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High Expression of Human 15-Lipoxygenase Induces NF-κB-Mediated Expression of Vascular Cell Adhesion Molecule 1, Intercellular Adhesion Molecule 1, and T-Cell Adhesion on Human Endothelial Cells

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ABSTRACT

Expression of 15-lipoxygenase (15-LO) is induced over 100-fold in early fatty streak lesions. 15-LO activity leads to the production of specific lipid hydroperoxides, which can have major effects on the expression of proinflammatory genes involved in atherogenesis. We have used retrovirus-mediated gene transfer to achieve stable high expression of 15-LO in human endothelial ECV304 cells. These cells were used to study the effects of 15-LO on the expression of vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), activation of nuclear factor kappa B (NF-κB), and T-cell adhesion on endothelial cells. NF-κB activation was greatly potentiated by increased 15-LO activity in the stably transduced cells, and both VCAM-1 and ICAM-1 were significantly induced in these cells in response to tumor necrosis factor-α (TNF-α) and phorbol 12-myristate 13-acetate (PMA) stimulation, as studied by flow cytometry. The induction of ICAM-1 was sensitive to antioxidants in a dose-dependent manner. The adherence of Jurkat T cells on the 15-LO-expressing endothelial cells was markedly induced after PMA stimulation. These results indicate that 15-LO activity may be involved in the early pathogenesis of atherosclerosis by inducing VCAM-1 and ICAM-1 expression and by increasing T-cell adhesion on the endothelium. Antiox. Redox Signal. 1, 83–96.

INTRODUCTION

ATHEROSCLEROSIS IS A CHRONIC INFLAMMATORY DISEASE characterized by accumulation of intra- and extracellular lipids, foam cell formation, proliferation of arterial smooth muscle cells, and accumulation of connective tissue components. Among the earliest events in atherogenesis is the increased adherence and migration of monocytes and T lymphocytes into the subendothelial space (Ross, 1993). Oxidized low-density lipoprotein (LDL) seems to be one of the key pathogenetic factors in this process (Ylä-Herttuala, 1998). Several lines of evidence indicate that 15-lipoxygenase (15-LO) activity may be involved in LDL oxidation and

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atherogenesis (Sigal, 1991): 15-LO is expressed in atherosclerotic lesions and its expression is greatly induced in early fatty streak lesions (Ylä-Herttuala *et al.*, 1990, 1991; Hiltunen *et al.*, 1995), specific enzyme activity of 15-LO can be detected in atherosclerotic lesions (Kühn *et al.*, 1994; Kühn *et al.*, 1997), 15-LO can induce LDL oxidation (Sparrow *et al.*, 1988; Parthasarathy *et al.*, 1989; McNally *et al.*, 1990; Rankin *et al.*, 1991; Derian and Lewis, 1992), transfer of human 15-LO cDNA into iliac arteries of moderately hypercholesterolemic rabbits resulted in the appearance of oxidized LDL (Ylä-Herttuala *et al.*, 1995), LO inhibitors can reduce atherosclerosis in cholesterol-fed rabbits (Sendobry *et al.*, 1997; Bocan *et al.*, 1998), 12/15-LO knockout mice have reduced capacity to oxidize LDL (Sun and Funk, 1996), and atherosclerosis is accelerated in 15-LO transgenic mice (Harats *et al.*, 1997). However, apart from the possible role of 15-LO in LDL oxidation, other mechanisms for the proatherogenic role of 15-LO have remained unclear (Ylä-Herttuala, 1998; Sigal, 1991). Also, the role of 15-LO as a proatherogenic enzyme has been challenged (Sparrow and Olszewski, 1992; Shen *et al.*, 1996).

While several studies have addressed the role of 15-LO in the oxidative modification of LDL, very little is known about the role of 15-LO in regulating gene expression of proatherogenic molecules, even though lipid hydroperoxides are implied in the amplification of inflammatory signals (Andalibi *et al.*, 1993) and reactive oxygen species activate several transcription factors, including NF-κB (Andalibi *et al.*, 1993; Schreck *et al.*, 1992; Sen and Packer, 1996). In this study, we have used retrovirus-mediated gene transfer (Gilboa *et al.*, 1986) of human reticulocyte 15-LO cDNA into human endothelial cells to achieve stable high expression of 15-LO. These cells were used to study the role of 15-LO in the modulation of the expression of adhesion molecules. Our results show that the transduced cells express 15-LO mRNA and protein and have high 15-LO enzyme activity. The expression of 15-LO markedly potentiated the activation of NF-κB in response to various stimuli, and induced NF-κB-mediated expression of vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1). The induction was antioxidant sensitive in a dose-dependent manner. Also, the adherence of Jurkat T cells on the 15-LO-expressing cells after phorbol 12-myristate 13-acetate (PMA) stimulation was markedly induced as compared to control cells.

MATERIALS AND METHODS

Materials

The cell lines Ψ2, PA317, ECV304, and Jurkat T clone E6-1 were obtained from American Type Culture Collection (ATCC, Rockville, MD). Dulbecco's modified Eagle's medium (DMEM), medium 199, RPMI 1640, fetal bovine serum (FBS), newborn calf serum, and penicillin-streptomycin were from Gibco (Life Technologies Ltd., Paisley, Scotland, UK), and polyethylene glycol (PEG) 6000 was from Fluka Chemie AG (Buchs, Switzerland). Polybrene, geneticin, phenylmethylsulfonyl fluoride (PMSF), butylated hydroxytoluene (BHT), SDS molecular weight markers, 4-chloro-1-naphthol, linoleic acid, triheptadecanoin, PMA, and H_2O_2 were purchased from Sigma Chemical Co. (St. Louis, MO). Oligo(dT)cellulose was from Boehringer Mannheim (Mannheim, Germany), and [α-^{32}P]dCTP and Hybond-N nylon filters were from Amersham International (Amersham, UK). Prime-a-Gene Labeling System, NF-κB probe, and T4 polynucleotide kinase were obtained from Promega Inc. (Madison, WI). X-ray films were purchased from Fuji Photo Film Co., Ltd. (Japan) and nitrocellulose filters were from Schleicher & Schuell (Dassel, Germany). Polyclonal rabbit anti-human recombinant 15-LO antibody (Sigal *et al.*, 1990) was kindly provided by Dr. Elliott Sigal, and affinity-purified horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Bio-Rad Laboratories (Richmond, CA). TNF-α was from Genentech Inc. (CA), thapsigargin was from Calbiochem (La Jolla, CA), and poly(dI-dC) and NAP-5 columns were from Pharmacia (Stockholm, Sweden). [γ-^{32}P]dATP was from DuPont NEN (Boston, MA), NF-κB mutant oligonucleotide was from Santa Cruz Biotechnology Inc. (CA), and α-lipoate (racemate mixture) was from ASTA Medica (Frankfurt, Germany). Monoclonal VCAM-1 antibody and fluorescein isothiocyanate (FITC)-coupled

