

# Linoleic Acid Increases Lectin-Like Oxidized LDL Receptor-1 (LOX-1) Expression in Human Aortic Endothelial Cells

Fritz Maingrette<sup>1</sup> and Geneviève Renier<sup>2</sup>

**Results from in vitro studies suggest that selected fatty acids, and especially linoleic acid (LA), can elicit endothelial dysfunction (ED). Because LA is increased in all LDL subfractions in patients with type 2 diabetes, this alteration may contribute to ED associated with diabetes. Lectin-like oxidized LDL receptor-1 (LOX-1) is the major endothelial receptor for oxidized LDL (oxLDL), and uptake of oxLDL through LOX-1 induces ED. To evaluate whether LA may contribute to the upregulation of endothelial LOX-1 in diabetes, we studied the effect of LA on LOX-1 expression in cultured human aortic endothelial cells (HAECs). Treatment of HAECs with LA increased, in a time- and dose-dependent manner, endothelial LOX-1 protein expression. Pretreatment of HAECs with antioxidants and inhibitors of NADPH oxidase, protein kinase C (PKC), and nuclear factor- $\kappa$ B (NF- $\kappa$ B) inhibited the stimulatory effect of LA on LOX-1 protein expression. Furthermore, in LA-treated HAECs, increased expression of classic PKC isoforms was observed. LA also led to a significant increase in LOX-1 gene expression and enhanced the binding of nuclear proteins extracted from HAECs to the NF- $\kappa$ B regulatory element of the LOX-1 gene promoter. Finally, LA enhanced, through LOX-1, oxLDL uptake by endothelial cells. Overall, these results demonstrate that LA enhances endothelial LOX-1 expression through oxidative stress-sensitive and PKC-dependent pathways. This effect seems to be exerted at the transcriptional level and to involve the activation of NF- $\kappa$ B. Upregulation of LOX-1 by LA may contribute to ED associated with type 2 diabetes. *Diabetes* 54: 1506–1513, 2005**

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DiI-oxLDL, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate-labeled oxLDL; ED, endothelial dysfunction; EGM, endothelial growth culture medium; HAEC, human aortic endothelial cell; LA, linoleic acid; LOX-1, lectin-like oxidized LDL receptor-1; NAC, N-acetyl-L-cysteine; oxLDL, oxidized LDL; NF, nuclear factor; PKC, protein kinase C; SR, scavenger receptor.

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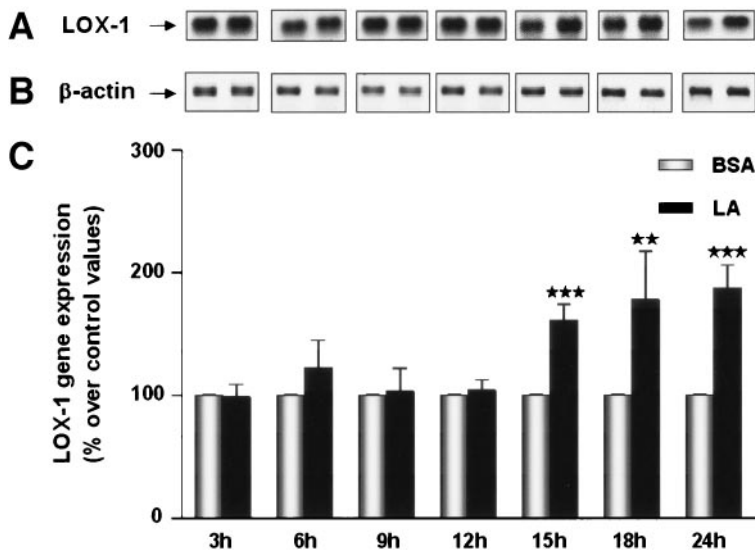
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**E**ndothelial dysfunction (ED), a characteristic feature of early-state atherosclerosis, is well documented in patients with diabetes and in individuals with insulin resistance or at high risk for developing type 2 diabetes (1,2). Critical factors that may induce activation and injury to the endothelium in the diabetic state include oxidized LDL (oxLDL) (3) and selected unsaturated fatty acids, such as linoleic acid (LA) (4). One potential key determinant of oxLDL-induced ED is lectin-like oxidized LDL receptor-1 (LOX-1), a newly identified limiting factor for oxLDL uptake by endothelial cells (5). Data showing that LOX-1 expression is enhanced by proatherogenic factors relevant to human diabetes, including oxLDL (6), high glucose (7), advanced glycation end products (8), and C-reactive protein (9), and is upregulated in endothelium and aortas of diabetic animals (8) support a role of this receptor in the pathogenesis of diabetic vascular dysfunction. Substantial amounts of data have been accumulated to show that LA is a pro-oxidative and proinflammatory molecule (10–12) that can induce endothelial cell activation and dysfunction. These data and the observation that patients with type 2 diabetes show increased concentrations of LA in all LDL subfractions (13) support the significance of this fatty acid in ED associated with diabetes. In the present study, we investigated the role of LA in the regulation of endothelial LOX-1, the molecular mechanisms involved in this effect, and the role of LOX-1 in LA-induced oxLDL uptake by endothelial cells.

