Effect of Linoleic Acid on Endothelial Cell Inflammatory Mediators

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Selected lipids may influence the inflammatory cascade within the vascular endothelium. To test this hypothesis, endothelial cells were treated with linoleic acid (18:2, n − 6) for 12 hours and/or tumor necrosis factor-α (TNF) for 4 hours. For a combined exposure to 18:2 and TNF (18:2 + TNF), cells were first preenriched with 18:2 for 8 hours before exposure to TNF for an additional 4 hours. Exposure to 18:2 increased cellular oxidative stress, activated nuclear factor-κB (NF-κB), increased interleukin-8 (IL-8) production, and elevated intercellular adhesion molecule-1 (ICAM-1) levels. A combined exposure to 18:2 + TNF resulted in decreased NF-κB activation compared with TNF treatment alone. In addition, preexposure to 18:2 altered TNF-mediated IκB-α signaling. Within the first 15 minutes of a 90-minute period, cytoplasmic levels of IκB-α decreased more rapidly in cells treated with 18:2+TNF compared with TNF, suggesting translocation and activation of NF-κB in cultures that were pretreated with 18:2 before TNF exposure. A combined exposure to 18:2+TNF had various effects on IL-8 production and ICAM-1 levels depending on the time of exposure. For example, 18:2+TNF treatment increased ICAM-1 levels at 12 hours but decreased ICAM-1 levels at 24 hours compared with treatment with TNF alone. These data suggest that selected fatty acids such as 18:2 can exert proinflammatory effects and, in addition, may markedly alter TNF-mediated inflammatory events.

ATHEROSCLEROSIS remains one of the leading causes of death in the Western world. The primary step in the formation of atherosclerotic lesions involves adherence of circulating monocytes to the endothelium with subsequent migration to the arterial intima, where they differentiate into macrophages. In fact, the adherence of circulating leukocytes to the arterial endothelium is one of the earliest detectable events in experimental atherosclerosis induced by hypercholesterolemia.[1-3] There is evidence implicating inflammatory cytokines such as tumor necrosis factor (TNF) or interleukin-8 (IL-8) and adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) in neutrophil adhesion and transmigration into vascular tissues.[4,5] For example, TNF production by activated leukocytes is increased in atherosclerosis patients, and the presence of this cytokine is detected in atherosclerotic lesions.[6,7] In addition, both plaque macrophages and blood monocytes from patients with carotid atheromatous plaques can produce IL-8,[8] suggesting an important role of this cytokine in atherogenesis. Inflammatory cytokines may affect growth and gene expression in the vessel wall,[9] as well as act as potent chemoattractants and activators for neutrophils.[10]

Several clinical and animal studies in vivo and in vitro implicate the intake and composition of dietary fats in endothelial cell adhesion molecule expression.[11-13] Moreover, there is strong evidence that lipids, including selected fatty acids, can cause endothelial dysfunction or injury.[14] It also has been proposed that the hydrolysis of triglyceride-rich lipoproteins can contribute a significant amount of free fatty acids in proximity to the vascular endothelium.[15] Recent evidence suggests that linoleic acid (18:2, n − 6) may play a critical role in the pathogenesis of atherosclerosis.[14] An increase in 18:2 levels has been reported in the phospholipid fractions of human coronary arteries in cases of sudden cardiac death due to ischemic heart disease.[16] Additionally, concentrations of 18:2 in adipose tissue were positively correlated with the degree of coronary artery disease.[17] With in vitro experiments, 18:2 and its oxidative derivatives have been shown to disrupt endothelial barrier function as determined by increased endothelial permeability to albumin or lipoprotein remnants.[18-20] Furthermore, recent evidence indicates that 18:2 can activate the oxidative stress–sensitive nuclear factor-κB (NF-κB) and stimulate NF-κB–dependent transcription.[21,22]

Endothelial cells can produce numerous cytokines including IL-1, IL-6, and IL-8 during inflammation, which themselves exert proinflammatory actions.[23,24] For example, cytokines can transcriptionally activate E-selectin, VCAM-1, and ICAM-1 genes in vascular endothelium. Expression of genes encoding for inflammatory cytokines and for adhesion molecules is regulated by NF-κB. These genes contain sequences in their promoter regions that are recognized by this transcription factor.[25-27]

Because 18:2 is a potent activator of NF-κB,[21,22] it appears that selected fatty acids may play a role in atherogenesis through modulation of the inflammatory response of the vascular endothelium. Therefore, the present study focuses on the hypothesis that 18:2-induced upregulation of adhesion molecules and cytokine levels is involved in the inflammatory stages of endothelial cell dysfunction.

