Linoleic Acid-Stimulated Vascular Adhesion Molecule-1 Expression in Endothelial Cells Depends on Nuclear Factor-κB Activation

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Endothelial activation is an important step in atherogenesis. In addition to established cardiovascular risk factors, such as hypercholesterolemia, hypertension, diabetes mellitus, and homocysteinemia, high plasma levels of triglyceride-rich lipoproteins may be an important cause of endothelial activation as well. Free fatty acids hydrolyzed from core triglycerides of these particles can exert both pro- and anti-inflammatory effects on the vascular wall. ω-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been shown to inhibit cytokine-induced endothelial activation. In contrast, we and others have previously shown that the ω-6 fatty acid linoleate activates transcription factor nuclear factor-κB (NF-κB) in endothelial cells. In this study, we show that linoleic acid stimulates vascular adhesion molecule-1 (VCAM-1) protein and mRNA expression in cultured human endothelial cells, as assessed by immunoflourescence and Northern blotting. Release of shedded soluble VCAM-1 from cultured human endothelial cells was also increased by the addition of linoleic acid, as determined by enzyme-linked immunosorbent assay (ELISA). By use of cultured rat aortic endothelial cells transfected with an IκB super-repressor (ΔN2 cells), we provide evidence that NF-κB signalling is required in the linoleic acid-induced VCAM-1 expression in endothelial cells, whereas other transcription factors appear to be involved in the increased endothelial plasminogen activator inhibitor-1 (PAI-1) production in response to linoleic acid. These findings suggest that diets rich in linoleic acid may be proinflammatory and thus atherogenic by activating vascular endothelial cells.

LIPID COMPONENTS OF the diet have well-established influences on coronary heart disease (CHD). Dietary lipids, in particular, substances formed during the oxidation of low-density lipoprotein (LDL) particles, as well as fatty acids, influence endothelial gene expression by interference with both pro- and anti-inflammatory transcription factors, such as NF-κB, AP-1, and peroxisome proliferator activated receptors (PPARs) (for a review, see Kunsch and Medford[1]).

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 physiologic stress, such as strenous exercise, fasting, and diabetes.[2] There is increasing evidence that monounsaturated and ω-3 fatty acids may protect from atherosclerotic and other diseases.[3,4] In contrast, a diet rich in ω-6 fatty acids may contribute to an increased incidence of atherosclerosis, hyperinsulinemia, and tumorigenesis.[5] Recent evidence from in vivo and in vitro studies suggest that, in particular, linoleic acid (18:2, ω-6), an essential fatty acid in the human diet, may play a critical role in the pathogenesis of atherosclerosis by sustaining subclinical arterial inflammation (for a review, see Toborek and Hennig[6]).

Linoleic acid activates NF-κB in endothelial cells and stimulates endothelial intercellular adhesion molecule-1 (ICAM-1) and interleukin (IL)-8 production.[7-9] Pre-enrichment of cultured endothelial cells with linoleic acid followed by exposure to tumor necrosis factor (TNF)-α doubled IL-6 production compared with TNF-α exposure alone.[10] Cultured smooth muscle cells incubated with linoleic acid strongly upregulate the mRNA expression for the core proteins of the proteoglycans versican, decorin, and syndecan 4, which might have implications for plaque size and stabilization, as well as for the retention of lipoproteins. Darglitazone, a PPARγ ligand, neutralizes the linoleic acid-mediated induction of the decorin gene.[11]

This study was performed to characterize the effects of linoleic acid on endothelial vascular cellular adhesion molecule-1 (VCAM-1) expression. VCAM-1 (CD 106) is believed to play a key role in the initiation of atherogenesis, being the receptor for the mononuclear cell $\alpha_4\beta_1$-integrin and thus mediating monocyte adhesion to atherosclerotic lesions.