monoclonal ICAM-1 antibody were obtained from Immunotech (Cedex, France), FITC-labeled goat anti-mouse IgG monoclonal antibody was from Coulter (Miami, FL), and calcein acetoxymethyl ester (calcein-AM) was from Molecular Probes (Eugene, OR).

Vector construction

The retroviral vector pLLORNL (Benz *et al.*, 1995; Ylä-Herttuala *et al.*, 1995) is derived from the pLDRNL vector described by Miyanohara *et al.* (1988). The human LDL receptor cDNA in the pLDRNL vector was replaced with human reticulocyte 15-LO cDNA (Sigal *et al.*, 1988) digested with *Bgl*II at site 2,245. Thus, this construct does not contain the polyadenylation signal present in the original cDNA clone (Sigal *et al.*, 1988). In the pLLORNL vector, the 15-LO cDNA is expressed from the long terminal repeat (LTR) of Moloney murine leukemia virus (van Beveren *et al.*, 1980), Tn5 neomycin phosphotransferase gene (Beck *et al.*, 1982) is expressed from an internal Rous sarcoma virus (RSV) promoter, and a second Moloney murine leukemia virus LTR provides transcriptional termination sequences. The retroviral vector pLZRNL (Sharkey *et al.*, 1990; Ylä-Herttuala *et al.*, 1995) containing the *Escherichia coli lacZ* gene (Kalnins *et al.*, 1983) coding for β-galactosidase was used as a control vector.

Cell lines and culture techniques

Ecotropic Ψ2 (Mann *et al.*, 1983) and amphotropic PA317 (Miller and Buttimore, 1986) mouse fibroblast packaging cell lines were routinely grown in DMEM supplemented with 5% newborn calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. ECV304 cells were grown in medium 199 supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Growth media were supplemented with 400 μg/ml of the neomycin-analog geneticin for selection and culture of neomycin-resistant Ψ2 and PA317 clones, and with 600 μg/ml geneticin for ECV304 clones.

Transfection and transduction

Transfections and transductions were made as described (Ausubel *et al.*, 1992). Briefly, the ecotropic Ψ2 packaging cell line was first transfected with the retroviral plasmid pLLORNL by the calcium phosphate precipitation method. The virus stock obtained from the growth medium of the transfected Ψ2 cells was filtered and concentrated by PEG precipitation method or by centrifugation. The concentrated virus stock was used to transduce the amphotropic PA317 packaging cell line in the presence of polybrene (8 μg/ml). Stably transduced geneticin-resistant PA317 clones were isolated, expanded, and analyzed for 15-LO mRNA production, protein expression, and enzymatic activity. One of the PA317 15-LO clones was chosen for the production of amphotropic virus. This virus stock was concentrated from the growth medium of the PA317 clone by PEG precipitation and was used for the transduction of ECV304 cells. Geneticin-resistant ECV304 clones were isolated, expanded, and analyzed for 15-LO expression. The same procedure was done with the pLZRNL plasmid. The clones were analyzed for β-galactosidase expression, and were used as controls.

Determination of virus titers and detection of helper virus

Virus titers of the concentrated amphotropic virus stocks were 4–60 $\times 10^4$ cfu/ml as determined using a bioassay on 209F cells. All these stocks were free of helper virus as detected through horizontal spread of drug resistance (Ausubel *et al.*, 1992; Ylä-Herttuala *et al.*, 1995).

mRNA isolation and Northern blotting

Poly(A)$^+$ RNA was isolated from the cells using oligo(dT)-cellulose (Ausubel *et al.*, 1992). Ethanol-precipitated mRNA samples were electrophoresed on 1% agarose/formaldehyde gels and transferred to a nylon membrane. Random-primed ^{32}P-cDNA-probes were synthesized using [α-^{32}P]dCTP and Promega's Prime-a-Gene Labeling System according to manufacturers instructions. Northern blot hybridizations were done according to Sambrook *et al.* (1989), and mRNA signals were detected by autoradiography.

Protein isolation, SDS-PAGE, and Western blotting

A total of 7–20 × 10^6 cells were trypsinized, washed in PBS, and collected by centrifugation. The cell pellets were suspended in 3 ml of 10% sucrose/PBS containing 1 m*M* PMSF and homogenized on ice with tissue homogenizer 2 × 30 sec. Homogenized cells were centrifuged at 10,000 × *g* for 1 hr at +5°C in a Beckman L60 ultracentrifuge using a SW55 rotor (Beckman Instruments, Inc., Palo Alto, CA). The supernatant was analyzed for protein content according to Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard, and stored at −70°C. SDS-PAGE and immunoblotting were done as described (Ylä-Herttuala *et al.*, 1989) with slight modifications. SDS-PAGE was performed using 10% gels in 0.025 *M* Tris/0.19 *M* glycine buffer pH 8.3 containing 0.1% SDS (150 V, 75 min). Before the electrophoresis, the samples were heated for 4 min in a boiling water bath. The sample buffer contained 0.063 *M* Tris-HCl, 2% SDS, 10% glycerol, 10 μ*M* BHT, and 0.001% bromophenol blue pH 6.8. Four micrograms of protein was loaded per lane. Transfer to nitrocellulose membranes for Western blotting was done at 4°C for 2 hr (200 mA). After the transfer, nonspecific binding sites on the membranes were blocked by incubation with 5% nonfat dried milk proteins at 4°C for 3 hr. After blotting and blocking, the membranes were incubated overnight with the polyclonal rabbit anti-human recombinant 15-LO antibody (1:500 dilution) (Sigal *et al.*, 1990) in PBS containing 1% nonfat dried milk proteins and 0.05% Tween-20. The membranes were washed 3 × 15 min with the same buffer without antibody, incubated with horseradish peroxidase-conjugated secondary antibody (1:3000 dilution) at 4°C for 3 hr, and washed 5 × 10 min. 4-Chloro-1-naphthol was used as a color substrate to detect protein bands.