MATERIALS AND METHODS

Human Umbilical Vein Endothelial Cell Cultures

Human umbilical veins were cannulated and washed with Hanks balanced salt solution. Dispase (2 mg/mL medium 199 [M199]) was infused into the veins and incubated at 4°C for up to 18 hours. The dispase and endothelial suspension was flushed from the veins three times using Hanks solution and then centrifuged for 15 minutes at 250 × *g* at room temperature. The cell pellet was resuspended in enriched M199 culture medium, which included 20% heat-inactivated fetal calf serum, 1% each of penicillin/streptomycin, glutamine, and antibiotic-antimycotic, heparin (300 μg/mL; Gibco BRL, Grand Island,

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NY), HEPES (6 mg/mL; Sigma Chemical, St Louis, MO), and endothelial cell growth supplement (40 μg/mL; Collaborative Research, Bedford, MA). Human umbilical vein endothelial cells (HUVECs) were plated and grown until confluent in 5% CO_2 at 37°C. Cells were determined to be endothelial in origin by their cobblestone morphology and uptake of fluorescent labeled acetylated low-density lipoprotein (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate; Molecular Probes, Eugene, OR). Cells from passage 2 were used in the present study. For experimental studies, HUVECs were cultured in 20% fetal calf serum and treated with oleic acid (18:1, n − 9), linoleic acid (18:2, n − 6), or linolenic acid (18:3, n − 3) at concentrations of up to 180 μmol/L in 10% serum for 12 or 24 hours. In several experiments, treatment with 18:2 was combined with TNF (20 ng/mL = 100 U/mL) for the remaining 4 hours of the 18:2 enrichment period. Linoleic acid (>99% pure) was obtained from Nu-Chek Prep (Elysian, MN). Preparations of experimental media with 18:2 and/or TNF were made as described previously.[18,22]

Measurement of Intracellular Oxidative State

Oxidative stress in viable cells was measured by 2,7-dichlorofluorescein (DCF) fluorescence. An oxidation-sensitive compound, 2′,7′-dichlorofluorescin diacetate (DCF-DA; Molecular Probes), is converted to a nonfluorescent polar derivative by cellular esterases following incorporation into cells. Dichlorofluorescin is then oxidized to the fluorescent DCF by peroxidase and peroxides, including hydrogen peroxide. DCF fluorescence in 18:2- and/or TNF-treated cell cultures was quantified using imaging methods as described by Goodman and Mattson.[28] Briefly, the cells were loaded with DCF (50 μmol/L in H_2O) during the remaining 50 minutes of the experiment, followed by three washes in Hanks buffer. The relative fluorescence intensity of the cells was assessed by a confocal laser-scanning microscope system consisting of a Nikon Diaphot microscope (Melville, NY) using 488-nm excitation and 510-nm emission filters. The average pixel intensity was measured within each field and expressed in relative units of DCF fluorescence.

Nuclear Extracts and Electrophoretic Mobility Shift Assays

Nuclear protein extracts from HUVECs were prepared according to the method of Dignam et al,[29] and an electrophoretic mobility shift assay (EMSA) was performed using a commercially available kit from Promega (Madison, WI). Nuclear protein (5 μg) was incubated with the ^{32}P-end-labeled 22-basepair oligonucleotide probe containing the NF-κB consensus sequence (underlined) (5′-AGTTGAGGGGACTTTC-CAGGC-3′) at room temperature for 20 minutes. The resulting DNA protein complexes were resolved on a 5% nondenaturing polyacrylamide gel.

Measurement of IκB

HUVECs were grown until confluent on 60-mm plates, and cytosolic extracts were prepared and then electrophoresed as described by Sambrook et al.[30] Briefly, cell monolayers were harvested by scraping, washed in cold phosphate-buffered saline (PBS), and incubated in 200 μL lysis buffer (10 mmol/L HEPES, pH 7.9, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol, 0.5 mmol/L PMSF, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 μg/mL leucine thiol, and 0.1% Nonidet P-40) for 15 minutes on ice. The crude nuclei were then collected by microcentrifugation, and the supernatants (cytosolic extracts) were collected and stored at −70°C. Cytosolic extracts were electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose in 25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol at 370 mA for 1.5 hours at 4°C. Western blots were analyzed for IκB-α using a primary antibody, rabbit polyclonal anti-human IκB-α (isotype IgG; Biotechnology, Santa Cruz, CA) (1:500) in blocking buffer. Immunoreactive proteins were detected according to the enhanced chemiluminescence protocol (Western blot kit; Schleicher and Schuell, Keene, NH) using 1:4,000 alkaline phosphatase–linked goat anti-rabbit secondary antibody. Blots were exposed to film for 2 hours.

