochrome P450 arachidonic acid epoxygenase highly expressed in heart. J Biol Chem 271:3460–3468, 1996.
28. Bauersachs J, Christ M, Ertl G, Michaelis UR, Fisslthaler B, Busse R, Fleming I: Cytochrome P450 2C expression and EDHF-mediated relaxation in porcine coronary arteries is increased by cortisol. Cardiovasc Res 54:669–675, 2002.

29. Bylund J, Ericsson J, Oliw EH: Analysis of cytochrome P450 metabolites of arachidonic and linoleic acids by liquid chromatography-mass spectrometry with ion trap MS. Anal Biochem 265: 55–68, 1998.
30. Bylund J, Kunz T, Valmsen K, Oliw EH: Cytochromes P450 with bisallylic hydroxylation activity on arachidonic and linoleic acids studied with human recombinant enzymes and with human and rat liver microsomes. J Pharmacol Exp Ther 284:51–60, 1998.
31. Capdevila JH, Dishman E, Karara A, Falck JR: Cytochrome P450 arachidonic acid epoxygenase: stereochemical characterization of epoxyeicosatrienoic acids. Methods Enzymol 206:441–453, 1991.
32. Oliw EH, Bylund J, Herman C: Bisallylic hydroxylation and epoxidation of polyunsaturated fatty acids by cytochrome P450. Lipids 31:1003–1021, 1996.
33. Zeldin DC, DuBois RN, Falck JR, Capdevila JH: Molecular cloning, expression and characterization of an endogenous human cytochrome P450 arachidonic acid epoxygenase isoform. Arch Biochem Biophys 322:76–86, 1995.
34. Puntarulo S, Cederbaum AI: Production of reactive oxygen species by microsomes enriched in specific human cytochrome P450 enzymes. Free Radic Biol Med 24:1324–1330, 1998.
35. Dai Y, Rashba-Step J, Cederbaum AI: Stable expression of human cytochrome P4502E1 in HepG2 cells: characterization of catalytic activities and production of reactive oxygen intermediates. Biochemistry 32:6928–6937, 1993.
36. Park JY, Shigenaga MK, Ames BN: Induction of cytochrome P4501A1 by 2,3,7,8-tetrachlorodibenzo-p-dioxin or indolo(3,2-b)carbazole is associated with oxidative DNA damage. Proc Natl Acad Sci U S A 93:2322–2327, 1996.
37. White RE, Coon MJ: Oxygen activation by cytochrome P-450. Annu Rev Biochem 49:315–356, 1980.
38. Porter TD, Coon MJ. Cytochrome P-450: Multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms. J Biol Chem 266:13469–13472, 1991.
39. Davydov DR: Microsomal monooxygenase in apoptosis: another target for cytochrome c signaling? Trends Biochem Sci 26:155–160, 2001.
40. Sun Y, Zhang J, Lu L, Chen SS, Quinn MT, Weber KT: Aldosterone-induced inflammation in the rat heart : role of oxidative stress. Am J Pathol 161:1773–1781, 2002.
41. Altavilla D, Deodato B, Campo GM, Arlotta M, Miano M, Squadrito G, Saitta A, Cucinotta D, Ceccarelli S, Ferlito M, Tringali M, Minutoli L, Caputi AP, Squadrito F: IRFI 042, a novel dual vitamin E-like antioxidant, inhibits activation of nuclear factor-kappaB and reduces the inflammatory response in myocardial ischemia-reperfusion injury. Cardiovasc Res 47:515–528, 2000.
42. Woo Lee Y, Joo PH, Hennig B, Toborek M: Linoleic acid induces MCP-1 gene expression in human microvascular endothelial cells through an oxidative mechanism. J Nutr Biochem 12:648–654, 2001.
43. Herbst U, Toborek M, Kaiser S, Mattson MP, Hennig B: 4-Hydroxynonenal induces dysfunction and apoptosis of cultured endothelial cells. J Cell Physiol 181:295–303, 1999.
44. Hennig B, Toborek M, Joshi-Barve S, Barger SW, Barve S, Mattson MP, McClain CJ: Linoleic acid activates nuclear transcription factor-kappa B (NF-kappa B) and induces NF-kappa B-dependent transcription in cultured endothelial cells. Am J Clin Nutr 63:322–328, 1996.
45. Park SH, Park JH, Kang JS, Kang YH: Involvement of transcription factors in plasma HDL protection against TNF-alpha-induced vascular cell adhesion molecule-1 expression. Int J Biochem Cell Biol 35:168–182, 2003.
46. Eyries M, Agrapart M, Alonso A, Soubrier F: Phorbol ester induction of angiotensin-converting enzyme transcription is mediated by Egr-1 and AP-1 in human endothelial cells via ERK1/2 pathway. Circ Res 91:899–906, 2002.
47. Akai M, Ishizaki T, Matsukawa S, Shigemori K, Miyamori I: Leukotoxin (9, 10-epoxy-12-octadecenoate) impairs energy and redox state of isolated perfused rat lung. Free Radic Biol Med 25:596–604, 1998.
48. Ozawa T, Sugiyama S, Hayakawa M, Satake T, Taki F, Iwata M, Taki K: Existence of leukotoxin 9,10-epoxy-12-octadecenoate in lung lavages from rats breathing pure oxygen and from patients with the adult respiratory distress syndrome. Am Rev Respir Dis 137:535–540, 1988.
49. Moran JH, Mitchell LA, Bradbury JA, Qu W, Zeldin DC, Schnellmann RG, Grant DF: Analysis of the cytotoxic properties of linoleic acid metabolites produced by renal and hepatic P450s. Toxicol Appl Pharmacol 168:268–279, 2000.
50. Haastrup A, Gadegbeku CA, Zhang D, Mukhin YV, Greene EL, Jaffa AA, Egan BM: Lipids stimulate the production of 6-keto-prostaglandin f(1alpha) in human dorsal hand veins. Hypertension 38:858–863, 2001.
51. Nakanishi M, Ishizaki T, Demura Y, Okamura S, Ameshima S, Sasaki F, Matsukawa S, Miyamori I: Leukotoxin, 9,10-epoxy-12-octadecenoate, causes pulmonary vasodilation by stimulation of vascular eNOS and iNOS. Lung 178:137–148, 2000.
52. Node K, Huo Y, Ruan X, Yang B, Spiecker M, Ley K, Zeldin DC, Liao JK: Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. Science 285:1276–1279, 1999.

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