MATERIALS AND METHODS

Materials

Linoleic acid bound to bovine serum albumin (BSA) was purchased from Sigma (St Louis, MO) in a ready-made solution at 100 mg/mL, containing 2 mol of linoleic acid/mol of albumin, endotoxin-free, and cell culture tested by the manufacturers. [α-^{32}P]deoxycytidine triphosphate (dCTP), [γ-^{32}P]adenosine triphosphate (ATP), and Rediprime

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labelling system were from Amersham Pharmacia Biotech (Buckinghamshire, UK). Sera and culture media were purchased from Life Technologies (Paisley, Scotland). FALCON culture slides were from Becton Dickinson (Stockholm, Sweden). AP-1 and NF-κB double-stranded consensus oligonucleotides and human recombinant c-Jun protein were purchased from Promega (Madison, WI). Monoclonal fluorescein isothiocyanate (FITC)-conjugated antihuman VCAM-1 antibody, human TNF-α, and antihuman soluble VCAM-1 enzyme-linked immunosorbent assay (ELISA) kit were from R & D Systems (Minneapolis, MN). Supershift antibodies directed against NF-κB1 (p50) and Rel A (p65) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rat plasminogen activator inhibitor-1 (PAI-1) cDNA cloned into the Eco RI site of pBluescript SK (-) (Stratagene, La Jolla, CA) was kindly provided by Drs Zeheb and Gelehrter (Department of Human Genetics, University of Michigan Medical School, Ann Arbor, MI).[12] A cocktail of single-stranded oligonucleotides consisting of an equimolar mix of 6 probes recognizing specific domains of human VCAM-1 were purchased from Ingenius (Novagen, Madison, WI).

Cell Culture

The endothelium-derived cell line EA.hy926 (generously provided by Dr C.-J.S. Edgell, the University of North Carolina, Chapel Hill, NC) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with high glucose, 10% fetal calf serum (FCS), HAT (100 μmol/L hypoxanthine, 0.4 μmol/L aminopterin, and 16 μmol/L thymidine), penicillin, and streptomycin as described.[13] Rat aortic endothelial cells (RAEC) isolated and stably transfected with a non-phosphorylatable IκB construct (IκBα deletion mutant lacking amino acids 1-37) cloned in MRE-pNeo expression vector downstream of a synthetic, Zn-inducible promoter, were provided by Dr Cecilia Giachelli (Department of Bioengineering, University of Washington, Seattle, WA). In this study, subclone ΔN2 was used, which had been shown to lose NF-κB inducibility upon treatment with 100 μmol/L $ZnSO_4$ for 12 hours. Cells were grown in RPMI 1640 medium containing 10% Zn-depleted FCS and 100 U/mL each of penicillin and streptomycin.[14]

Immunofluorescence

Confluent EA.hy926 cells grown on glass culture slides were incubated with TNF-α (10 ng/mL) or linoleate (100 μmol/L each) for 24 hours. After washing in phosphate-buffered saline (PBS), cells were fixed by acetone for 10 minutes and air-dried. Cells were incubated with a FITC-labeled VCAM-1 antibody according to manufacter's instructions (dilution 1:5, 2 hours at room temperature). The cells were washed twice in PBS again before being observed using a fluorescence microscope.

Isolation and Analysis of VCAM-1 and PAI-1 mRNA Expression by Northern Blotting

Confluent 100-mm plates of EA.hy926 or ΔN2 RAECs were incubated with TNF-α (10 ng/mL) or linoleate (100 μmol/L) for 8 hours. Concerning the experiments with ΔN2 RAECs, half of the dishes had been pretreated with 100 μmol/L $ZnSO_4$ for 12 hours, which has been shown to eliminate NF-κB DNA binding activity.[13] Total RNA was isolated from the cells according to the RNeasy handbook (Qiagen, Hilden, Germany). Northern blotting and hybridization on DuPont GeneScreen Plus nylon membranes were performed using ExpressHyb Hybrization solution (Clontech, Palo Alto, CA) according to the manufacturer's protocol. A total of 100 ng of single-stranded oligonucleotides recognizing human VCAM-1 was labeled with T4 kinase and [γ-^{32}P]ATP and purified using Pharmacia NICK columns. A total of 25 ng of cDNA probe for rat PAI-1 was labeled with [α-^{32}P]dCTP using the Rediprime random prime labelling system. Blots were hybridized with 10^6 dpm of probe/mL of hybridization solution.