ICAM-1 Determination

ICAM-1 levels were determined by flow cytometry. Briefly, HUVECs were grown to confluence on six-well tissue culture plates and treated with 18:2 and/or TNF for a predetermined time. HUVECs were then washed with Hanks buffer and harvested by trypsin/EDTA. Endothelial cells (5×10^5) were incubated on ice with saturating amounts of monoclonal mouse anti-human CD54-FITC (ICAM-1; Immunotech, Westbrook, ME) or IgG1 isotype control (Becton Dickinson Immunocytometry System, San Jose, CA). Samples were washed twice with ice-cold PBS, fixed in 4% paraformaldehyde to prevent homotypic aggregation, and analyzed with 10,000 cells per sample in a fluorescence-activated cell sorter (Becton Dickinson). Following correc-

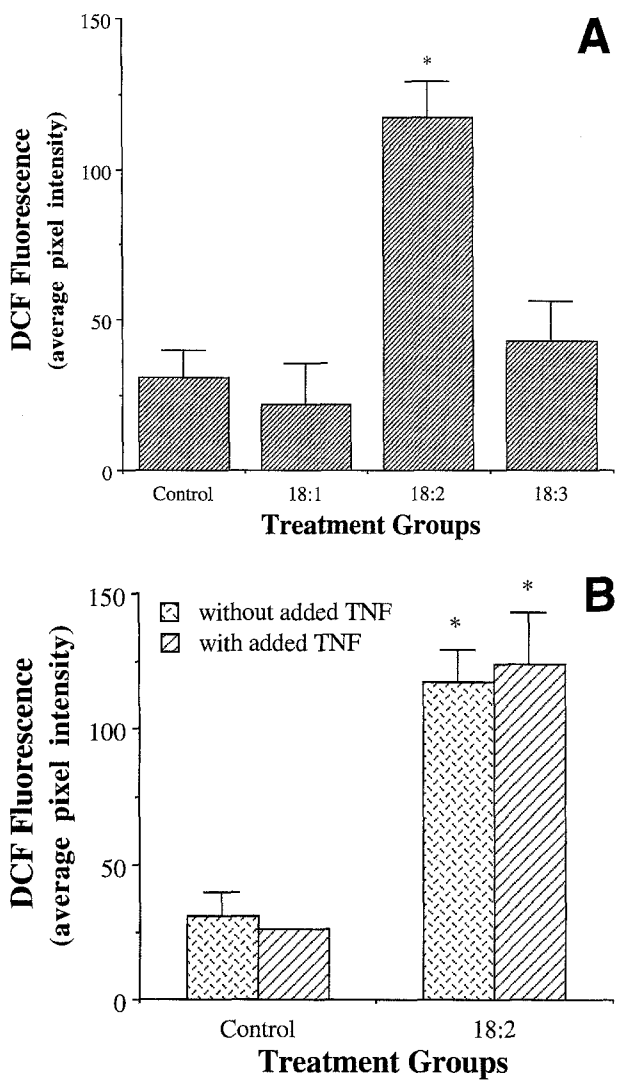

Fig 1. (A) Effect of 18:1, 18:2, or 18:3 on cellular oxidation as measured by DCF fluorescence. (B) Effect of 18:2 and/or TNF on cellular oxidation as measured by DCF fluorescence. HUVECs were exposed to 18:2 (180 μmol/L) for 12 hours, with added exposure to TNF (20 ng/mL) for the remaining 4 hours of the enrichment period. Values are the mean ± SEM. *$P < .001$ *v* control.

tion for unspecific binding (isotype control), specific mean fluorescence intensity was expressed.

IL-8 Determination

IL-8 production by HUVECs was measured by a commercially available enzyme-linked immunosorbent assay method (R&D Systems, Minneapolis, MN). Following the various treatments, media from HUVEC cultures were collected and used to measure the IL-8 concentration as described by the manufacturer.

Protein Determination

Protein content was determined by modifying the Lowry method for lipid-containing samples as described by Lees and Paxman.[31]

Statistical Analysis

Data were analyzed statistically by ANOVA. One-way or multiway ANOVA were used to compare the mean response among treatments. For each end point, the treatment means were compared in pairs using Fisher's least-significant difference procedure.[32] A statistical probability value of P less than or equal to .05 was considered significant.

