Cholesterol Attenuates Linoleic Acid–Induced Endothelial Cell Activation

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Vascular endothelial cell activation and dysfunction are critical early events in atherosclerosis. Even though very low or high levels of cholesterol can compromise cellular functions, cholesterol is a critical membrane component and may protect the vascular endothelium from oxidative stress and polyunsaturated fatty acid–mediated inflammatory responses. We have previously shown that the parent omega-6 fatty acid linoleic acid can markedly activate vascular endothelial cells. We now propose that membrane cholesterol can modify and inhibit linoleic acid–mediated endothelial cell dysfunction. To test this hypothesis, pulmonary artery endothelial cells were incubated with cholesterol (0 to 100 μmol/L) for 24 hours and then treated with 90 μmol/L of linoleic acid (18:2n-6) for 6 to 24 hours. In control cells, treatment with linoleic acid reduced intracellular glutathione levels and induced the DNA binding activity of nuclear factor-κB (NF-κB) leading to the upregulation of interleukin-6 (IL-6). In addition, the expression of endothelial nitric oxide synthase (eNOS) was altered, with linoleic acid increasing eNOS activity. In contrast, enrichment with cholesterol enhanced glutathione levels and reduced the linoleic acid–induced activation of NF-κBand the production of IL-6. Prior exposure to 50 μmol/L cholesterol also prevented the fatty acid–induced increase in eNOS activation. Cholesterol loading activated peroxisome proliferator–activated receptor-gamma (PPAR-γ), a nuclear receptor that can decrease inflammatory responses. Furthermore, the PPAR-γ agonist thiazolidinedione markedly downregulated the NF-κB activation mediated by linoleic acid. Our data suggest that signaling pathways linked to endothelial cell activation by prooxidant and proinflammatory insults may be influenced by cellular cholesterol levels.

INJURY OR DYSFUNCTION of the vascular endothelium is considered to be one of the early events in the pathology of atherosclerosis. The endothelium interacts with the blood and underlying tissues, serves as both a prothrombic and antithrombic surface, and releases regulatory factors important in modulating vascular tone. Metabolic alterations and dysfunction of the endothelium can be induced by numerous activating molecules, such as certain lipids, prooxidants, and inflammatory cytokines. Little is known about the role of specific fatty acids in atherosclerosis and especially in vascular endothelial cell function. The significance of saturated fatty acids in atherosclerosis has been questioned.[1,2] In fact, data from subjects with varying degrees of coronary atherosclerosis support the hypothesis that high serum polyunsaturated fatty acid levels (eg, linoleic acid), when insufficiently protected by antioxidants, may indicate a higher risk of atherosclerosis.[3,4] Research with a population from Israel, a country with one of the highest dietary polyunsaturated/saturated fat ratios in the world, has concluded that diets rich in omega-6 (or n-6) fatty acids may contribute to an increased incidence in atherosclerosis, hyperinsulinemia, and tumorigenesis.[5] There appears to be a positive correlation between linoleic acid levels in the phospholipid fractions of human coronary arteries and ischemic heart disease.[6] In addition, linoleic acid can increase expression of CD36, a scavenger receptor for oxidized low-density lipoprotein (LDL),[7,8] and concentrations of linoleic acid in adipose tissue were positively correlated with the degree of coronary artery disease.[9]

We have demonstrated that selected unsaturated fatty acids (eg, linoleic acid) can disrupt endothelial barrier function.[10,11] Linoleic acid is a major fatty acid found in commonly consumed oils, such as corn oil or safflower oil. Mechanisms of linoleic acid–mediated endothelial cell dysfunction are not fully understood, but may include an imbalance in cellular oxidative stress/antioxidant status. Nuclear factor-κB (NF-κB) is a major transcription factor activated by oxidative stress and is critical in the regulation and expression of inflammatory genes, such as inflammatory cytokines (eg, interleukin-6 [IL-6]) and vascular cell adhesion molecules (eg, VCAM-1). We have evidence that of all the 18-carbon fatty acids tested, linoleic acid contributes most markedly to cellular oxidative stress.[12] We also found that LDL derived from rabbits fed a high corn oil diet displayed proinflammatory properties by activating NF-κB in cultured endothelial cells.[13]

Impaired nitric oxide bioactivity has been suggested to be pathogenic and to contribute to vascular dysfunction. Nitric oxide is produced locally by endothelial nitric oxide synthase (eNOS) within the vessel wall, and impaired eNOS expression and activity may influence the pathology of atherosclerosis. There is evidence that the cellular redox state may regulate eNOS expression and that reactive oxygen species and cholesterol are important regulators of eNOS function.[14]