## RESEARCH DESIGN AND METHODS

**Reagents.** FCS was purchased from Wisent (St. Bruno, PQ, Canada). Hank's balanced salt solution, penicillin-streptomycin, glycine, SDS, and Trizol reagent were obtained from Invitrogen Life Technologies (Burlington, ON, Canada). Human aortic endothelial cells (HAECs), endothelial growth culture medium (EGM), and an EGM bullet kit were obtained from Cedarlane Laboratories Limited (Hornby, ON, Canada). RPMI-1640 was obtained from Gibco (Grand Island, NY). Sodium salt LA, fatty acid-free BSA fraction V, isopropanol, vitamin C, and monoclonal antibody against  $\beta$ -actin were purchased from Sigma (St. Louis, MO). Ficoll and horseradish peroxidase-conjugated anti-mouse IgG were obtained from Amersham Biosciences (Buckinghamshire, U.K.). Monoclonal antibodies against LOX-1, p50, p65, and protein kinase C (PKC)- $\alpha$ , - $\beta$ , - $\beta$ , and - $\gamma$  were bought from Santa Cruz Biotechnology (Santa Cruz, CA). IgG<sub>1</sub> neutralizing antibody was purchased from R&D Systems (Minneapolis, MN). Calphostin C, GF109203X, BAY11-7085, N-acetyl-L-cysteine (NAC), curcumin, apocynin, thenoyltrifluoroacetone, Mn(III)tetrakis (4-benzoic acid), and diphenylethidium chloride were obtained from Calbiochem (La Jolla, CA).

**Cells.** Commercially available HAECs (passage 3) were grown to confluence in EGM under recommended conditions. The EGM was supplemented with



**FIG. 1.** Effect of LA on endothelial LOX-1 mRNA expression. HAECs were incubated for 3–24 h in the presence of 0.2 mmol/l LA. At the end of these incubation periods, cells were lysed and total RNA was extracted and analyzed by PCR for LOX-1 (A) and  $\beta$ -actin (B) mRNA expression. Levels of LOX-1 mRNA were normalized to the levels of  $\beta$ -actin mRNA (C). Results are means  $\pm$  SE of four different experiments. \*\* $P < 0.01$  vs. BSA; \*\*\* $P < 0.001$  vs. BSA.

10% FCS containing 0.01  $\mu$ g/ml human epidermal growth factor, 0.1% gentamicin sulfate amphotericin-B, 1  $\mu$ g/ml hydrocortisone, and 12  $\mu$ g/ml bovine brain extract protein content. Cells were used at passages 3–5. Evidence that these cells express von Willebrand factor, adhesion molecules, and cytokines and demonstrate plasminogen activator inhibitor-1 activity supports their relevance as a model for the study of native vascular endothelium. Confluent HAECs were incubated for different time periods in serum-free EBM supplemented with 1% penicillin-streptomycin in the presence of 0.2 mmol/l LA bound to 2% BSA. Serum-free EBM supplemented with 2% BSA was used as a control. Under these experimental conditions, the fatty acid-to-albumin molar ratio was  $\sim 1$ , a value that is within physiological levels.

**Analysis of mRNA expression.** Expression of the LOX-1 gene in HAECs was measured by the PCR technique. Total RNA for use in the PCR was extracted from cells by an improvement of the acid-phenol technique of Chomczynski and Sacchi. Briefly, cells were lysed with Trizol reagent and chloroform was added to the solution. After centrifugation, the RNA present in the aqueous phase was precipitated and resuspended in diethyl pyrocarbonate water. cDNA was synthesized from RNA by incubating total cellular RNA with 0.1  $\mu$ g oligodT (Pharmacia) for 5 min at 98°C and then by incubating the mixture with reverse transcription buffer for 1 h at 37°C. The cDNA obtained was amplified by using 0.8  $\mu$ mol/l of two synthetic primers specific for human LOX-1 (5'-TTACTCTCCATGGTGGTGGCC-3') (5'-AGCTTCTCTGCTTGTGTGCC-3') and human  $\beta$ -actin (5'-TCGAATCTGAGAAGAGC TATGAGCTGCCG-3') (5'-TCGCATCCGTGCCACCAGACAGCACTGTGTG-3') used as the internal standard in the PCR mixture. A 193-base pair human LOX-1 cDNA fragment and a 201-base pair human  $\beta$ -actin cDNA fragment were amplified enzymatically by 40 and 20 repeated cycles, respectively. An aliquot of each reaction mixture was then subjected to electrophoresis on 1% agarose gel containing ethidium bromide. The intensity of the bands was measured by an image analysis scanning system (Alpha Imager 2000, Packard Instrument Company). Titrating the cDNA samples ensured that the signal lies on the exponential part of the standard curve.

**Western blot.** HAEC protein extracts (8  $\mu$ g) were applied to 10% SDS-PAGE and transferred to a nitrocellulose membrane using a Bio-Rad transfer blotting system at 100 V for 1 h. Nonspecific binding was blocked with 5% milk for 1 h at room temperature. After washing with PBS-Tween 0.1%, blots were incubated overnight at 4°C with anti-LOX-1 or anti- $\beta$ -actin antibodies. After further washing, membranes were incubated for 1 h at room temperature with a horseradish peroxidase-conjugated donkey anti-mouse IgG (1/5,000). Antigen detection was performed with an enhanced chemiluminescence detection system (Amersham).

For PKC isoforms analysis, HAECs were recovered and homogenized (Dounce: 15 strokes) in 500  $\mu$ l ice-cold buffer A (20 mmol/l Tris [pH 7.5], 0.5 mmol/l EDTA, 0.5 mmol/l EGTA, 25  $\mu$ g/ml aprotinin, and 25  $\mu$ g/ml leupeptin). The membrane and cytosolic fractions were separated by ultracentrifugation (100,000g for 30 min at 4°C). After recovery of high-speed supernatants containing cytosolic PKC, the corresponding membrane pellets were homogenized in 500  $\mu$ l buffer A containing 0.5% Triton X-100 and assayed for classic PKC isoforms by Western blot analysis.