RESULTS

Oxidative Stress in Cells Treated With Individual Fatty Acids and With 18:2 and/or TNF

Figure 1A shows the effects of 18:1, 18:2, and 18:3 on cellular oxidation as measured by DCF fluorescence. Only exposure to 18:2 resulted in increased oxidative stress under the current experimental settings. Therefore, 18:2 was selected to further study fatty acid–mediated effects on inflammatory mediators in cultured endothelial cells.

Figure 1B shows the effects of 18:2 and/or TNF on DCF fluorescence. HUVECs were enriched with 18:2 for 12 hours, including TNF exposure for the latter 4 hours. TNF exposure for 4 hours alone or in combination with 18:2 did not contribute to further oxidative stress compared with control cultures or 18:2-enriched cells.

NF-κB and IκB Studies

In HUVECs enriched with 18:2, NF-κB DNA binding was slightly activated over control levels (Fig 2). Furthermore, enrichment with 18:2 attenuated TNF-mediated NF-κB DNA binding.

IκB-α specifically binds to the Re1A subunit of NF-κB, thereby sequestering this transcription factor in the cytoplasm and rendering it transcriptionally inactive. Consequently, the presence of IκB-α in the cytoplasm can be indicative of inactivation of NF-κB. In contrast, decreased levels of IκB-α may suggest activation and nuclear translocation of NF-κB. Figure 3A and B shows that IκB-α markedly fluctuated depending on the time of exposure to TNF. In cells pretreated with 18:2 for 12 hours, 15 minutes of exposure to TNF markedly decreased IκB-α levels in TNF- and 18:2+TNF-treated cells. In 18:2+TNF-treated cells, IκB-α recovered more rapidly than with TNF exposure alone at 30 minutes.

IL-8 Levels

Figure 4 shows IL-8 levels in media from HUVEC cultures treated with 18:2 with or without stimulation by TNF. Enrichment with 18:2 or TNF significantly increased IL-8 production compared with control cultures. However, exposure to combined treatments did not affect the already induced TNF-

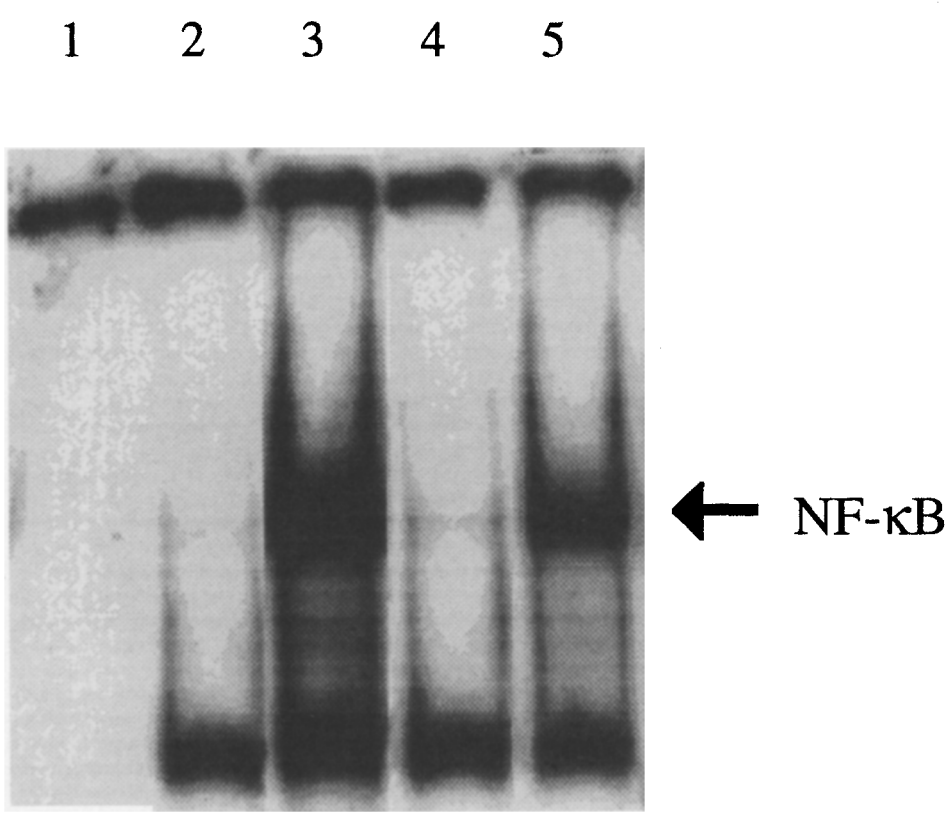

Fig 2. Effect of 18:2 and/or TNF on NF-κB activation. HUVECs were exposed to 18:2 (180 μmol/L) for 12 hours, with added exposure to TNF (20 ng/mL) for the remaining 4 hours of the enrichment period. Lane 1, probe; lane 2, control; lane 3, TNF; lane 4, 18:2; lane 5, 18:2+TNF.