Even though excess circulating cholesterol, and especially as a component of LDL, is associated with the risk of atherosclerosis, changes in cellular cholesterol levels may markedly compromise cell function. Cholesterol is an essential component of biomembranes and is necessary for maintenance of membrane structure and function. For example, cholesterol has been shown to be a critical determinant of membrane remodeling in cultured endothelial cells.[15] There is evidence that cholesterol plays a direct role as a modulator of receptor function,[16] in cell signaling,[17] and that changes in cholesterol levels may lead to the reorganization of signaling molecules.[18] In addition, it is

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likely that cholesterol stabilizes vascular endothelial cells by interfering with signaling pathways involved in the inflammatory process, and hence cholesterol may provide protection against oxidative and proinflammatory insults. Interestingly, recent data support the notion that activation of the peroxisome proliferator–activated receptor (PPAR) is an antiatherogenic phenomenon.[19] Whether cholesterol can modulate linoleic acid–induced endothelial cell activation in part through PPAR-γ signaling is not known. Thus, the focus of the present study was to understand the mechanism by which cholesterol status modifies the effects of linoleic acid–mediated endothelial cell dysfunction. Our data suggest that signaling pathways linked to endothelial cell activation, ie, metabolic events mediated by prooxidant and proinflammatory insults, are linked to cellular cholesterol levels.

MATERIALS AND METHODS

Cell Culture and Experimental Media

Endothelial cells were isolated from porcine pulmonary arteries and cultured as previously described.[20] 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 low density lipoprotein (DiI-Ac-LDL; Molecular Probes, Eugene, OR). The basic culture medium consisted of M199 (Gibco Laboratories, Grant Island, NY) containing 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT). The experimental media were composed of M199 enriched with 10% FBS and supplemented with 0 to 100 μmol/L cholesterol as an albumin complex as described by Hennig and Boissonneault[21] with slight modifications. After enrichment with or without cholesterol for 24 hours, cultures were exposed to linoleic acid (90 μmol/L) for up to 12 hours. Some cultures were pre-enriched for 24 hours with the PPAR-γ agonist thiazolidinedione (10 μmol/L; Sigma, St Louis, MO). Linoleic acid (>99% pure) was obtained from Nu-Chek Prep (Elysian, MN). Preparations of experimental media with linoleic acid were performed as described earlier.[12,20] Thus, fatty acids were introduced into the media bound to serum albumin. Assuming albumin concentrations of 30 μmol/L (in 5% serum) to 60 μmol/L (in 10% serum) in our culture media, the fatty acid concentrations are within physiological and metabolic relevance. Considering the amount of serum (5% to 10%) present in our culture media and albumin being the vehicle for introducing solubilized cholesterol, concentrations of 25 to 100 μmol/L were chosen. We have also shown that endothelial cell exposure to such concentrations results in cellular cholesterol enrichment and that levels of up to 100 μmol/L are nontoxic to the vascular endothelium.[21]

Cholesterol Measurement

The cells were scraped in 1 mL of phosphate-buffered saline (PBS) to which was added 3 times the volume of hexane:isopropanol (3:2), which was then vortexed and incubated at room temperature for 30 minutes. The organic phase was saved while the aqueous phase was re-extracted. The organic phases were combined and dried to completeness with nitrogen. Each sample was solubilized in 1 mL of 1% Triton X-100 in chloroform, and dried to completeness with nitrogen. Each sample was then solubilized in 500 μL of water, and the total cholesterol was determined as described by Uittenbogaard et al[22] using a Wako kit (Wako Pure Chemical Industries, Osaka, Japan).

Glutathione Measurement

Determination of glutathione was performed by enzymatic recycling method described by Baker et al,[23] 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 (dry ice-ethanol) and thawing, and centrifuged at 10,000 × *g* for 5 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 mmol/L phosphate buffer containing 0.225 mmol/L DTNB, 0.302 mmol/L NADPH, and glutathione reductase at the 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 3 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 Assays

Nuclear extracts from endothelial cells were prepared according to the method of Beg et al.[24] Binding reactions were performed in a 20-μL volume containing 7 μg of nuclear protein extracts, 10 mmol/L Tris-Cl, pH 7.5, 50 mmol/L NaCl, 1 mmol/L EDTA, 0.1 mmol/L dithiothreitol (DTT), 10% glycerol, 0.5 μg of poly[dI-dC] (nonspecific competitor), and 40,000 cpm of ^{32}P-labeled specific oligonucleotide probe. Double-stranded oligonucleotides containing a tandem duplicate of the NF-κB binding site (underlined) (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and the consensus sequence for PPAR-γ (5′-AACTAGGTCAAAGGTCA-3′) were radiolabeled with [γ-^{32}P]-adenosine triphosphate (ATP) (Amersham Pharmacia Biotech, Piscataway, NJ) using T4 polynucleotide kinase. Resultant protein-DNA complexes were resolved on native 5% polyacryamide gels using 0.25x TBE buffer (50 mmol/L Tris-Cl, 45 mmol/L boric acid, 0.5 mmol/L EDTA, pH 8.4). Competition studies were performed by the addition of a molar excess of unlabeled oligonucleotide to the binding reaction. Rabbit polyclonal anti–NF-κB p65 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and employed in supershift experiments.