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared essentially as described.[7] All buffers were kept on ice unless stated otherwise. 2-Mercaptoethanol (5 mmol/L) and the protease inhibitors leupeptin (0.7 μg/mL), aprotinin (16.7 μg/mL), and phenylmethyl sulfonyl fluoride (PMSF) (0.5 mmol/L) were added to all buffers just before use. A total of 3 μg of protein from nuclear extracts was incubated on ice with 2 μg poly(dI-dC) and 1 μg acetylated bovine serum albumin (BSA) in binding buffer (giving the final concentrations stated below) for 10 minutes. The oligonucleotide probe (50,000 cpm in 5 μL) was added, and the reaction mixture (25 μL) was incubated for 25 minutes at room temperature. Final concentrations in binding reactions were as follows: 10% glycerol, 10 mmol/L Hepes (pH 7.9), 60 mmol/L KCl, 5 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, 1 mmol/L dithiothreitol (DTT), and 1 mmol/L PMSF. DNA-protein complexes were separated from unbound DNA probe on native 7% polyacrylamide gels (acrylamide:bisacrylamide (wt/wt) 80:1) in low ionic strength buffer (22.5 mmol/L Tris, 22.5 mmol/L borate, 0.5 mmol/L EDTA, pH 8) by electrophoresis at 200 V for 2 hours. The sequences of the double-stranded oligonucleotide probes (labeled with T4 kinase and [γ-^{32}P]ATP and purified using Pharmacia NICK columns) were as follows: NF-κB 5′-AGT TGA GGG GAC TTT CCC AGG C -3′; AP-1 5′- CGC TTG ATG AGT

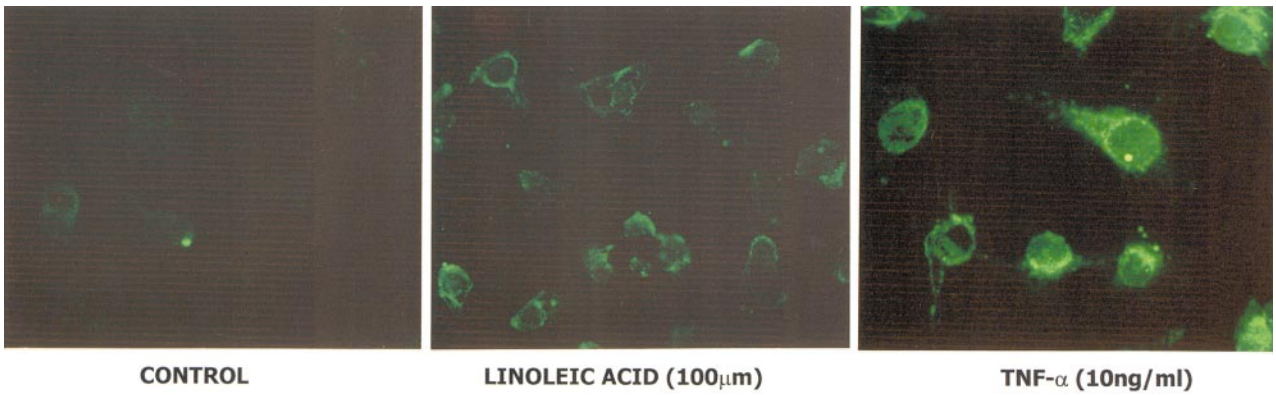

Fig 1. Immunofluorescence staining detecting increased VCAM-1 expression on human cultured endothelial cells (Ea.hyb926) 24 hours after incubation with linoleic acid (100 μmol/L) (B) or TNF-α (10 ng/mL) (C) over untreated control cells (A).