Analysis of hydroxy fatty acids

Cells were trypsinized from confluent 10-cm culture dishes, centrifuged, resuspended in 0.5 ml of PBS, and broken either by mechanical shearing with five strokes in a glass tissue grinder (PA317 cells) or by three freeze-thaw cycles (5 min in dry ice-ethanol, 5 min at 37°C) (ECV304 cells). The broken cell suspensions were incubated at 37°C for 15 min with 50 μ*M* linoleic acid. After incubation, 25 μg of BHT was added to the cell suspensions as an antioxidant and 25 μg of triheptadecanoin as an internal standard. Lipids were extracted, hydrogenated, saponified, esterified, and acetylated, and the hydroxy fatty acids were detected by gas chromatography as described (Nikkari *et al.*, 1995). After lipid extraction, the protein pellet was dissolved in 5% SDS/0.5 *N* NaOH overnight at 37°C, and the amount of protein was measured according to Lowry *et al.* (1951) using BSA as a standard.

Nuclear extraction and electrophoretic mobility shift assay

Cells were activated with either TNF-α (100 ng/ml), PMA (100 n*M*), thapsigargin (1 μ*M*), or H_2O_2 (250 μ*M*) for 2 hr. Nuclear extracts were prepared (Sen *et al.*, 1996a), and electrophoretic mobility shift assays (EMSAs) were performed (Roy *et al.*, 1998) as previously described. The specificity of the NF-κB band was demonstrated by treating the nuclear protein extract with an excess of a cold competitor probe or a cold mutant probe (1 bp altered) for 15 min before incubation with a labeled consensus NF-κB probe (Sen *et al.*, 1996b).

Flow cytometric detection of adhesion molecule expression

Cells were activated with either PMA (100 n*M*) or TNF-α (100 ng/ml) for 24 hr. For another set of experiments, cells were pretreated for 48 hr with different concentrations of α-lipoate. The expression of VCAM-1 and ICAM-1 was analyzed by flow cytometry as described (Roy *et al.*, 1998).

Adhesion of Jurkat T cells to endothelial cells

Jurkat T cells were fluorescently labeled with calcein acetoxymethyl ester (calcein-AM) as described (Roy *et al.*, 1998). ECV304 clones were seeded at a density of 10^4 cells/well in 96-well tissue culture plates. After 24 hr of seeding, the cells were activated with 100 n*M* PMA for 24 hr and the cell–cell adhesion assay was performed as described previously (Roy *et al.*, 1998).

RESULTS

15-LO mRNA expression

The isolated 15-LO clones were shown to contain a proviral transcript of ~5.7 kb on Northern blots when hybridized with a random-primed ^{32}P-labeled 15-LO cDNA probe (Figs. 1A and 2A). None of the control cell lines—a β-galactosidase-producing PA317 clone and untransduced PA317 and ECV304 cells—contained such a transcript, and no endogenous 15-LO expression could be detected. Hybridization with a random-primed cDNA probe for the neomycin phosphotransferase gene resulted in mRNA signals at exactly the same sites, where the 15-LO mRNA signal was detected (Figs. 1B and 2B). This observation confirmed that the whole proviral DNA between the LTRs was transcribed as a full-length transcript, since the 15-LO cDNA insert does not contain a poly(A) signal. In some clones, a smaller mRNA signal of ~2.4 kb was detected when hybridized with the probe for the neomycin phosphotransferase gene (Figs. 1B and 2B). In these clones, the neomycin phosphotransferase gene was transcribed from the internal RSV promoter.

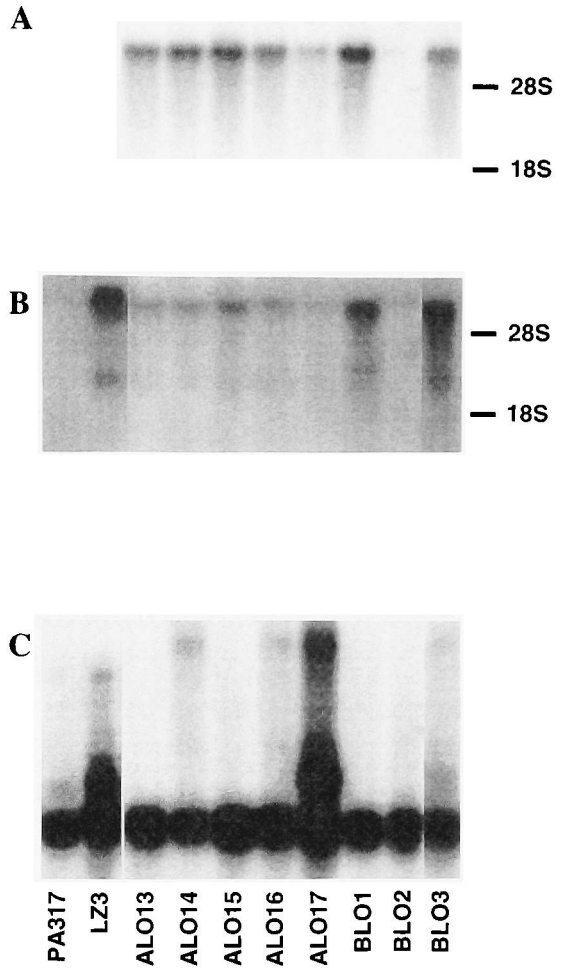

FIG. 1. Expression of 15-LO mRNA in transduced PA317 cells. Northern blot analysis of poly(A)$^+$ RNA samples from PA317 clones. From left: Untransduced control PA317 cells, control lacZ clone LZ3, and 15-LO clones ALO13 to BLO3. Hybridization with random primed cDNA probe for 15-LO (**A**), neomycin phosphotransferase (**B**), and glyceraldehyde-3-phosphate dehydrogenase (gAPDH) (**C**).