**Uptake of 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate-labeled oxLDL.** Native LDL (density 1.019–1.063) was isolated from plasma obtained from healthy donors by sequential ultracentrifugation

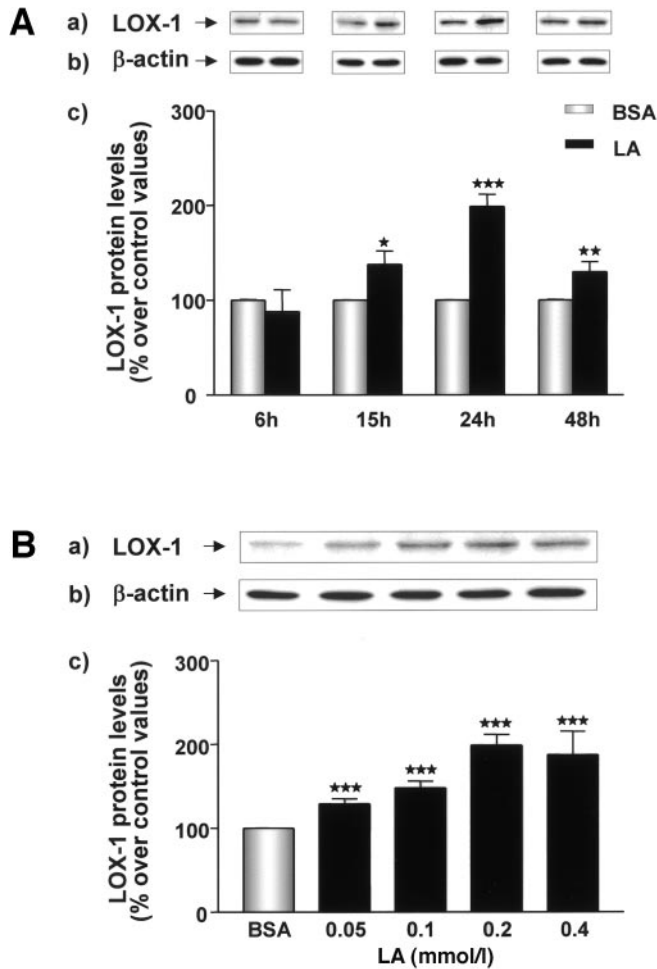
using potassium bromide for density adjustment. Native LDL was extensively dialyzed for 24 h at 4°C against 5 mmol/l Tris/50 mmol/l NaCl to remove EDTA. 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-labeled oxLDL (DiI-oxLDL) was obtained from Biomedical Technologies (Stoughton, MA). To examine cellular uptake of oxLDL, HAECs were seated in eight-well culture slides (Falcon) and incubated for 3 h in medium containing 5% of lipoprotein-deficient serum with DiI-oxLDL (80  $\mu$ g/ml) in the presence or absence of a 500-fold excess of unlabeled oxLDL. At the end of the incubation period, cells were washed, mounted on coverslips with mounting medium, and examined by fluorescence microscopy. To measure amounts of DiI-oxLDL accumulated in cells, HAECs were seated in 12-well plates receiving the same treatment as mentioned above. At the end of the incubation period, DiI was extracted by isopropanol, and the fluorescence was determined at 520/564 nm. Results were normalized to total cell protein concentrations (Bradford).

**DNA binding assay.** The isolation of the nuclei was performed as follows. Briefly,  $5 \times 10^7$  HAECs were washed and collected with 10 ml ice-cold PBS/phosphatase inhibitor buffer (125 mmol/l sodium fluoride, 250 mmol/l  $\beta$ -glycerophosphate, 250 mmol/l para-nitrophenyl phosphate, and 25 mmol/l sodium orthovanadate). The cells were transferred to a prechilled 15-ml tube and centrifuged at 3,000 rpm for 5 min at 4°C. The pellet was resuspended in 1 ml ice-cold hypotonic buffer (20 mmol/l HEPES, pH 7.5, 5 mmol/l sodium fluoride, 10  $\mu$ mol/l sodium molybdate, and 0.1 mmol/l EDTA). After a 15-min incubation on ice, 50  $\mu$ l Nonidet-P40 (NP-40) (10%) was added, and the homogenate was mixed and centrifuged for 30 s at 4°C in a microcentrifuge. The nuclear pellet was resuspended in 50  $\mu$ l lysis buffer (1 mmol/l dithiothreitol, 2 mol/l potassium chloride, 25 mmol/l HEPES, 0.1 mmol/l EDTA, 1  $\mu$ g/ml aprotinin, and 2 mmol/l phenylmethylsulfonyl fluoride) and rocked on ice for 30 min. Nuclei were collected by centrifugation for 20 min at 13,000 rpm. Aliquots (50  $\mu$ l) of the supernatants were frozen at  $-80^\circ\text{C}$ , and the protein concentration was determined. DNA retardation (mobility shift) electrophoresis assays were performed as follows. Briefly, 5- $\mu$ g nuclear extracts were incubated for 15 min in the presence of  $5 \times$  binding buffer (125 mmol/l HEPES, pH 7.5, 50% glycerol, 250 mmol/l sodium chloride, 0.25% NP-40, and 5 mmol/l dithiothreitol). End-labeled double-stranded consensus sequences of the LOX-1 promoter nuclear factor (NF)- $\kappa$ B-enhancing elements (20,000 cpm per sample) were then added to the samples for 30 min. Samples were analyzed on a 4% PAGE containing 0.01% NP-40. The specificity of the nuclear protein binding was assessed by incubating the nuclear proteins isolated from HAECs with a labeled DNA probe in the presence of a 1,000-fold molar excess of unlabeled DNA probe.