IL-6 Bioassay

After exposure to cholesterol and/or linoleic acid, the media were removed from the culture plates and frozen immediately at −80°C until IL-6 analysis. IL-6 production and release into the medium was determined using the murine hybridoma cell line B9 (kindly supplied by Dr L.A. Aarden, Emeryville, CA) as described by Hennig et al.[25] The B9 cell line viability is IL-6–dependent, and thus, the incorporation of ^{3}H-thymidine by viable cells is a reflection of the quantity of IL-6 produced by endothelial cells.

Reverse-Transcriptase Polymerase Chain Reaction

PPAR-γ mRNA levels were determined using a semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR). Treated endothelial cells were lysed, and total RNA was extracted using RNA-STAT-60 (Tel TEST, Friendswood, TX) according to the manufacturer's protocol. To each 60-mm dish, 1 mL of RNA STAT 60 was added and incubated for 30 minutes at 4°C. At the end of the incubation period 200 μL chloroform was added for RNA extraction, and samples were incubated for 5 minutes and centrifuged at 12,000 × *g* for 15 minutes at 4°C. The upper aqueous phase was collected and 600 μL of isopropanol was added, mixed by inverting, and incubated at −70°C for 20 minutes. At the end of the incubation period, tubes were centrifuged at 12,000 × *g* at 4°C for 15 minutes, and the supernatant was discarded. The pellets were washed twice with 75% ethanol, dissolved in 20 μL diethyl phrocarbonate (DEPC) water and stored at −70°C. Isolated RNA was quantitated by determining the absorbance at 260 nm. Superscript II reverse transcriptase (Gibco Laboratories) was used for reverse transcription of total RNA to total cDNA. PCR

reaction was performed using Taq DNA polymerase (Gibco Laboratories) using Perkin-Elmer (Shelton, CT) GeneAmp PCR system 9700. Specific primers were designed using the software package Oligo 5.0 (National Biosciences, Plymouth, MN) and were synthesized by MWG-Biotech Inc. (High Point, NC). The primer sequences used for PPAR-γ were 5′(-AGC CCT TCA CCA CTG TTG ATT T-)3′ and 5′(-GCG GGA AGG ACT TTA TGT ATG AGT-)3′, respectively. Oligonucleotide primers used to amplify the porcine house-keeping gene, β-actin, were as published by Barchowsky et al.[26] The primer sequences for PPAR-γ resulted in a product size of 579 bp. The annealing temperature was 61°C for PPAR-γ. Expression of IL-6 and PPAR-γ were studied, using 41 and 40 cycles, respectively. Cycling times were optimized to ensure that the amplification cycles were below the plateau levels. The amplified PCR products were electrophoresed on a 2% TBE agarose gel, stained with SYBER Gold (Molecular Probes), and visualized using phosphoimaging technology (FLA-2000; Fuji, Stamford, CT).

Nitric Oxide Synthase Activity Assay

NO synthase activity was determined by measuring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline after separation of these amino acids by anion exchange chromatography using a modification of the method of Davda et al.[27] After enrichment with cholesterol and treatment with linoleic acid, subconfluent cells were washed and harvested with ice-cold PBS. A whole cell extract was prepared by freezing (dry ice-ethanol) and thawing in 200 μL of lysis buffer (50 mM Tris-HCl, pH 7.8, 20 μmol/L BH4, 3.0 mmol/L DTT, 10 mmol/L (3-[(3-cholamidopropyl) dimethyl-ammoniol-1 propanesulfonate (CHAPS) containing protesase inhibitors (1 μmol/L pepstation A, 2 μmol/L leupeptin, 1 μmol/L bestatin, and 1 μmol/L phenylmethylsulfonyl fluoride [PMSF]) and used for NO synthase activity assay. Each sample (150 μL) was incubated for 30 minutes at 37°C with 150 μL of HEPES buffer (40 mmol/L, pH 7.4) containing the cofactors (final concentration: 2 mmol/L NADPH, 2 μmol/L BH4, 10 mmol/L flavin mononucleotide (FMN), 0.5 mmol/L $CaCl_2$), 15 nmol/L calmodulin, and the substrate 2 μmol/L cold L-arginine combined with [^{3}H]L-arginine (Amersham). The NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME; negative control) was added to a sample aliquot of 150 μL to a final concentration of 4 mmol/L. The reaction was quenched by addition of 1.2 mL of stop buffer (20 mmol/L HEPES, pH 5.5, containing 2 mmol/L EDTA and 2 mmol/L EGTA). The reaction mixture was loaded onto a column containing 1.0 mL of Dowex AG 50WX-8 (Na-form; Sigma, St Louis, MO) added as a 1:1 slurry in water. The columns were then washed twice with 0.5 mL of stop buffer, and the elutes were combined. Ten milliliters of scintillation cocktail was added to the vials, and the radioactivity was quantified by liquid scintillation spectroscopy. NOS activity is expressed as picomoles of [^{3}H]L-citrulline produced per milligram of protein. The protein in the cell extract was determined using Bio-Rad DC reagent (Bio-Rad Laboratories, Hercules, CA).