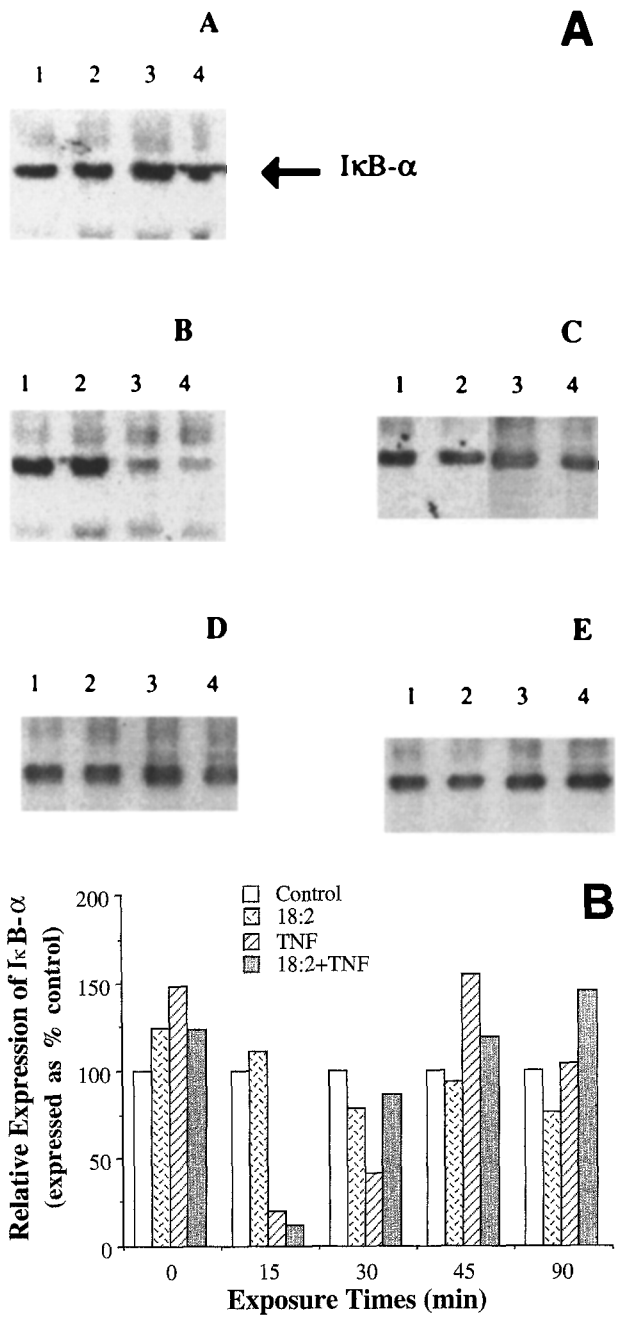

Fig 3. (A) Effect of 18:2 and/or TNF on IκB-α levels. HUVECs were enriched with 18:2 (180 μmol/L) for 12 hours, with added exposure to TNF for various times at the end of the 18:2 enrichment period. TNF exposure ranged from 0 to 90 minutes. Lane 1, control; lane 2, 18:2 (180 μmol/L); lane 3, TNF (20 ng/mL); lane 4, 18:2+TNF. TNF exposure: A, 0 minutes; B, 15 minutes; C, 30 minutes; D, 45 minutes; E, 90 minutes. (B) Scanning densitometry of Western blots from (A). HUVECs were enriched with 18:2 (180 μmol/L) for 12 hours, with added exposure to TNF (20 ng/mL) for various times at the end of the 18:2 enrichment period. TNF exposure ranged from 0 to 90 minutes.

mediated IL-8 production. IL-8 levels significantly decreased by 24 hours following 18:2 enrichment alone or in joint exposure to TNF compared with the effect of these treatments at 12 hours.