**DNA probes.** Double-stranded oligonucleotides containing the NF- $\kappa$ B (5-CGTCTGCCCTTCCCTCT-3 and 5-GAGAAGAGGGGAAAGG-3) consensus sequence of the human LOX-1 gene promoter (14) were synthesized with the aid of an automated DNA synthesizer. After annealing, the oligonucleotides were labeled with [ $\gamma$ - $^{32}\text{P}$ ]ATP using the Boehringer Mannheim 5' end-labeling kit (Indianapolis, IN).

**Determination of cell viability.** Cell viability after treatment with LA was assessed by trypan blue exclusion and was consistently found to be  $>80\%$ .

**Determination of total protein concentration.** Total protein content was estimated according to the Bradford method using a colorimetric assay (Bio-Rad, Mississauga, ON, Canada).



**FIG. 2.** Time- and dose-dependent effect of LA on endothelial LOX-1 protein expression. HAECs were cultured for 6–48 h with 0.2 mmol/l LA (A) or for 24 h with 0.05–0.4 mmol/l LA (B). At the end of the incubation period, cells were lysed and LOX-1 protein expression was determined by Western blot analysis. LOX-1 protein levels were normalized to the levels of β-actin protein. Data are means ± SE of four different experiments. \**P* < 0.05 vs. BSA; \*\**P* < 0.01 vs. BSA; \*\*\**P* < 0.001 vs. BSA.

**Statistical analysis.** All values were expressed as means ± SE. Data were analyzed by one-way ANOVA, followed by the Turkey test. A *P* value < 0.05 was considered statistically significant.

**RESULTS**

**Effect of LA on endothelial LOX-1 mRNA expression.**

Treatment of HAECs for 15–24 h with 0.2 mmol/l LA significantly increased LOX-1 mRNA levels [LOX-1 mRNA levels [percent increase over control values] at 15 h: 161 ± 13, *P* < 0.001; 18 h: 178 ± 40, *P* < 0.01; 24 h: 187 ± 19, *P* < 0.001] (Fig. 1A). Under these experimental conditions, no modulation of the mRNA expression of β-actin was observed (Fig. 1B). LOX-1 mRNA levels normalized to the levels of β-actin mRNA are presented in Fig. 1C.

**Effect of LA on endothelial LOX-1 protein expression.**

To determine whether the increased LOX-1 mRNA expression induced by LA resulted in enhanced protein expression, HAECs were treated with 0.2 mmol/l LA for 6–48 h and LOX-1 protein expression was determined by Western blot analysis. Exposure of HAECs for 15–48 h to LA significantly increased LOX-1 protein levels (Fig. 2A-a). Maximal effect was observed at 24 h. LOX-1 protein levels normalized to the levels of β-actin (Fig. 2A-b) are illus-

trated in Fig. 2A-c. In contrast, treatment of HAECs for 24 h with 0.2 mmol/l palmitic acid, oleic acid, or docosahexaenoic acid did not affect LOX-1 protein expression (data not shown). LA-induced LOX-1 protein expression was dose dependent (Fig. 2B-a). Induction of LOX-1 protein was already observed at 0.05 mmol/l LA and reached a maximum at 0.2 mmol/l. LOX-1 protein levels normalized to the levels of β-actin (Fig. 2B-b) are illustrated in Fig. 2B-c.

**Role of oxidative stress in LA-induced LOX-1 protein expression.**

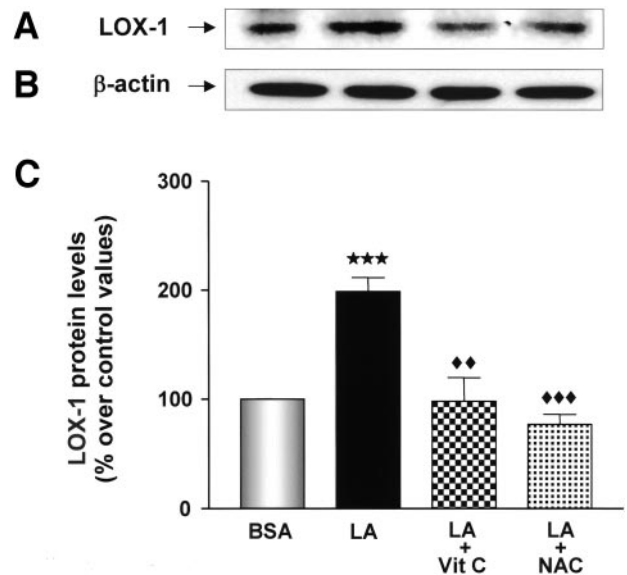
To evaluate whether oxidative stress is involved in the upregulation of LOX-1 by LA, HAECs were preincubated for 1 h with vitamin C (10 μmol/l) or NAC (10 mmol/l) before exposure to LA. Pretreatment of HAECs with these antioxidants totally prevented LA-induced LOX-1 protein expression (Fig. 3A). LOX-1 protein levels normalized to the levels of β-actin (Fig. 3B) are illustrated in Fig. 3C. (LOX-1 protein levels [percent increase over control values] are as follows: LA: 199 ± 13; LA + vitamin C: 98 ± 22, *P* < 0.01 vs. LA; LA + NAC: 77 ± 9, *P* < 0.001 vs. LA).