Statistical Analysis

The data were analyzed using SYSTAT 7.0 (SPSS Inc, Chicago, IL). Comparisons between treatments were made by 1-way analysis of variance (ANOVA) with post-hoc comparisons of the means made by Fischer's least significant difference procedure. Statistical probability of $P < .05$ was considered significant.

RESULTS

Supplementation With Linoleic Acid Does Not Affect Cellular Cholesterol Levels

Figure 1 shows the effect of cholesterol loading on changes in cellular cholesterol levels. Enrichment of culture media with 25 to 100 μmol/L cholesterol for up to 24 hours resulted in a concentration-dependent increase in cellular cholesterol levels. A subsequent 6-hour exposure to linoleic acid in cultures enriched with 50 μmol/L cholesterol did not alter cellular cholesterol levels (data not shown).

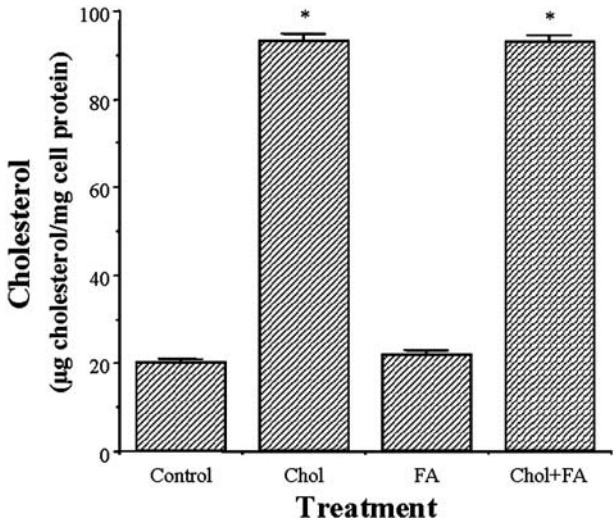

Fig 1. Effect of cholesterol loading on endothelial cell total cholesterol levels. Cultures were incubated first in cholesterol-free media (10% FBS, lipoprotein-deficient) for 16 hours before culture supplementation with 25, 50, or 100 μmol/L cholesterol for up to 24 hours. Values are mean ± SEM, n = 6. *Significantly different from control (unloaded) cultures.

Moderate Cholesterol Supplementation Attenuates Linoleic Acid–Induced Depletion of Cellular Glutathione

Supplementation of culture media with cholesterol resulted in an increase in cellular glutathione only at 50 μmol/L but not at 25 or 100 μmol/L cholesterol (data not shown). These cholesterol concentrations did not affect endothelial cell viability. A 6-hour exposure to 90 μmol/L linoleic acid resulted in a marked decrease in cellular glutathione, which was blocked in cultures that were first enriched for 24 hours with 50 μmol/L cholesterol (Fig 2).

Moderate Cholesterol Attenuates Linoleic Acid–Induced Activation of NF-κB

Similar to cellular glutathione, NF-κB activation, as analyzed by electrophoretic-mobility-shift assay (EMSA), also was dependent on the amount of cholesterol supplementation. 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 (eg, IL-6) and adhesion molecules, such as VCAM-1. Figure 3 shows that no activation of NF-κB was observed by cellular enrichment with 50 μmol/L cholesterol, compared to untreated control cultures. Linoleic acid markedly activated NF-κB. This activation of NF-κB was reduced by pretreating endothelial cells with 50 μmol/L cholesterol.