ICAM-1 Expression

ICAM-1 is constitutively expressed on endothelial cells, as seen in untreated (control) cultures. However, expression of this adhesion molecule on HUVECs was upregulated as a result of cellular enrichment with 18:2 for 24 hours (Fig 5A) and especially with TNF (Fig 5B). Combined exposure to 18:2+TNF for 12 hours resulted in increased ICAM-1 levels as compared with 18:2 or TNF alone. However, treatment with 18:2+TNF for 24 hours decreased ICAM-1 expression on HUVECs compared with individual treatment with 18:2 or TNF.

DISCUSSION

Much evidence supports the role of a dysfunctional endothelium in the pathogenesis of atherosclerosis.[33,34] Furthermore, lipids have been implicated in the development or exacerbation of the chronic inflammatory environment that defines this disease. The vascular endothelium can be exposed to significant levels of free fatty acids derived from lipoprotein lipase–mediated hydrolysis of triglyceride-rich lipoproteins. Such fatty acid levels may greatly exceed the plasma concentration of free fatty acids, which can range from 90 to 1,200 μmol/L but can be as high as 2,500 μmol/L under conditions that impose physiological stress such as strenuous exercise, fasting, and diabetes. Since the fatty acid composition of cellular membrane and plasma lipids reflects that of dietary lipids,[35] insight into the relationship between fatty acids and endothelial cell reactivity may contribute to a greater understanding of dietary lipids and the inflammatory events leading to atherosclerosis. We have compared several 18-carbon fatty acids and found that linoleic acid most markedly disrupted the endothelial barrier function (reviewed in Hennig et al[14]). Furthermore, linolenic acid had no effect on endothelial barrier function,[36] even though this fatty acid is more unsaturated than linoleic acid. In fact, when

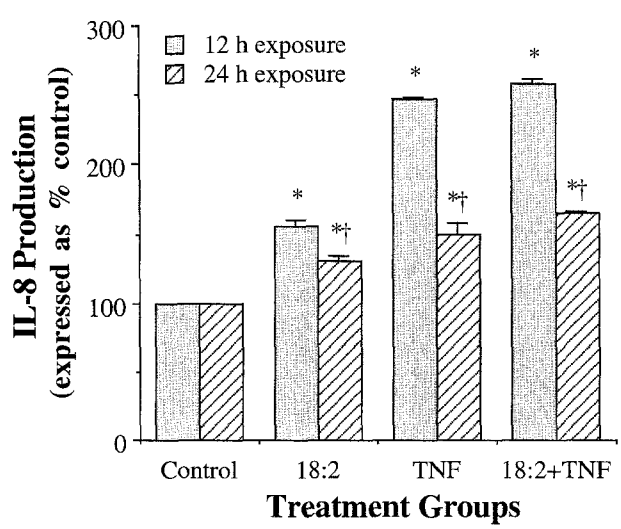

Fig 4. Effects of 18:2 and/or TNF on IL-8 production. HUVECs were enriched with 18:2 for 12 or 24 hours, with added TNF for the remaining 4 hours of the enrichment period. Values are the mean ± SEM; n = 3. *Significantly different from control; †significance in treatment groups at 24 hours *v* corresponding treatments at 12 hours.