**Role of NADPH oxidase in LA-induced LOX-1 protein expression.**

To ascertain the enzymatic superoxide sources activated by LA, HAECs were preincubated for 1 h with the NADPH oxidase inhibitors apocynin (10 μmol/l) and diphenyleneiodonium chloride (10 μmol/l) or with the mitochondrial electron transport chain inhibitors thenoyl-trifluoroacetone (10 μmol/l) and Mn(III)tetrakis (4-benzoic acid) (100 μmol/l) prior to treatment with 0.2 mmol/l LA. Pretreatment of HAECs with NADPH oxidase inhibitors prevented LA-induced LOX-1 protein expression, whereas mitochondrial electron transport chain inhibitors were ineffective (Fig. 4).

**Role of PKC and NF-κB in LA-induced LOX-1 protein expression.**

To examine the signaling pathways mediating the effect of LA on LOX-1, HAECs were treated for 1 h



**FIG. 3.** Effect of antioxidants on LA-induced LOX-1 protein expression. HAECs were preincubated for 1 h in the presence of vitamin C (Vit C) (10 μmol/l) or NAC (10 mmol/l) and then treated for 24 h with 0.2 mmol/l LA. At the end of the incubation period, cells were lysed and LOX-1 protein expression was determined by Western blot analysis. LOX-1 protein levels (A) normalized to the levels of β-actin protein (B) are presented on the graph bar (C). Data are means ± SE of four different experiments. \*\*\**P* < 0.001 vs. BSA, ♦♦*P* < 0.01 vs. LA; ♦♦♦*P* < 0.001 vs. LA.

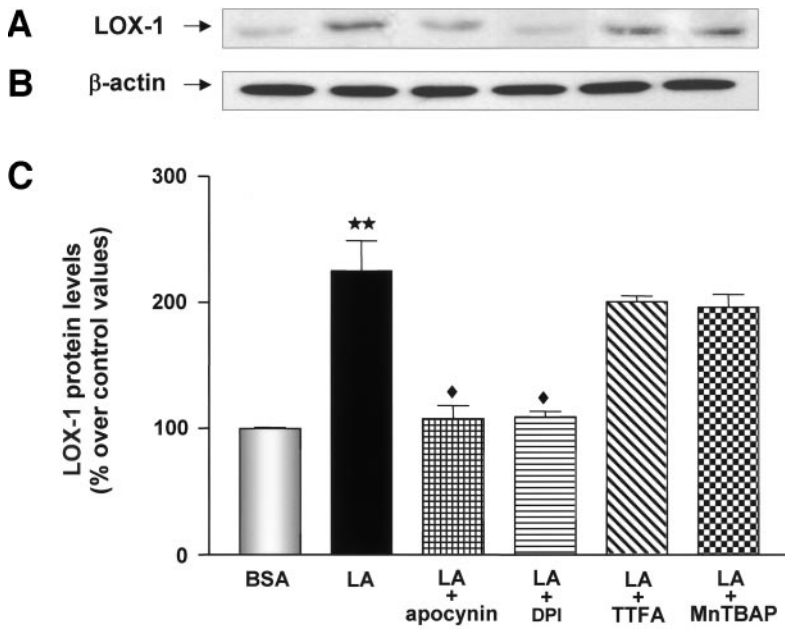


FIG. 4. Effect of NADPH oxidase and mitochondrial electron transport chain inhibitors on LA-induced LOX-1 protein expression. HAECs were preincubated for 1 h with the NADPH oxidase inhibitors apocynin (10  $\mu$ mol/l) and diphenyleneiodonium (DPI) chloride (10  $\mu$ mol/l) or with the mitochondrial electron transport chain inhibitors thenoyl-trifluoroacetone (TTFA) (10  $\mu$ mol/l) and Mn(III)tetrakis (4-benzoic acid) (MnTBAP) (100  $\mu$ mol/l) before treatment with 0.2 mmol/l LA. At the end of the incubation period, cells were lysed and LOX-1 protein expression was determined by Western blot analysis. LOX-1 protein levels (A) normalized to the levels of  $\beta$ -actin protein (B) are presented on the graph bar (C). Data are means  $\pm$  SE of three different experiments. \*\* $P$  < 0.01 vs. BSA;  $\blacklozenge$   $P$  < 0.05 vs. LA.

with the pan-specific PKC inhibitor calphostin C (0.1  $\mu$ g/ml), the NF- $\kappa$ B inhibitor BAY11-7085 (40  $\mu$ mol/l), or the activator protein-1 inhibitor curcumin (15  $\mu$ mol/l). As shown in Fig. 5, pretreatment of the cells with calphostin C and BAY11-7085, but not with curcumin, totally abolished the stimulatory effect of LA on LOX-1 protein expression.

**Effect of LA on PKC expression.** To determine the expression of classic PKC isozymes in LA-treated cells, HAECs were treated for 15 h with 0.2 mmol/l LA, and expression of these isoforms was determined by Western blot analysis. As illustrated in Fig. 6, LA increased the

expression of PKC- $\alpha$  (1.9-fold), PKC- $\beta_1$  (2.3-fold), PKC- $\beta_{II}$  (3.5-fold), and PKC- $\gamma$  (3.7-fold). Under these experimental conditions, no modulation of  $\beta$ -actin, used as an internal control, was observed.