15-LO protein expression

Immunoblotting analysis of the PA317 15-LO clones with the polyclonal rabbit anti-human recombinant 15-LO antibody showed the presence of 15-LO protein in the isolated 15-LO clones, whereas 15-LO protein could not be detected in the control cell lines (Fig. 3). No signal was obtained when the primary antibody was omitted (data not shown). The size of the 15-LO protein present in the isolated 15-LO clones was estimated from immunoblots using molecular weight markers. Crude rabbit reticulocyte 15-LO was also included as a control. The size of the crude 15-LO protein and the 15-LO protein in the transduced clones was ~75 kD (Fig. 3), which is in good agreement with previous observations (Sigal *et al.*, 1990; Kühn *et al.*, 1993).

15-LO enzyme activity

To detect 15-LO activity, the isolated virus-producing PA317 clones were incubated with linoleic acid and the production of 13-hydroxy-octadecadienoic acid (13-HODE) was measured by gas chromatography. Untransduced PA317 cells and one isolated PA317 clone producing β-galactosidase were used as controls. As shown in Fig. 4, the production of 13-HODE in the untransduced control clone and the β-galactosidase-producing clone was comparable. The 15-LO clones produced about 10–40 times more 13-HODE than the controls, except for one clone, which had a production level comparable to that of the controls. Production of the side products 2- and 9-HODE in the 15-LO clones was only one- to three-fold higher than in the controls. On average, the percent-

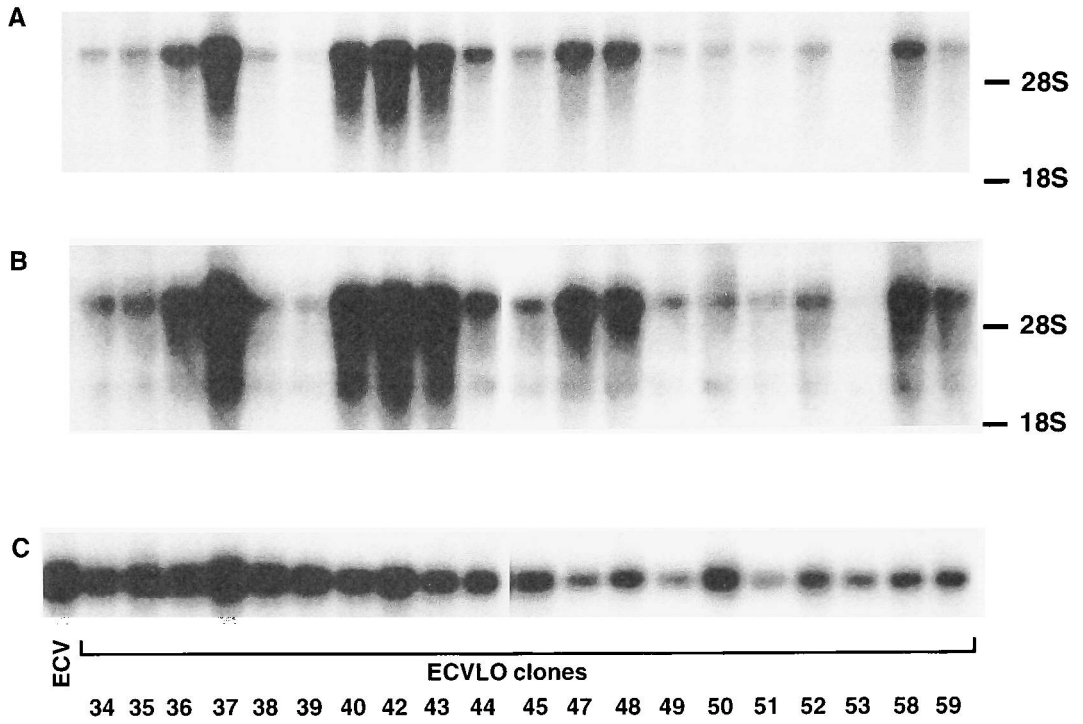

FIG. 2. Expression of 15-LO mRNA in transduced ECV304 cells. Northern blot analysis of poly(A)$^+$ RNA samples from ECV304 clones. *From left:* Untransduced ECV304 and ECV304 15-LO clones (34–59). Hybridization with random-primed cDNA probe for 15-LO (**A**), neomycin phosphotransferase (**B**), and human β-actin (**C**).

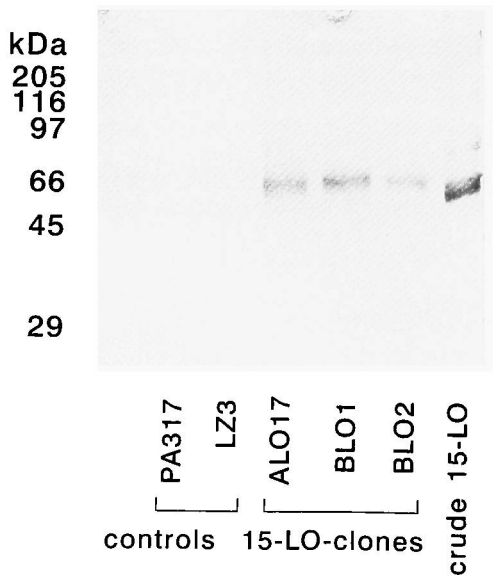

FIG. 3. Expression of 15-LO protein in transduced PA317 cells. Western blot analysis of protein samples from PA317 clones. *From left*: Control PA317 cells, control lacZ clone LZ3, and 15-LO clones (ALO17 to BLO2). Crude rabbit 15-LO sample was included as an additional control. The Western blot was done using a polyclonal rabbit anti-human recombinant 15-LO antibody (dilution 1:500). Four micrograms protein was loaded per lane.

age of the main product 13-HODE of all the HODEs detected (2-, 9-, and 13-HODE) was 81–89% in the good producers. The gas chromatographic method used in this study (Nikkari *et al.*, 1995) does not give information about the enantiomeric composition of the 15-LO reaction product. However, the enantiomeric specificity has been verified in similarly transduced rabbit aortic smooth muscle cells where the major reaction product after incubation with arachidonic acid was 15-*S*-hydroxyeicosatetraenoic acid (15-*S*-HETE), as detected by chiral phase high-performance liquid chromatography (HPLC) (Dr. Hartmut Kühn, personal communication).