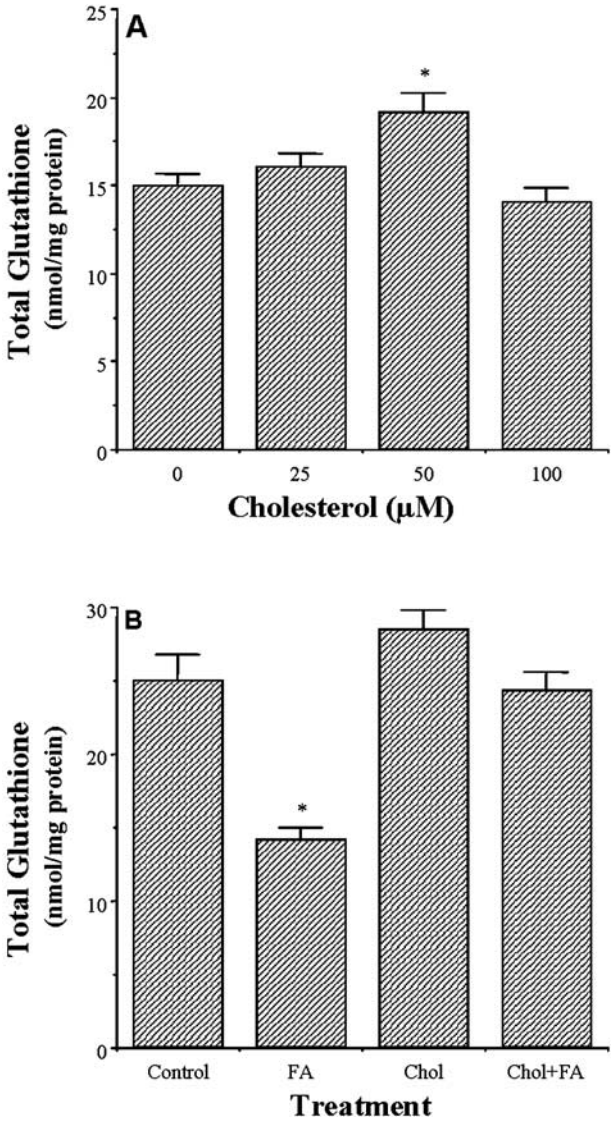

Fig 2. Effect of cholesterol supplementation and exposure to linoleic acid on total glutathione levels. In the cholesterol plus fatty acid group (Chol+FA), cells were supplemented with 50 μmol/L cholesterol (Chol) for 24 hours and then treated with 90 μmol/L linoleic acid (FA) for 6 hours. Control cultures (Control) were incubated with media supplemented with 10% FBS. Values are mean ± SEM, n = 6. *Significantly different from control cultures.

Moderate Cholesterol Treatment Induces the Expression and DNA Binding Activity of PPAR-γ

Endothelial cell exposure to culture media supplemented with 50 μmol/L cholesterol for 24 hours resulted in a marked increase in the expression (Fig 4) and DNA binding activity (Fig 5) of PPAR-γ. Linoleic acid, on the other hand, only slightly increased PPAR-γ expression compared to control cultures (Fig 4). Exposure to cholesterol for 24 hours followed by cotreatment with linoleic acid for an additional 6 hours increased PPAR-γ gene expression compared with linoleic acid treatment alone (Fig 4). Similar to the PPAR-γ agonist thiazolidinedione, cholesterol increased PPAR-γ activity (Fig 5).

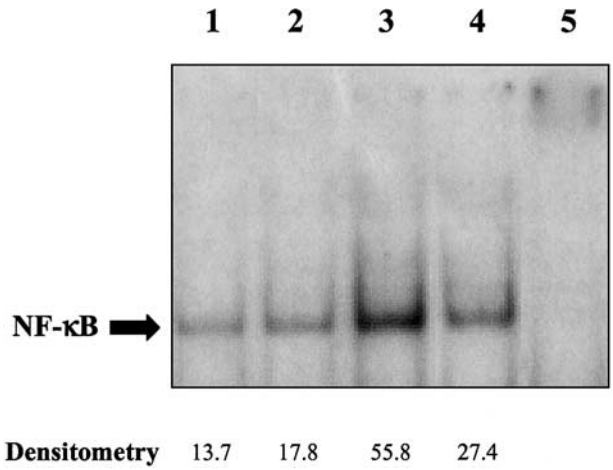

Fig 3. Effect of cholesterol loading and treatment with linoleic acid on activation of NF-κB. Endothelial cells were enriched with cholesterol for 24 hours. In the cholesterol plus fatty acid group, cells were supplemented with 50 μmol/L cholesterol for 24 hours and then treated with 90 μmol/L linoleic acid for 6 hours. Lane 1, control; lane 2, cholesterol (50 μmol/L); lane 3, linoleic acid (90 μmol/L); lane 4, cholesterol + linoleic acid. Equal amounts of protein (nuclear extracts) per treatment were applied to each gel, and the NF-κB band was confirmed by supershift assay to be the transcriptionally active p65/p50 heterodimer (lane 5). Densitometric quantification (relative units) is listed below each NF-κB band.