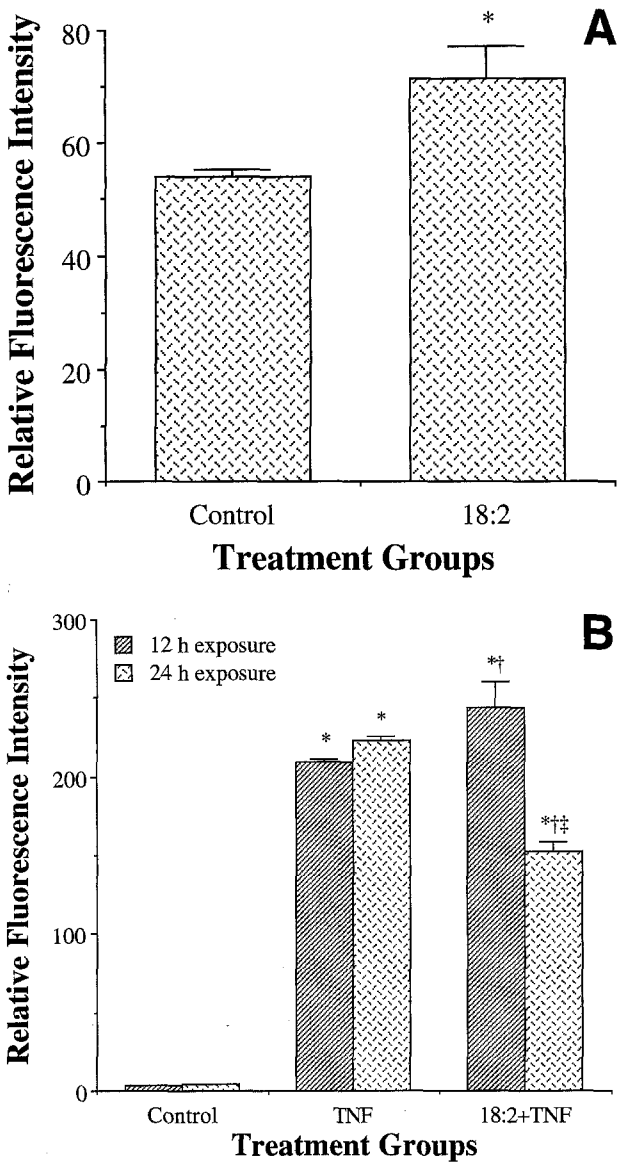

Fig 5. (A) Effect of 18:2 on ICAM-1 expression in HUVECs. Cells were enriched with 18:2 for 24 hours. HUVECs were stained for ICAM-1 or isotype control and analyzed by flow cytometry. Data are expressed as mean fluorescence intensity and corrected for unspecific binding. (B) Effects of TNF or 18:2+TNF on ICAM-1 expression in HUVECs. Cells were enriched with 18:2 for 12 or 24 hours, with added TNF for the remaining 4 hours of the enrichment period. Cells were stained for ICAM-1 or isotype control and analyzed by flow cytometry. Data are expressed as relative fluorescence intensity and corrected for unspecific binding. Values are the mean ± SEM; n = 3. *Significantly different from control; †significant difference for 18:2+TNF group *v* TNF group; ‡significant difference for 18:2+TNF at 12 hours *v* 24 hours.

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.[37] For these and other reasons, the present study focused on linoleic acid as a potential mediator of inflammatory mediators.

Recent evidence suggests that linoleic acid (18:2, n − 6) may play a critical role in the pathogenesis of atherosclerosis.[16,17,38] In our study, enrichment with 18:2 markedly increased oxidative stress as determined by DCF. This observation is in agreement with our previously reported data.[22] The effects of 18:2 on cellular oxidation may be due to its oxidizability, as well as its cellular accumulation. 18:2 supplementation of endothelial cells has been shown to induce oxidative stress at 6 hours but not at 24 hours of fatty acid exposure,[22] indicating that 18:2-mediated oxidation is related to cellular levels of antioxidants. To support this hypothesis, a maximum depletion of glutathione (GSH) was seen at 6 hours after fatty acid treatment, followed by a significant increase in GSH synthesis.[39] In the present study, all measurements (including oxidative stress) were performed 12 hours after experimental treatments. We reported previously that elevated oxidative stress was mediated by TNF and 18:2 at 1.5 and 6 hours, respectively. This, then, may explain why at 12 hours additional TNF did not further enhance cellular oxidative stress mediated by 18:2. However, the combined exposure of 18:2+TNF already caused a significant elevation in cellular cytokine production and adhesion molecule expression at 12 hours, suggesting that an amplified oxidative stress signal occurred much earlier.

There is ample evidence indicating that increased oxidative stress results in the activation of NF-κB important for the inducible expression of numerous cellular genes including cytokines, cytokine receptors, and adhesion molecules.[40-42] Recent evidence has demonstrated the presence of activated NF-κB,[43] as well as IL-8 and ICAM-1,[8,44-47] in human atherosclerotic lesions. In cell culture model systems, we found that 18:2 can be a potent activator of NF-κB and that 18:2-mediated NF-κB DNA binding activity can induce gene expression, as measured by chloramphenicol acetyltransferase assay of an NF-κB–responsive promoter construct.[21,22] In the present study, we provide evidence that endothelial cell treatment with 18:2 can induce production of IL-8 and ICAM-1, which is regulated by NF-κB.

Both IL-8 and ICAM-1 are important mediators of the inflammatory process. For example, neutrophil migration across HUVEC monolayers was shown to be partly blocked by a monoclonal antibody against IL-8,[48] and both adherence and migration were inhibited by anti–ICAM-1 antibody.[5,49] In addition, IL-8 might have a direct injury effect on endothelial cells. It was demonstrated that IL-8 increased endothelial (HUVEC) permeability.[50] Thus, it is possible that 18:2 and its mediated production of IL-8 may cause massive damage to the endothelium by participating in the inflammatory process and atherogenesis.