On the basis of preliminary enzyme activity studies, clone ALO17 was chosen for the production of amphotropic virus, which was concentrated from the growth medium and was used for the transduction of ECV304 cells. Four ECV304 clones were chosen for enzyme activity analysis: three clones showing high mRNA expression and one clone showing low mRNA

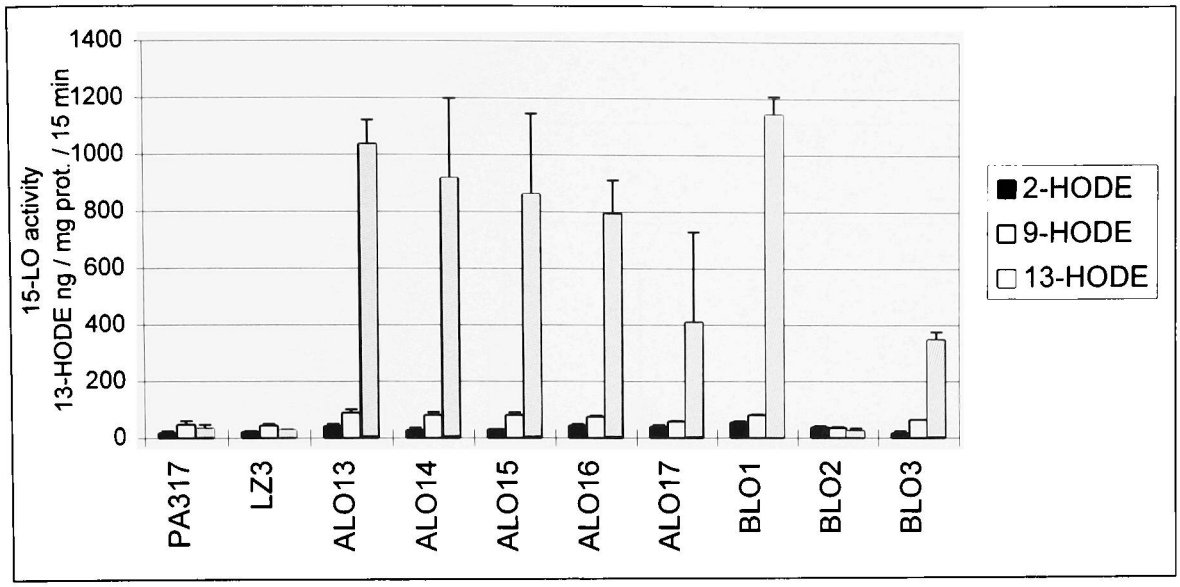

FIG. 4. 15-LO enzyme activity in transduced PA317 cells. *From left*: control PA317 cells, control lacZ clone LZ3, and 15-LO clones (ALO13 to BLO3). Enzyme activity was measured as the amount of 13-HODE produced in ng/mg protein per 15 min after incubation of the cells with 50 μM linoleic acid at 37°C. Each point is the mean ± SD of duplicate determinations. The amount of 13-HODE produced was measured as described in Materials and Methods.

expression by Northern hybridization. The one clone showing low 15-LO mRNA expression also had low 15-LO enzyme activity, whereas the other clones showing high 15-LO mRNA expression had 10–20 times higher 15-LO enzyme activity than the β-galactosidase-producing control clone ECVLZ5 (Fig. 5). The percentage of 13-HODE of all the HODEs detected in the high producers was 81–100%. Clone ECVLO37 was chosen for further studies.

15-LO mRNA expression levels in the transduced cells correlate with protein expression and enzyme activity. As shown in Fig. 1A, PA317 clone BLO2 had a very weak hybridization signal for 15-LO probe, clone ALO17 had somewhat stronger signal, and the signal in clone BLO1 was the strongest. Likewise, clone BLO2 showed the weakest signal for 15-LO protein production and enzyme activity, whereas clone BLO1 had the highest production levels among these three clones (Figs. 3 and 4). No endogenous 15-LO mRNA or protein expression could be detected in the transduced cells.

NF-κB activation

When nuclear protein extracts of the human ECV304 clone overexpressing 15-LO (ECVLO) were examined by EMSA using a labeled NF-κB-binding oligonucleotide, a distinct NF-κB retardation band was observed (Fig. 6). In these cells, stimulation with TNF-α (100 ng/ml), PMA (100 nM), thapsigargin (1 μM), or H_2O_2 (250 μM) markedly potentiated NF-κB activation as compared to a β-galactosidase-expressing clone (ECVLZ). Competition assays with cold consensus and mutant NF-κB oligonucleotides confirmed the specificity of the NK-κB band.

Flow cytometric detection of adhesion molecule expression

Flow cytometric analysis showed that the level of constitutive ICAM-1 expression was similar in ECVLO cells and in the corresponding β-galactosidase-expressing ECVLZ control cells. However, the inducibility of NF-κB-regulated ICAM-1 expression in response to PMA (100 nM, 24 hr) or TNF-α (100 ng/ml, 24 hr) stimulation was markedly higher in ECVLO cells (Fig. 7). This is consistent with our previous observation that activation of NF-κB in response to different stimuli is potentiated in the presence of 15-LO overexpression. We further studied whether this inducible ICAM-1 expression in ECVLO cells was antioxidant-sen-