PPAR-γ Agonist Attenuates Linoleic Acid–Induced Activation of NF-κB

As shown above, a 6-hour exposure to linoleic acid activated NF-κB. Pre-exposure to the PPAR-γ agonist thiazolidinedione markedly downregulated the fatty acid–mediated activation of NF-κB (Fig 6).

Moderate Cholesterol Affects eNOS Activity

eNOS activity was increased after a 6-hour exposure to 90 μmol/L linoleic, but was not affected by cholesterol treatment alone (Fig 7). On the other hand, the fatty acid–induced increase in eNOS activity was blocked by prior cell exposure to 50 μmol/L cholesterol.

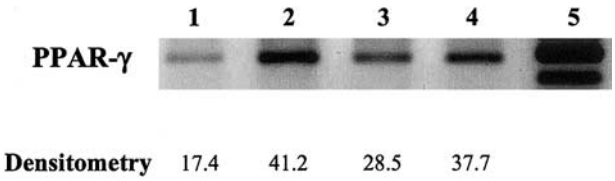

Fig 4. Effect of cholesterol loading and treatment with linoleic acid on PPAR-γ gene expression as analyzed by RT-PCR. Endothelial cells were treated either with cholesterol (50 μmol/L; 24 hours) or linoleic acid (90 μmol/L; 6 hours). In the cholesterol plus fatty acid group, cells were supplemented with 50 μmol/L cholesterol for 24 hours and then treated with 90 μmol/L linoleic acid for 6 hours. Lane 1, control; lane 2, cholesterol; lane 3, linoleic acid, lane 4, cholesterol + linoleic acid; lane 5, molecular weight marker. Densitometric quantification (relative units) is listed below each PPAR band.

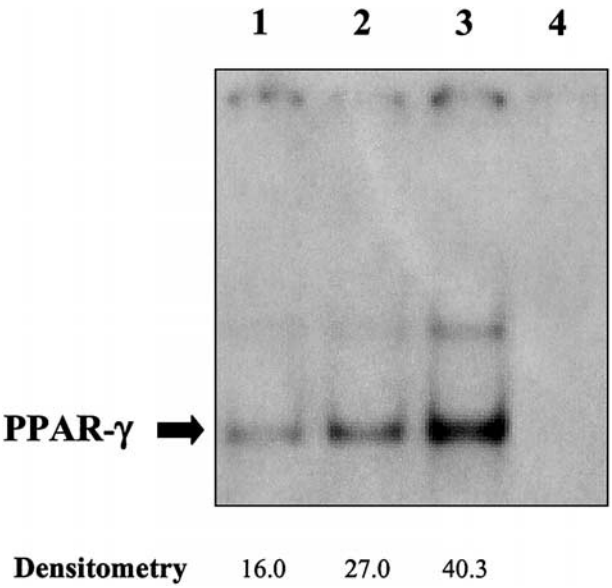

Fig 5. Effect of cholesterol (50 μmol/L; 24 hours) or thiazolidinedione (10 μmol/L; 24 hours) treatment on PPAR-γ DNA binding activity. Lane 1, control; lane 2, cholesterol; lane 3, thiazolidinedione; lane 4, cold quench (unlabeled competitor). Densitometric quantification (relative units) is listed below each PPAR band.

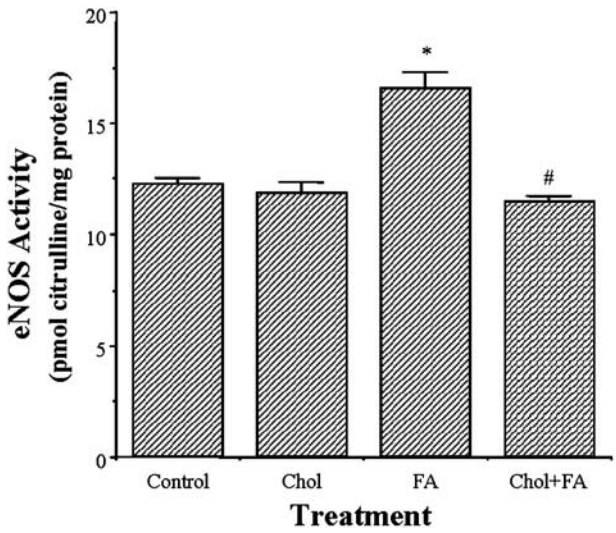

Fig 7. Effect of cholesterol loading and treatment with linoleic acid (FA) on eNOS activity. Endothelial cells were incubated with media supplemented with 10% FBS (Control) or enriched with cholesterol (50 μmol/L; 24 hours) or linoleic acid (90 μmol/L; 6 hours). In the cholesterol plus fatty acid group, cells were supplemented with 50 μmol/L cholesterol for 24 hours and then treated with 90 μmol/L linoleic acid for 6 hours. Values are mean ± SEM, n = 6. *Significantly different from control cultures. #Significantly lower than cultures treated only with linoleic acid (LA).