Lipids may not only induce direct injury effects in the vascular endothelium but may also modulate the biological effects of inflammatory cytokines such as TNF.[22] Recent evidence also implicates lipid mediators and lipid peroxidation products in the regulation of nuclear transcription factors involved in cytokine gene expression.[38,51] Therefore, in some experiments, HUVECs were exposed to TNF and 18:2+TNF. Although both 18:2 and TNF activated NF-κB in cultured HUVECs, combined treatment with 18:2+TNF attenuated the increased activity of this transcription factor observed as a

result of the individual treatments. It is not clear if attenuation of NF-κB activity by 18:2+TNF is due to a maximal response occurring at an earlier time point compared with 18:2 or TNF treatment alone, or to a downregulation of oxidative stress–sensitive genes by the early exposure to 18:2.

The effect of combined exposure to 18:2+TNF on the induction of oxidative stress and expression of IL-8 or ICAM-1 may be explained by specific autoregulatory mechanisms of NF-κB activation.[52] NF-κB is sequestered in the cytoplasm as an inactive complex by inhibitory molecules known as IκBs. Studies have indicated that IκB-α regulates NF-κB through a novel autoregulatory loop.[53-55] Induction of NF-κB results in phosphorylation of the IκB-α subunit and its subsequent proteasomal degradation. However, the activated NF-κB translocated to the nucleus can upregulate IκB-α mRNA due to NF-κB sites in the IκB-α promoter. The newly synthesized IκB-α is translated, and its accumulation in the cytosol once more binds the NF-κB, inactivating it and downregulating the transcription of κB-dependent genes. The present data suggest that early activation of NF-κB by 18:2 alone with a subsequent additional challenge by TNF may have evoked a subsequent autoregulatory step to protect the cells from NF-κB–activated transcription of cytokine, adhesion molecule, and a host of other genes that play a putative role in chronic inflammation. Thus, this autoregulatory loop is likely an inducible protective mechanism by endothelial cells against further insult.

Since activation of IκB reflects an inactive cytosol-sequestered NF-κB, IκB-α was studied following increasing time exposure to TNF alone or in combination with 18:2. IκB-α is a known target of TNF, resulting in a transient activation of NF-κB.[56] The present results clearly confirmed an IκB-α transient pattern of activation induced by TNF. IκB-α was considerably decreased in HUVECs following a short exposure to TNF alone for 15 minutes or in combination following enrichment with 18:2. However, the recovery of IκB-α in 18:2+TNF-treated cells was increased by 48% as compared with TNF alone for 30 minutes. By 90 minutes of TNF exposure, IκB-α levels in the 18:2+TNF group remained slightly higher than the control levels; however, IκB-α levels remained decreased in cultures exposed to TNF alone. The more rapid recovery of IκB-α observed in cells exposed to TNF following 18:2 enrichment may explain the observed downregulation of the TNF-mediated increase of NF-κB at a later time point. In addition, the regulatory mechanisms of NF-κB/IκB-α activation could have influenced the production of IL-8 or ICAM-1 in cells exposed to 18:2+TNF as compared with individual treatment with 18:2 or TNF.

Although both IL-8 and ICAM are involved in leukocyte recruitment and their production is regulated by NF-κB, a direct relationship between these two inflammatory mediators is unclear. For example, it has been reported that IL-8 expression is not dependent on the early-response cytokines (IL-1 and TNF) or expression of ICAM-1.[57] However, in patients with sepsis, Nakae et al[58] reported that soluble ICAM-1 released from the membrane into the circulation significantly correlated with the levels of endotoxin, TNF, and IL-8. In the present study, IL-8 and ICAM-1 were both increased at 12 hours of 18:2 and/or TNF treatment. However, medium IL-8 levels decreased at 24 hours independently of treatment. In contrast, such an effect was not observed with ICAM-1, except in combined treatment with 18:2+TNF. In addition, in comparison to treatment with TNF alone, the combined exposure to 18:2+TNF decreased ICAM-1 expression but did not affect medium IL-8 levels. These results indicate that both IL-8 and ICAM-1 are upregulated and coexpressed under proinflammatory conditions. However, their expression might be regulated by separate mechanisms, partially related to the induction of oxidative stress and NF-κB activation. These processes may have significant implications in the combined involvement of lipids and inflammatory mediators in atherogenesis.

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