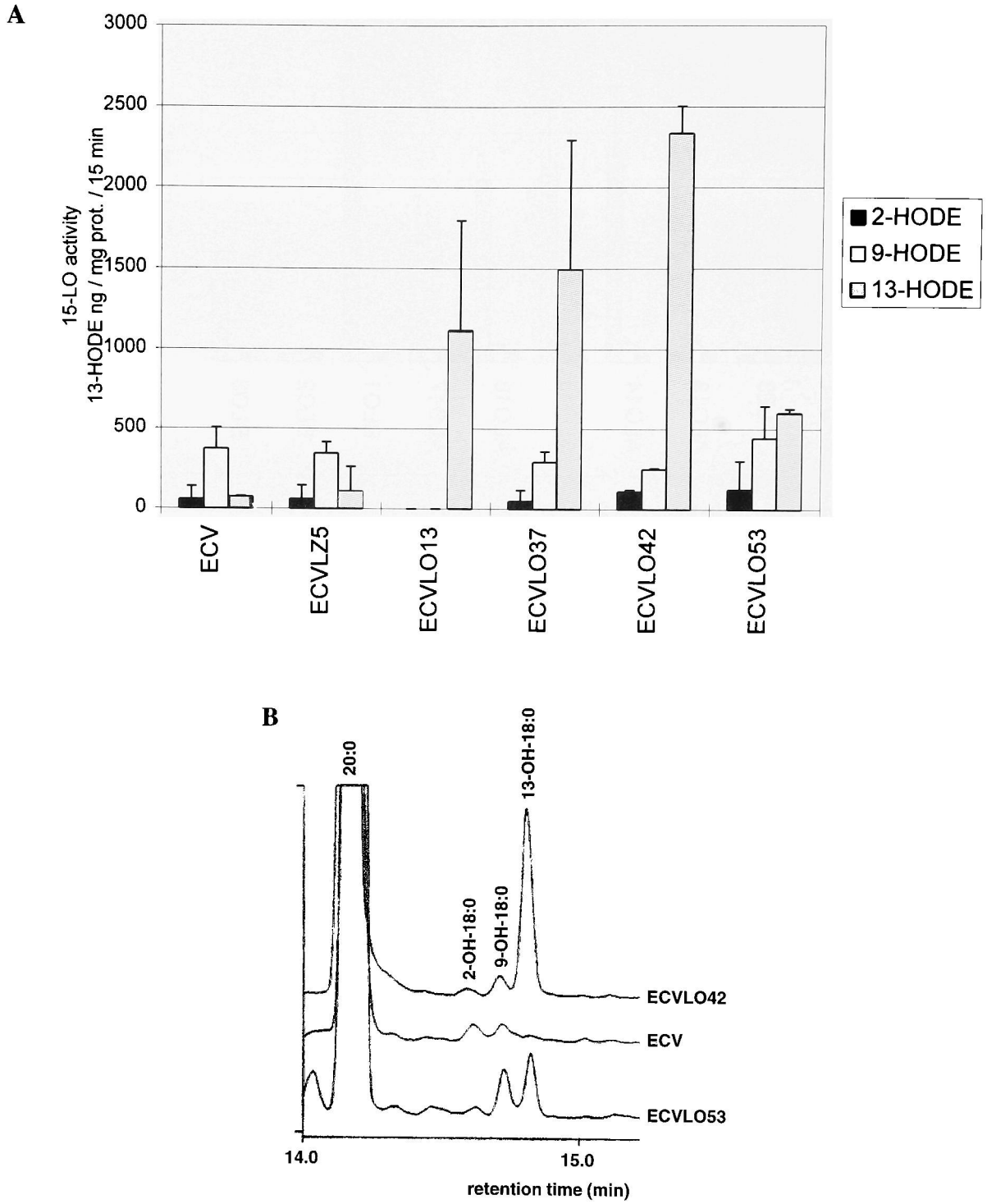

FIG. 5. 15-LO enzyme activity in transduced ECV304 cells. A. *From left*: Control ECV304 cells, control lacZ clone ECVLZ5, and 15-LO clones ECVLO13, ECVLO37, ECVLO42, and ECVLO53. Enzyme activity was measured as the amount of 13-HODE produced in ng/mg protein per 15 min after incubation of the cells with 50 μM linoleic acid at 37°C. Each point is the mean ± SD of duplicate determinations. The amount of 13-HODE produced was measured as described in Materials and Methods. **B.** Gas chromatographic analysis after incubation of endothelial cells with linoleic acid. Hydroperoxy fatty acids were hydrogenated, methylated, and acetylated before gas chromatography. 20:0, docosanoic acid; 2-OH-18:0, 2-hydroxy octadecanoic acid; 9-OH-18:0, 9-hydroxy octadecanoic acid; 13-OH-18:0, 13-hydroxy octadecanoic acid. Samples are the same as in **A.**