Moderate Cholesterol Attenuates Linoleic Acid–Mediated Cellular IL-6 Production

Compared to control cells, exposing endothelial cells to linoleic acid caused an increase in cellular IL-6 production (Fig 8). In contrast, a 24-hour pre-exposure to 50 μmol/L cholesterol attenuated the linoleic acid–mediated IL-6 production by endothelial cells. Similar to IL-6 production, RT-PCR data, looking at the IL-6 message, were consistent with the biological assay (data not shown).

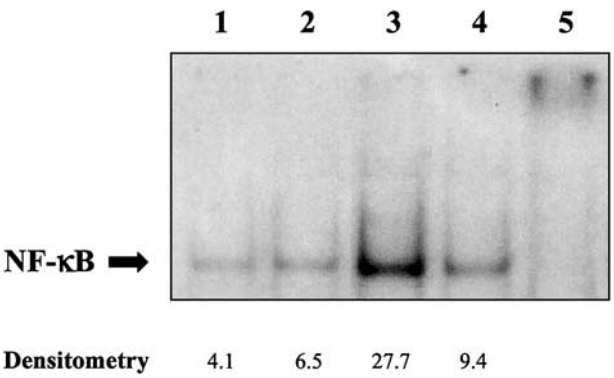

Fig 6. Effect of thiazolidinedione loading and treatment with linoleic acid on activation of NF-κB. Endothelial cells were enriched with the PPAR-γ agonist for 24 hours. In the thiozolidinedione plus fatty acid group, cell were supplemented with 10 μmol/L of the PPAR-γ agonist for 24 hours and then treated with 90 μmol/L linoleic acid for 6 hours. Lane 1, control; lane 2, thiazolidinedione; lane 3, linoleic acid, lane 4, thiazolidinedione + linoleic acid; lane 5, supershift p^{65}. Densitometric quantification (relative units) is listed below each NF-κB band.

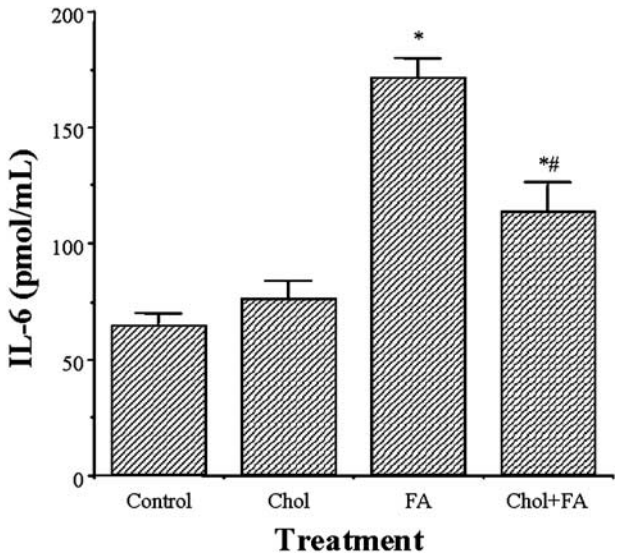

Fig 8. Effect of cholesterol supplementation and treatment with linoleic acid on IL-6 production by endothelial cells. Cells were enriched with 50 μmol/L cholesterol for 24 hours. Appropriate groups were treated with linoleic acid (90 μmol/L) for 6 hours. In the cholesterol plus fatty acid group (Chol+FA), cells were supplemented with 50 μmol/L cholesterol (Chol) for 24 hours and then treated with 90 μmol/L linoleic acid (FA) for 6 hours. Values are mean ± SEM, n = 6. *Significantly higher than control cultures. #Significantly lower than cultures treated only with linoleic acid (LA).

DISCUSSION

Factors implicated in the pathogenesis of atherosclerosis, thrombosis, and peripheral vascular diseases include chronic and cumulative metabolic alterations of the endothelium by certain lipids and inflammatory cytokines.[28-31] Most patients with coronary artery disease have increased postprandial triglyceride levels compared to healthy control subjects.[32,33] Furthermore, hypertriglyceridemia can lead to endothelial cell dysfunction associated with increased vascular superoxide anion production and a subsequent compromised nitric oxide bioavailability.[34] In addition, it has been reported that leukocyte, and especially monocyte, adhesion to the endothelial surface is stimulated by triglyceride-rich lipoproteins[35,36] and that endothelial cell activation during metabolic states of hypertriglyceridemia and postprandial lipemia is redox-sensitive.[37]

There is evidence that selected fatty acids, and especially omega-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.[38] 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.[10,39] 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.[40] Furthermore, our data strongly support the fact that selected unsaturated fatty acids (eg, linoleic acid) and inflammatory cytokines may cross-amplify vascular endothelial cell activation, an inflammatory response and atherosclerosis.[11,12]