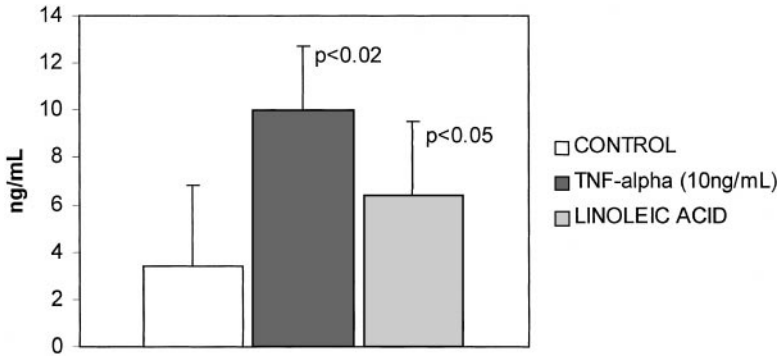

Fig 2. Stimulated release of soluble VCAM-1 from cultured human cultured endothelial cells (Ea.hyb926) after an incubation with linoleic acid (100 μmol/L) or TNF-α (10 ng/mL) for 24 hours. Mean values ± SD were as follows: control cells: 3.4 (± 3.4) ng/mL, n = 12; TNF-α–treated cells: 10.0 (± 2.7) ng/mL, n = 12, *P* < .02 as compared with control; linoleic acid-treated cells: 6.4 (± 3.1) ng/mL, n = 7, *P* < .05 as compared with control. Differences in continuous variables between the groups were tested by ANOVA and post hoc Tukey-HSD test.

CAG CCG GAA -3′. Unlabeled competitor oligonucleotides were added in 50× excess to confirm the specificity of the binding reactions.

Reverse Transcriptase-Polymerase Chain Reaction

For the cDNA synthesis, 2 μg of total RNA was added to 48 μL reagent mixture (10 mmol/L DTT, 0.5 mmol/L dNTPs, 1 U/μL RNAse I, and 10 U/μL M-MLV reverse transcriptase) and reverse-transcribed at 42°C for 50 minutes by random-priming method using Pd(N)6 hexamers. cDNA synthesis was performed using a Perkin-Elmer (Boston, MA) DNA thermal cycler model 9600 at an intial temperature of 30°C for 10 minutes, 42°C for 50 minutes, and 94°C for 2 minutes. The VCAM-1 oligonucleotide primers were designed from the DNA sequence of rat VCAM-1 (5′- TGT GAA GAT GGT CGC GAT C -3′; 5′- CGT CAG TGT GGA TGT AGC C -3′). β-Actin primers were from the rat β-actin control amplimer set from Clontech. PCR amplification was performed by adding 2 μL of cDNA with 50 μL of reagent mixture (0.2 mmol/L of 4 dNTP, 2 U Taq polymerase, and 0.4 μmol/L of each primer) and amplified at a 30-step cycle program (94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 2 minutes). A 15-μL aliquot of each reaction was electrophoresed on a 2% agarose gel, and the bands were analyzed by ethidium bromide staining.

Statistical Analysis

After testing for normal distribution, differences in continous variables between 2 treatment groups (see Fig 3) were tested by using an unpaired Student's *t* test, whereas differences between 3 treatment groups (see Fig 2) were analyzed by analysis of variance (ANOVA) and post hoc Tukey-HSD test. Calculations were performed using SPSS software (version 7.5 for Windows, Chicago, IL). A *P* value of less than .05 was considered to be significant.

RESULTS

Linoleic Acid Stimulates VCAM-1 Expression in Cultured Human Endothelial Cells

The effects of linoleic acid were first studied in cultured human endothelial cells (Ea.hy926 cells). As assessed by immunofluorescence, incubation with 100 μmol/L of linoleic acid for 24 hours increased VCAM-1 protein surface expression over untreated control cells (Fig 1A and B). Incubation with TNF-α was used as a positive control in the same experimental settings (Fig 1C). Incubation with 100 μmol/L linoleic acid also increased the release of shedded soluble VCAM-1, as measured by a quantitative ELISA kit (Fig 2). As assessed by Northern blotting (Fig 3), incubation with 100 μmol/L linoleic acid for 8 hours increased VCAM-1 mRNA expression over untreated control cells. All cells were confluent and had been serum-starved at 1% FCS for 24 hours prior to the start of the incubation time.