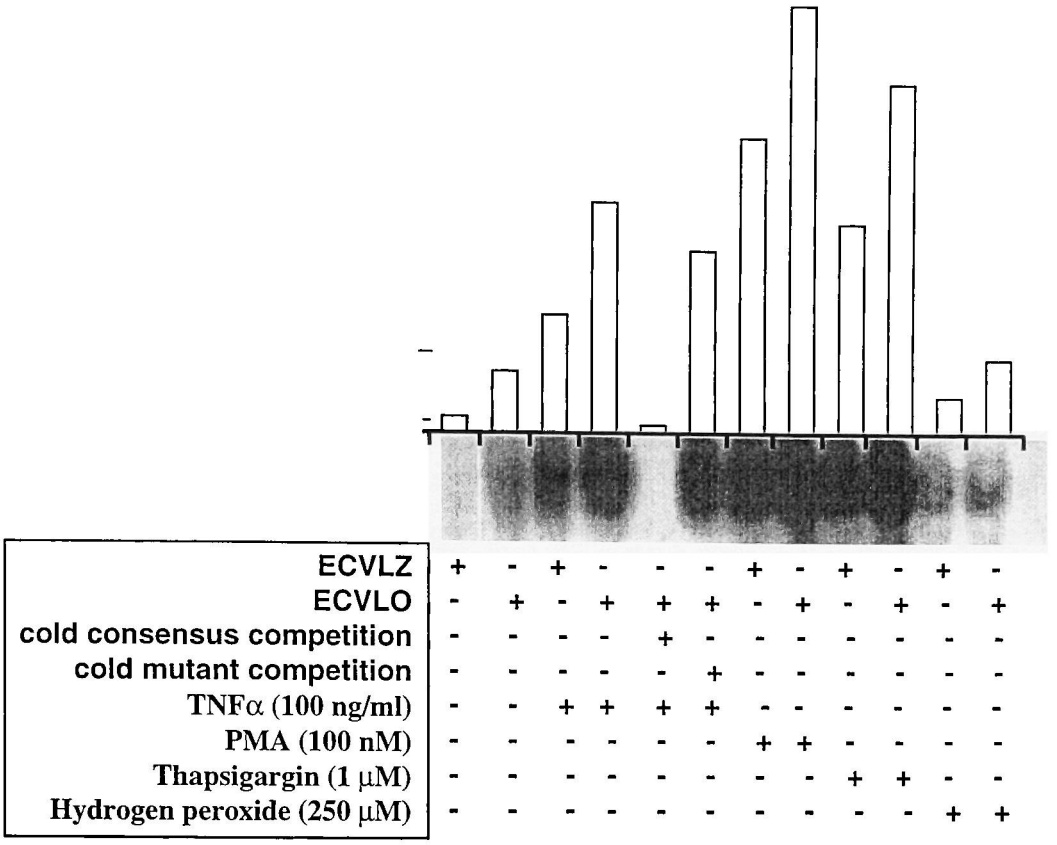

FIG. 6. Potentiation of NF-κB activation by overexpression of 15-LO in human endothelial cells in response to TNF-α, PMA, thapsigargin, and hydrogen peroxide. Cells were stimulated for 2 hr before nuclear extraction and EMSA. Competition assays with consensus and mutant NF-κB oligonucleotides confirm the specificity of the NF-κB band. Bars represent densitometric results obtained from scanning the respective autoradiograms. ECVLO, transduced ECV304 cells expressing human 15-lipoxygenase; ECVLZ, transduced ECV304 cells expressing *E. coli* β-galactosidase.

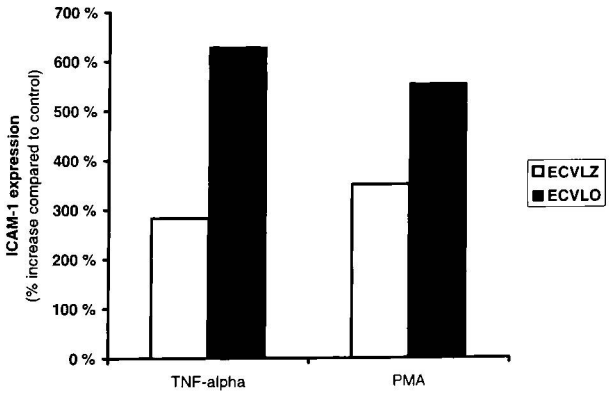

FIG. 7. Induction of ICAM-1 expression in ECVLO and ECVLZ cells in response to PMA and TNF-α stimulation. Cells were stimulated for 24 hr. Results were obtained by flow cytometric detection using FITC-labeled monoclonal antibody against ICAM-1. ICAM-1 expression is presented as percent increase compared to nonactivated cells. Histogram represents one of three essentially identical experiments.

sitive by pretreating the cells with different concentrations of an antioxidant α-lipoate. The results in Fig. 8 show that α-lipoate pretreatment had no effect on the constitutive ICAM-1 expression in ECVLO cells, but inhibited the PMA or TNF-α induced ICAM-1 expression in a dose-dependent manner. These results further support the hypothesis that oxidative stress in the ECVLO cells is involved in the NF-κB-induced ICAM-1 expression.

Under resting conditions, both ECVLO and ECVLZ control cells were almost devoid of VCAM-1 expression, but consistent with the ICAM-1 results, inducibility of VCAM-1 in response to PMA or TNF-α stimulation was much stronger in ECVLO cells as compared to the β-galactosidase-expressing control cells (Fig. 9).

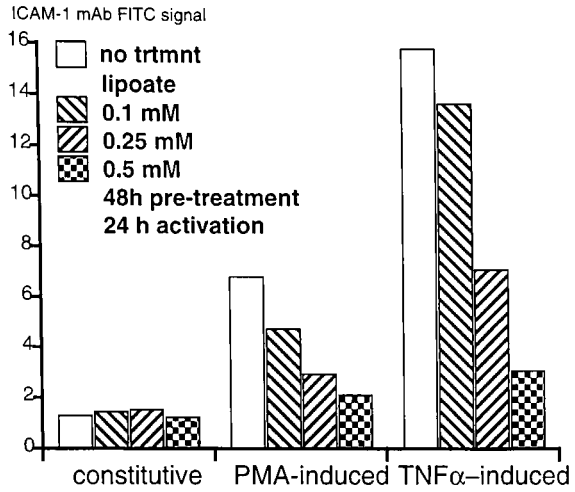

FIG. 8. Dose-dependent inhibition of PMA and TNF-α stimulated ICAM-1 induction in ECVLO cells by α-lipoate. Cells were pretreated with α-lipoate for 48 hr and then treated for 24 hr with the indicated compounds. Results were obtained by flow cytometric detection using FITC-labeled monoclonal antibody against ICAM-1.

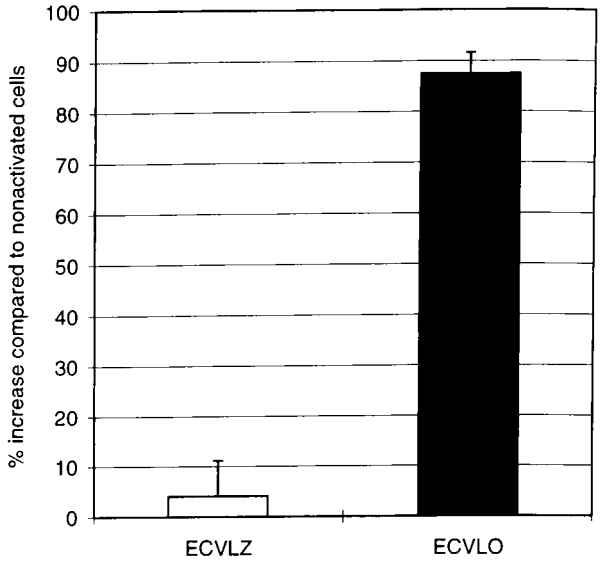

FIG. 10. Increased adherence of Jurkat T cells to ECVLO cells in response to PMA stimulation. Cells were stimulated with 100 n*M* PMA for 24 hr and then co-cultured with calcein-AM-labeled Jurkat T cells for 1 hr. The results are expressed as percent increase in the number of adhered Jurkat T cells to ECVLO and ECVLZ cells following PMA stimulation. Three separate experiments were done in triplicate.

Adhesion of Jurkat T cells to endothelial cells

The adherence of Jurkat T cells on ECVLO cells was markedly induced in response to PMA stimulation, whereas the adherence on ECVLZ cells was not affected (Fig. 10).