**Effect of LA on the binding of nuclear proteins to the regulatory NF- $\kappa$ B sequence of the LOX-1 gene promoter.** To determine whether the incubation of HAECs with LA might result in changes at the level of LOX-1 gene binding proteins, we measured the binding activity of nuclear proteins extracted from LA-treated HAECs to the NF- $\kappa$ B consensus sequence of the LOX-1 gene promoter. Experiments showed that incubation of these cells for 6 h with LA resulted in a significant increase in the binding of

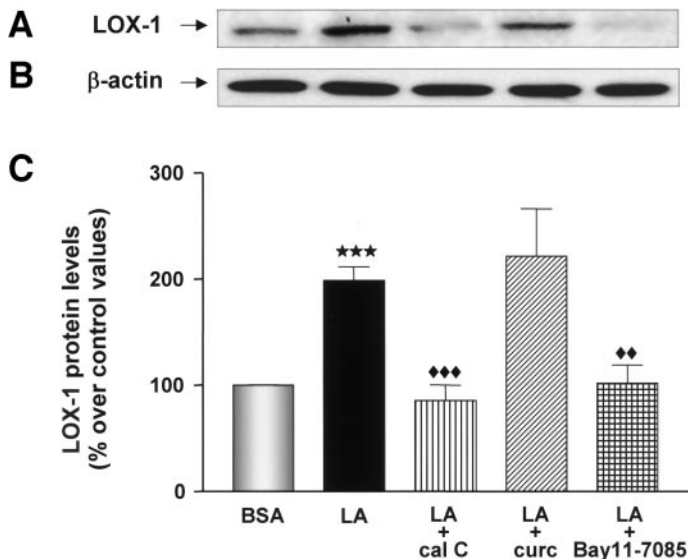


FIG. 5. Effect of PKC, NF- $\kappa$ B, and activator protein 1 (AP-1) inhibitors on LA-induced LOX-1 protein expression. HAECs were pretreated for 1 h with the pan-specific PKC inhibitor calphostin C (cal C) (0.1  $\mu$ g/ml), the NF- $\kappa$ B inhibitor BAY11-7085 (40  $\mu$ mol/l), or the AP-1 inhibitor curcumin (curc) (15  $\mu$ mol/l) and then exposed to 0.2 mmol/l LA for 24 h. At the end of the incubation period, cells were lysed and LOX-1 protein expression was determined by Western blot analysis. LOX-1 protein levels (A) were normalized to the levels of  $\beta$ -actin protein (B). Data illustrated on the graph (C) are means  $\pm$  SE of four different experiments. \*\*\* $P$  < 0.001 vs. BSA;  $\blacklozenge$   $P$  < 0.01 vs. LA;  $\blacklozenge$   $P$  < 0.001 vs. LA.

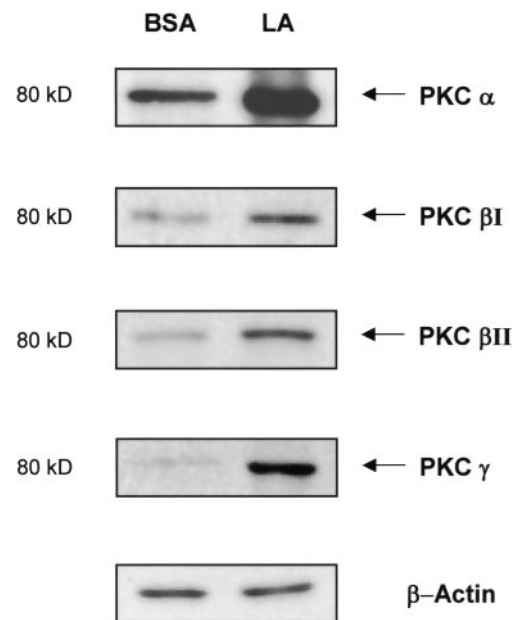


FIG. 6. Effect of LA on PKC expression. HAECs were incubated with 0.2 mmol/l LA for 15 h. Cell lysates were separated into cytosolic and membrane fractions and assayed for classic PKC isoforms by Western blot analysis as described in RESEARCH DESIGN AND METHODS. Data showing membrane PKC expression are presented. The figure illustrates the results of one representative experiment out of three.

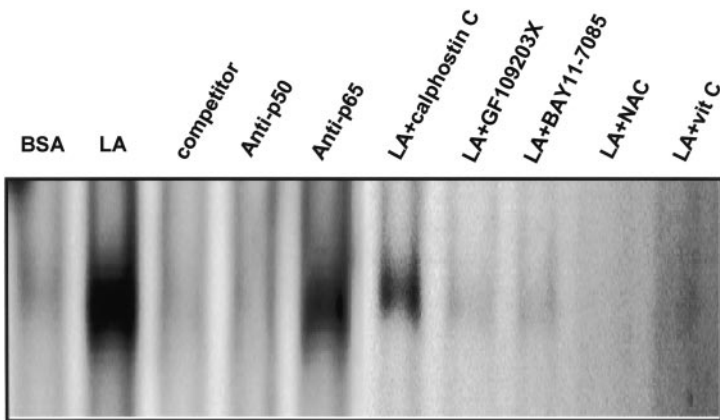


FIG. 7. Effect of LA on the binding of nuclear proteins to the regulatory NF- $\kappa$ B sequence of the LOX-1 gene promoter. HAECs were pretreated for 1 h with the pan-specific PKC inhibitor calphostin C (0.1  $\mu$ g/ml), the classic PKC selective inhibitor GF 109203X (20 nmol/l), the NF- $\kappa$ B inhibitor BAY 11-7085 (40  $\mu$ mol/l), or the antioxidants vitamin C (Vit C) (10  $\mu$ mol/l) or NAC (10 mmol/l), then stimulated for the next 6 h with 0.2 mmol/l LA. Nuclear proteins isolated from these cells were incubated with end-labeled NF- $\kappa$ B-specific consensus sequence of the LOX-1 gene promoter in the presence or absence of a 1,000-fold molar excess of the unlabeled DNA probe (competitor). Retardation was assessed by gel electrophoresis in 4% PAGE. In some experiments, nuclear proteins were incubated in the presence of anti-p50 or anti-p65 antibodies. Data are the results of one representative experiment out of three.

nuclear proteins to the regulatory NF- $\kappa$ B sequence of the LOX-1 gene promoter (Fig. 7). This binding complex was specifically competed in the presence of a 1,000-fold molar excess of the unlabeled NF- $\kappa$ B oligonucleotide. This effect was also reduced by pretreatment of HAECs with the p65 antibody, while preincubation of the cells with the p50 antibody calphostin C, GF109203X (a classic PKC selective inhibitor), BAY11-7085, NAC, or vitamin C totally abolished the stimulatory effect of LA on the binding of nuclear proteins to the NF- $\kappa$ B sequence of the LOX-1 gene promoter (Fig. 7).