Blocking NF-κB Activation Decreases Linoleic Acid-Mediated Stimulation of VCAM-1 mRNA Expression in Cultured RAEC, But Has Little Effect on PAI-1 Expression

Linoleic acid (18:2) activates transcription factor NF-κB in endothelial cells,[8-10] but the potential role of this phenomenon in atherogenesis is less well understood. To study whether the linoleic acid-induced VCAM-1 upregulation in endothelial cells was dependent on NF-κB activation, we used cultured RAEC (ΔN2) in which the NF-κB signalling pathway can be blocked by inducing the transcription of an IκB super-repressor. The addition of zinc-sulfate for 12 hours resulted in the expression of a mutant IκB-α protein, strongly decreasing the

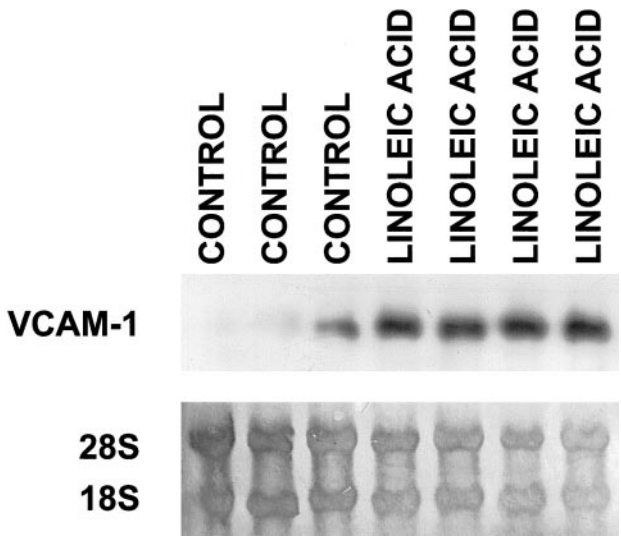

Fig 3. Northern blot showing the effects of linoleic acid (100 μmol/L) on VCAM-1 mRNA expression in human cultured endothelial cells (Ea.hyb926). Linoleic acid increased VCAM-1 mRNA significantly over controls. Densitometric analysis was performed using the Kodak Digital Science 1D Image Analysis Software (Eastman Kodak, Rochester, NY) (mean values expressed in arbitrary units ± SD): control, 61,274.3 (± 15,141.8); linoleic acid, 93,329 (± 13,525.5, *P* = .03). Differences in continous variables between the 2 groups were tested by using an unpaired Student's *t* test.