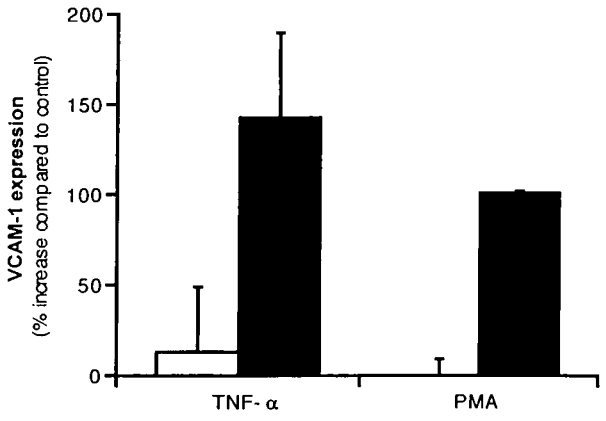

FIG. 9. Inducton of VCAM-1 expression in ECVLO and ECVLZ cells in response to PMA and TNF-α stimulation. Cells were stimulated for 24 hr. Results were obtained by flow cytometric detection using a monoclonal antibody against VCAM-1 and a FITC-labeled secondary antibody. VCAM-1 expression is presented as percent increase compared to nonactivated cells. Data are mean ± SD of at least three experiments.

DISCUSSION

In spite of the fact that 15-LO expression and enzyme activity in early atherosclerotic lesions have been clearly established (Kühn *et al.*, 1994, 1997) and that over 100-fold induction of 15-LO expression is seen in early fatty streak lesions (Hiltunen *et al.*, 1995), exact mechanisms for the proatherogenic role of 15-LO have remained unclear. Our working hypothesis was that, in addition to its possible role in LDL oxidation, 15-LO might have some other atherogenic effects in endothelial cells. This concept is also supported by a significant antiatherosclerotic effect of a 15-LO inhibitor in hypercholesterolemic rabbits, which is difficult to explain only on the basis of the inhibition of LDL oxidation (Sendobry *et al.*, 1997; Bocan *et al.*, 1998). In this study, we show that retrovirus-mediated stable gene transfer of human 15-LO leads to high expression of 15-LO mRNA, protein, and enzyme activity, potentiates VCAM-1 and ICAM-1 expression via NF-κB-mediated mechanism, and induces adhesion of T cells on the transduced endothelial cells.

Recent evidence suggests that reactive oxygen species and lipid hydroperoxides act as signaling molecules for the regulation of genes involved in atherogenesis. 9- and 13-HODEs have been shown to activate transcription factor peroxisome proliferator activated receptor γ (PPARγ) (Nagy *et al.*, 1998) and susceptibility of mice to atherosclerosis is genetically associated with the accumulation of lipid peroxidation products, induction of inflammatory genes, and the activation of NF-κB (Liao *et al.*, 1993, 1994). It has been clearly established that reactive oxygen species activate several transcription factors, including NF-κB (Schreck *et al.*, 1992; Andalibi *et al.*, 1993; Sen and Packer, 1996), which is present in atherosclerotic lesions in its activated form (Brand *et al.*, 1996). NF-κB regulates the expression of several genes expressed in atherosclerotic lesions, such as VCAM-1 (Cybulsky and Gimbrone, 1991; Li *et al.*, 1993a,b; O'Brien *et al.*, 1993) and ICAM-1 (Poston *et al.*, 1992; Printseva *et al.*, 1992). TNF-α activates NF-κB (Baeuerle and Henkel, 1994), and induces NF-κB-mediated expression of VCAM-1 (Rice and Bevilacqua, 1989) and ICAM-1 (Pober *et al.*, 1986; Roebuck *et al.*, 1995). It has also been shown that addition of the 15-LO product 13-hydroperoxyoctadecadienoic acid (13-HPODE) to endothelial cells in the presence of TNF-α increased the expression of VCAM-1 (Khan *et al.*, 1995). Also, 15-LO metabolites 15(*S*)-hydroperoxyeicosatetraenoic acid (15(*S*)-HPETE) and 12(*S*)-HETE induced endothelial adhesion molecule expression, and adhesion and transendothelial migration of monocyte-like HL-60 cells (Sultana *et al.*, 1996). However, these metabolites can be provided by cyclooxygenases as well as by 15-LO (Camacho *et al.*, 1995).

15-LO expression in the transduced ECV304 cells leads to increased oxidative stress as indicated by lowered α-tocopherol and ubiquinol levels in these cells as compared to the control ECVLZ cells (data not shown). This and blocking of the induction of ICAM-1 in the 15-LO-transduced cells by an antioxidant α-lipoate suggests that 15-LO is linked to the redox-sensitive signal transduction cascade. The constitutive expression of the adhesion molecules ICAM-1 and VCAM-1 were similar in the ECVLO and ECVLZ cells, but the expression of these adhesion molecules was induced in the ECVLO cells when stimulated with PMA or TNF-α. 15-LO inhibitor (eicosatetraenoic acid, ETYA) attenuated these findings (data not shown), although data obtained with relatively unspecific inhibitors should be interpreted with caution. Wölle *et al.* (1996) achieved similar results for VCAM-1 expression after transient transfection of 15-LO into bovine endothelial cells.

The adherence of Jurkat T cells on ECVLO cells was greatly induced after PMA stimulation, whereas the adherence on ECVLZ cells was not affected. VCAM-1 is a specific receptor for lymphocytes and monocytes, because its ligand, the very late activation antigen-4 (VLA-4) is expressed only on lymphocytes and monocytes, but not on neutrophils (Rice *et al.*, 1990). ICAM-1, on the other hand, is a ligand for lymphocyte function-associated antigen 1 (LFA-1) and Mac-1, which are expressed in all leukocytes (Makgoba *et al.*, 1988; Albelda, 1991). Substantial amounts of T lymphocytes are present in human atherosclerotic lesions, where they can have important effects on the inflammatory aspects of atherosclerosis. It is concluded that 15-LO may be involved in the early events of atherogenesis not only by inducing the oxidative modification of LDL, but also as a mediator of the adhesion of T cells on the endothelium via production of reactive oxygen species that can activate NF-κB and potentiate expression of VCAM-1 and ICAM-1 in response to various inflammatory stimuli. Inhibition of 15-LO activity could be one potential way to reduce atherogenesis in human arteries.

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