Changes in cellular cholesterol levels may markedly compromise cell function. Cholesterol is an essential component of biomembranes and is necessary for maintenance of membrane structure and function. The present study provides evidence that cholesterol can modulate endothelial cell activation mediated by linoleic acid. In fact, our data support the hypothesis that cholesterol may provide antioxidant properties.[41] In our cell culture model, the endothelial protective property of cholesterol appears to occur only at some critical concentration. Our data also suggest that at relatively low or high cholesterol concentrations, endothelial cells are susceptible to activation, which may occur via oxidative stress-sensitive signaling pathways. For example, we found that NF-κB was activated slightly when cells were enriched with 100 μmol/L cholesterol, which was not observed after enriching cultures with 50 μmol/L cholesterol. Similarly, cellular glutathione was enhanced after cellular enrichment with 50 μmol/L cholesterol but not with 25 or 100 μmol/L (data not shown). These data suggest that the cellular antioxidant defense is sensitive to varying cholesterol concentrations. Whether or not cholesterol itself can act as an antioxidant or has an indirect role either as a cell membrane stabilizer and/or signaling molecule, warrants further study.

Cholesterol appears to be a critical membrane component to protect vascular endothelial cells from inflammatory responses mediated by unsaturated fatty acids, such as linoleic acid. We found that treatment with linoleic acid reduced intracellular glutathione levels and induced the DNA binding activity of NF-κB leading to proinflammatory events such as the upregulation of IL-6. In addition, the activity of eNOS was increased after cellular exposure to linoleic acid. In contrast, enrichment with cholesterol upregulated glutathione levels and downregulated the linoleic acid–induced activation of NF-κB and production of IL-6. Moreover, our preliminary data suggest that linoleic acid can upregulate adhesion molecules such as VCAM-1 mRNA and that preincubation with 50 μmol/L cholesterol reduced the fatty acid–mediated expression of the VCAM-1 gene.

Our data also suggest that cholesterol can modulate oxidative stress-sensitive and inflammatory processes by regulating the expression of PPARs, such as PPAR-γ. PPARs, which are expressed in atherosclerotic lesions, can mediate pleiotropic effects such as stimulation of lipid oxidation, alteration in lipoprotein metabolism, and inhibition of vascular inflammation.[42] Most recently, the activation of PPAR-γ has been described as an antiatherogenic phenomenon.[19] There appears to be a modulatory role for PPARs in the control of the inflammatory response[43] by repressing NF-κB signaling and inflammatory cytokine production.[44] Recently, PPAR signaling also was demonstrated in the reverse cholesterol transport pathway in human macrophages,[45] suggesting that PPAR signaling is a broad function in the overall antiinflammatory and antiatherogenic outcome. Our data suggest that cholesterol can regulate PPAR expression and downregulate the inflammatory process mediated after endothelial cell exposure to linoleic acid by inhibiting the activation of the proinflammatory transcription factor NF-κB. This may have implications in understanding mechanisms of inflammation regulated by lipids such as cholesterol and unsaturated fatty acids.

As mentioned above, we found that linoleic acid can modulate eNOS activity. Prior exposure to 50 μmol/L cholesterol prevented the fatty acid–induced increase in eNOS activation, which might lead to peroxynitrate formation and to an increase in oxidative stress. Our data support findings by others that the cellular redox state may regulate eNOS expression and that reactive oxygen species and cholesterol are important regulators of eNOS function.[14] A decrease in NO levels was also observed in hypertensive rats, which was associated with a concomitant increase in superoxide anion production.[46] We have shown in this and other studies[11,47] that linoleic acid, the parent omega-6 fatty acid, can activate NF-κB, and we now report that the linoleic acid–mediated increase in eNOS activity can be blocked by prior supplementation of endothelial cells with 50 μmol/L cholesterol. Furthermore, there is evidence that lipids or lipoproteins susceptible to oxidative modification can deplete caveolae of cholesterol, thus resulting in the displacement of endothelial eNOS from caveolae and impaired eNOS activation.[22,48]

In summary, our data support the concept that specific dietary fatty acids (eg, linoleic acid) can activate vascular endothelial cells and are proinflammatory. We provide further evidence that a cellular imbalance in oxidative stress/antioxidant status is critical in signaling pathways of endothelial cell activation mediated by linoleic acid. Furthermore, our data show that moderate supplementation with cholesterol provides regulatory and protective properties to stabilize vascular endothelial

cells against prooxidant and proinflammatory insults. Mechanisms may include maintenance of membrane structure and function by interfering with signaling pathways involved in the nitric oxide bioactivity and the inflammatory process.

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