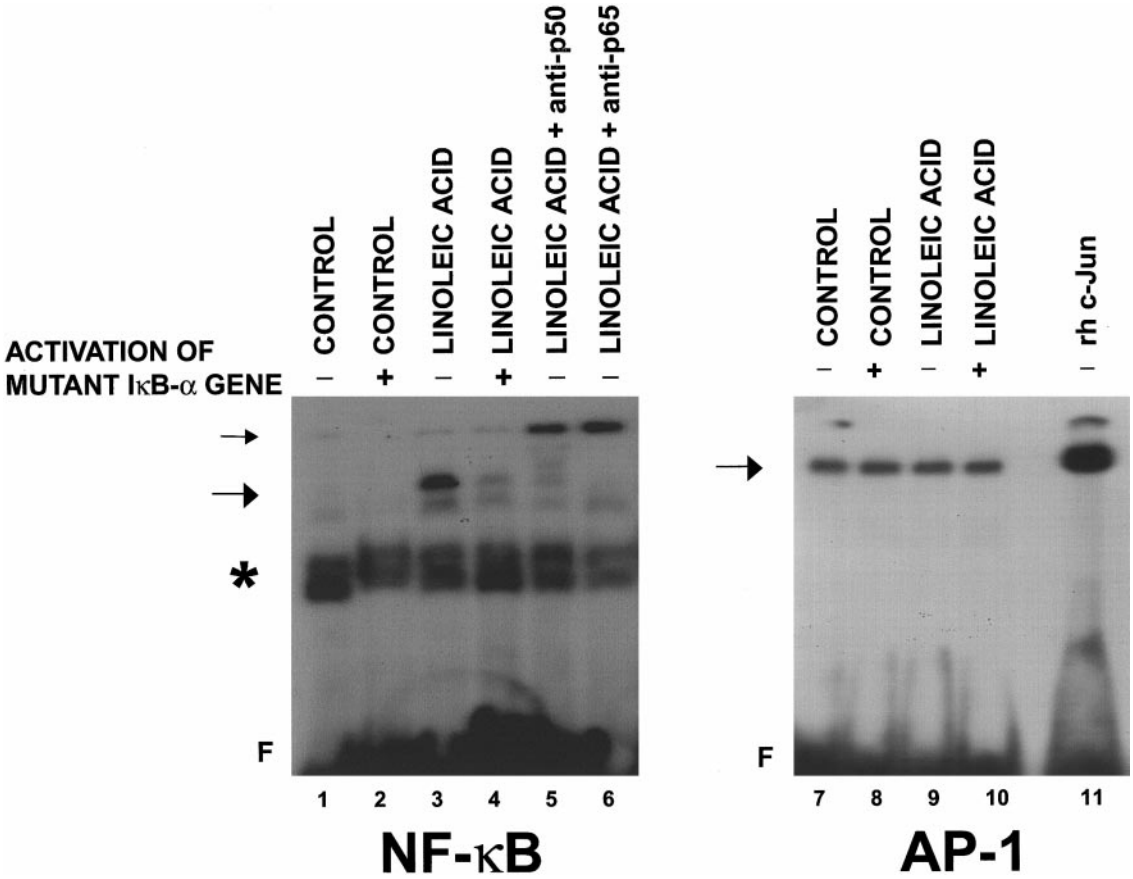

Fig 4. Effects of linoleic acid on transcription factors NF-κB and AP-1 in cultured RAEC (ΔN2), as assessed by electrophoretic mobility shift assay (EMSA). Autoradiographs showing that activation of a stable transfected mutant IκB-α gene (lacking serine residues 32 and 36, which prevents from phosphorylation and further degradation) markedly reduced NF-κB activition due to linoleic acid (lanes 3 and 4). As assessed by supershift analysis, the linoleic acid-induced NF-κB complex (large arrow) was mainly composed of the subunits NF-κB1 (p50) and RelA (p65) (lanes 5 and 6, small arrow, respectively). AP-1 DNA binding activity was found in all cells regardless of the addition of linoleate or the activation of the stable transfected mutant IκB- α gene. * Denotes unspecific DNA binding. F denotes free probe.

NF-κB activation due to linoleic acid (Fig 4, lanes 3 and 4), whereas AP-1 levels were unaffected (Fig 4, lanes 7 to 10). Reverse transcriptase-polymerase chain reaction (RT-PCR) showed that linoleic acid induced VCAM-1 mRNA in ΔN2 cells as well, and that this was dependent on the NF-κB pathway (Fig 5, lanes 1 to 8). Notably, increased PAI-1 mRNA expression induced by linoleic acid did not depend on NF-κB signalling (Fig 5, lanes 11 and 12).

DISCUSSION

In this study, we show that linoleic acid stimulates cultured endothelial cells to express VCAM-1. This upregulation depends on activation of the transcription factor NF-κB, a key regulator of the expression of inflammatory genes, such as cytokines, chemokines, and adhesion molecules.