**LA stimulates the uptake of oxLDL by endothelial cells through LOX-1.** To investigate whether LA might facilitate the uptake of oxLDL by endothelial cells and whether this effect involves LOX-1, HAECs were treated with 0.2 mmol/l LA for 23 h; then incubation was pursued for 1 h in the presence of saturating amounts of antibodies to LOX-1 or IgG<sub>1</sub> or in the presence of a 500-fold excess of unlabeled oxLDL. At the end of the incubation period, cells were incubated for 3 h with DiI-oxLDL (80  $\mu$ g/ml), and uptake of oxLDL was determined by measurement of extracted DiI-oxLDL and fluorescence microscopy. Incubation of HAECs with LA increased the uptake of oxLDL by these cells by more than twofold. This effect was totally blocked by pretreatment of the cells with anti-LOX-1 antibody or in the presence of excess unlabeled oxLDL. In contrast, no effect of the anti-IgG<sub>1</sub> antibody on LA-induced oxLDL uptake by endothelial cells was observed (Fig. 8).

**DISCUSSION**

The hypothesis that release of fatty acids during lipoprotein lipase-mediated triglyceride hydrolysis may be atherogenic by causing endothelial injury was first proposed in the 1970s by Zilversmit (15). Today, it is generally accepted that selected fatty acids, and especially n-6 unsaturated fatty acids derived from the hydrolysis of lipoproteins, may cause ED. Of all the fatty acids, LA appears to be the one with the most profound and deleterious effects on endothelial barrier function (4,16). This could be related to the very low basal activity of elongase and desaturase enzymes in endothelial cells, thus preventing arachidonic acid formation from LA in these cells (17). Mechanisms of LA-mediated ED are numerous. They include disruption of endothelial cell integrity with alterations of functions of gap-junctions proteins (18), cell morphology (19), membrane-bound enzymes (20), peroxysome (21), and induction of apoptotic cell death (22). Last but not least, accu-

mulating evidences suggest that increase in intracellular oxidative stress and calcium levels (10,23) could be the main mechanisms by which LA exerts its harmful effects on endothelial cells.

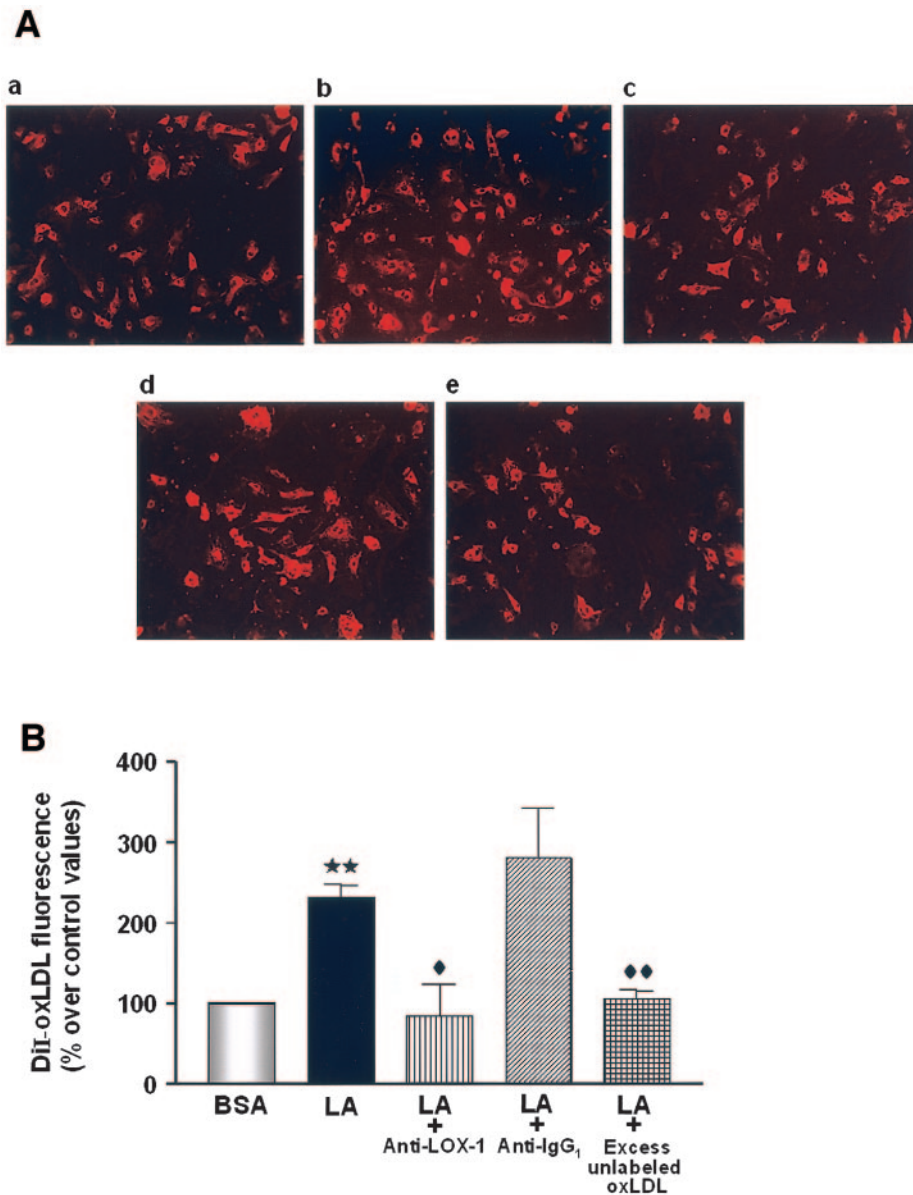
The significance of fatty acids, and especially LA, in the development of ED associated with human diabetes is supported by several observations. First, elevated levels of fatty acid-containing lipoproteins and circulating levels of free fatty acids are reported in diabetes (24,25). Second, upregulation of stearoyl-CoA desaturase by glucose may enhance the conversion of saturated to unsaturated fatty acids in the diabetic state (26). Third, lipoprotein lipase, the rate-limiting enzyme for triglyceride hydrolysis (15), is increased in patients with type 2 diabetes (27) and upregulated by fatty acids, including LA (28). Such high lipoprotein lipase activity in diabetes is likely to favor endothelial cell exposure to high levels of free fatty acids. Finally, increased concentrations of LA have been documented in all LDL subfractions in patients with type 2 diabetes (13).