Leukocyte and endothelial cell adhesion molecules are essential for emigration and extravasation of leukocytes through the endothelium into the arterial wall, an early step in atherogenesis. This process involves interactions between an array of cellular adhesion molecules, including the selectins (E-, P-, and L-selectin) and members of the immunoglobulin superfamily (eg, ICAM-1 and VCAM-1). The endothelial expression of VCAM-1 is an early manifestation of experimental cholesterol-induced atherosclerosis.[15,16] In addition, high levels of VCAM-1 are also detected in advanced human atherosclerotic plaques.[17,18] The observations made in this study suggest that other lipids besides cholesterol may also cause an upregulation of endothelial VCAM-1 expression.

Atherosclerosis is now widely regarded as an inflammatory disease. This study further supports the notion that transcription factor NF-κB plays an important role in the initiation and progression of a chronic arterial inflammation ultimately leading to atherosclerosis. Selected fatty acids hydrolyzed from triglyceride-rich lipoproteins by lipoprotein lipase may influence this inflammatory activity in the vascular wall. Saturated fatty acids appear to be more inert in terms of influencing vascular gene transcription, but their intake is positively correlated to plasma LDL cholesterol levels. ω-3 fatty acids, such as EPA and DHA, are regarded to protect from cytokine-induced endothelial dysfunction. DHA, but not EPA, attenuates induction of VCAM-1 in endothelial cells.[19,20] However, in vivo studies testing the effects of supplementation with ω-3

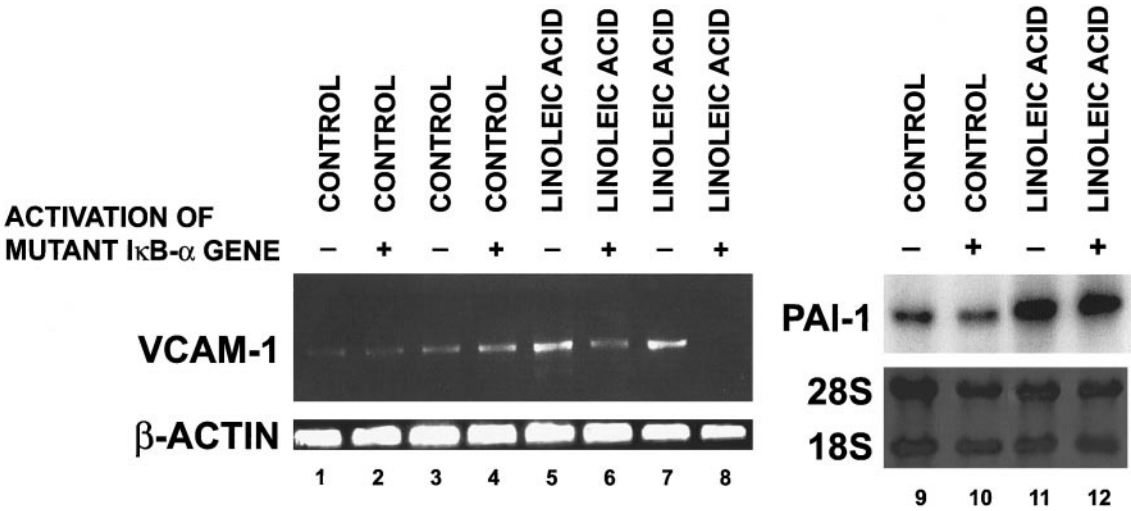

Fig 5. Upregulation of endothelial gene transcription by linoleic acid involves both NF-κB–dependent and indepenpent signalling pathways. As assessed by RT-PCR, linoleic acid (100 μmol/L) stimulated VCAM-1 mRNA expression in cultured RAEC. Blockage of NF-κB signalling by activation of a stable transfected, mutant IκB-α gene strongly decreased this proinflammatory effect of linoleic acid. As shown by Northern blotting, linoleate also upregulated PAI-1 mRNA expression in cultured RAEC, but this effect was independent of NF-κB signalling.

fatty acids (omacor) on plasma inflammatory parameters are still inconclusive.[21,22]

Oxidative stress is considered to be involved in the initial stages of atherosclerosis. Polyunsaturated fatty acids (PUFAs) are a potential source of oxidative stress in humans. In fact, data from subjects with varying degrees of coronary atherosclerosis support the hypothesis that high-serum PUFA levels, when insufficiently protected by antioxidants (eg, vitamin E), may indicate a higher risk of atherosclerosis.[23] A diet enriched with linoleic acid increases oxidative stress in vivo and affects nitric oxide metabolism in humans,[24] and similar results are found in cultured endothelial cells.[25] Lipolytic remnant products of triglyceride-rich lipoproteins produced after a meal rich in polyunsaturated fat are more injurious to arterial wall cells than those produced after a meal rich in saturated fat.[26]

As the linoleate-derivate arachidonic acid inhibits NF-κB activation and TNF-α–mediated VCAM-1 expression in cultured endothelial cells,[27,28] enzymes involved in the metabolic fate of linoleic acid are of special interest. Due to a very low-basal activity of endothelial cell elongases and Δ5 and Δ9 desaturases, arachidonic acid is not produced from 18:2 significantly in this cell type.[29] Consequently, 18:2 accumulates within endothelial cells.[29,30] A comparable situation may be found in diabetic patients who have a reduced activity of the insulin-dependent enzyme 5α-desaturase.[31] In fact, the content of linoleic acid within LDL particles and plasma levels of soluble VCAM-1 are markedly elevated in diabetic patients.[32,33]

Epidemiologic data support the notion that specific fatty acids have profound effects on endothelial function. Measurement of fatty acid composition of the total phospholipid fraction of human coronary arteries showed that as compared with controls (mostly traffic accident victims), the coronary arteries from victims of sudden cardiac death had significantly more atherosclerotic lesions, and the percentages of palmitic acid and linoleic acid were significantly higher and the percentages of arachidonic acid and of all the other major PUFAs, both ω-6 and ω-3, were significantly lower.[34] Furthermore, concentration of linoleic acid in adipose tissue was associated with coronary artery disease (CAD) confirmed by angiography, whereas the EPA concentration in platelets was inversely correlated to CAD in men, and the DHA concentration in platelets was inversely associated with CAD for women.[35] High levels of linoleic acid in the adipose tissue were also associated with the development of new atherosclerotic lesions in coronary arteries.[36]

Differences in the content of specific fatty acids within very–low-density lipoprotein (VLDL) particles may also contribute to the conflicting results as to whether or not these particles are directly atherogenic by sustaining vascular inflammation. β-VLDL increase monocyte adhesion to cultured endothelial cells in vitro,[37,38] and we have recently reported that VLDL from hypertriglyceridemic patients (type IV according to the Fredrickson classification) induces endothelial VCAM-1 expression in rat aorta after intravenous injection.[7] Chylomicrons isolated 4 hours after an oral lipid load induce E-selectin and VCAM-1 expression in cultured endothelial cells.[39]

Together with other PUFAs, linoleic acid upregulates PAI-1 expression in cultured hepatocytes[40] and endothelial cells.[41] Our findings support the notion that this effect is not dependent on NF-κB signalling. Instead, unknown transcription factor(s) recognizing a motif adjacent to the 4G/5G polymorphism in the human PAI-1 promoter may be involved.[42] Accordingly, we could not detect any effect on endothelial NF-κB activity by linolenic acid in a previous investigation,[7] whereas this fatty acid had pronounced increasing effects on PAI-1 gene transcription and expression comparable to linoleic acid.[41]

In conclusion, this study further supports the notion that linoleic acid can contribute to endothelial activation by upregu-

lating VCAM-1 expression. Dietary recommendations should take into account that selected PUFAs may influence vascular gene expression, possibly by increased oxidative stress leading to NF-κB activation. Further clinical studies on the associations between intake of different fatty acids, inflammatory parameters, and cardiovascular disease are warranted.

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