A role for LOX-1 as a major pathogenic determinant of ED associated with type 2 diabetes has recently been proposed. Consistent with this concept, it has been shown that endothelial LOX-1 is increased in diabetic animals and upregulated by metabolic and inflammatory factors dysregulated in diabetes, including glucose (7), advanced glycation end products (8), and C-reactive protein (9). The present study, which demonstrates that LA, at concentrations similar to those found in diabetic patients (29), upregulates endothelial LOX-1, further supports a role for this receptor in diabetes-related ED. Induction of LOX-1 by LA appears to be exerted at the transcriptional level, as reflected by the parallel increase in LOX-1 gene and protein expression.

Activation of the transcription factor NF- $\kappa$ B is critical for the expression of inflammatory genes involved in ED, and evidence exists that LA activates this transcription factor and induces NF- $\kappa$ B-dependent gene expression in endothelial cells (23,30). Consistent with these data, we found that LA enhances the binding of nuclear proteins to the NF- $\kappa$ B consensus sequence of the LOX-1 gene promoter (14) and that inhibition of NF- $\kappa$ B totally prevented LA-induced LOX-1 expression. On the whole, these data strongly suggest that activation of the LOX-1 gene by LA involves NF- $\kappa$ B.

Oxidative stress is a critical signal transduction pathway involved in NF- $\kappa$ B activation, and several evidences suggest that LA-mediated ED involves increased cellular

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**FIG. 8.** Effect of LA on oxLDL uptake by endothelial cells. HAECs were incubated for 23 h with LA (0.2 mmol/l), and then incubation was pursued for 1 h in the presence or absence of saturating amounts (20  $\mu$ g/ml) of LOX-1 or IgG<sub>1</sub> antibodies. At the end of the incubation period, cells were exposed for 3 h to DiI-oxLDL (80  $\mu$ g/ml) in the presence or absence of a 500-fold excess of unlabeled oxLDL. After washing, fluorescence of DiI was detected in the cytoplasm of HAECs by fluorescence microscopy (A): a) BSA-treated cells; b) LA-treated cells; c) LA-treated cells + LOX-1 antibody; d) LA-treated cells + IgG<sub>1</sub> antibody; e) LA-treated cells + excess unlabeled oxLDL. B: Fluorescence measured at 520/564 nm. Data are means  $\pm$  SE of three different experiments. \*\* $P$  < 0.01 vs. BSA; ◆ $P$  < 0.05 vs. LA; ◆◆ $P$  < 0.01 vs. LA.

oxidative stress. This assumption is mainly based on results from *in vitro* studies showing, in LA-treated endothelial cells, an increased production of reactive oxygen species and lipid hydroperoxides (23) with significant depletion in intracellular glutathione levels (11). Additional support for this hypothesis comes from the observations that LA induces, through oxidative stress, several endothelial cell inflammatory genes *in vitro* (10,31,32) and increases oxidative stress *in vivo* (33). Like many other endothelial antigens involved in endothelial cell activation/dysfunction, regulation of LOX-1 expression is redox-sensitive (34), and molecules that induce oxidative stress, such as homocysteine and angiotensin II, enhance LOX-1 expression (34,35). Our results showing that vitamin C and NAC inhibit the stimulatory effect of LA on LOX-1 protein expression clearly identify endothelial LOX-1 as a novel oxidative stress-sensitive target for LA. In agreement with previous studies (36,37), we found that NADPH oxidase inhibitors prevented LA-induced protein expression, thus indicating that NADPH oxidase is one of the enzymatic superoxide sources activated by LA. Considering the po-

tential key role of LOX-1 in ED, these results further support the concept that oxidative stress may constitute an important mechanism responsible for LA-mediated ED.

Superoxide per se is a strong stimulus for the activation of PKC (38). PKC activation has been implicated in ED associated with type 2 diabetes (1), and LA has been shown to activate PKC activity in various cell types (39–41). Considering the causal link between oxidative stress and PKC activation and the well-documented role of this kinase in NF- $\kappa$ B activation, we elaborated a tentative model in which sequential activation of the redox-sensitive PKC and NF- $\kappa$ B signaling pathways could mediate the regulatory effect of LA on endothelial LOX-1 gene expression. Our data, which demonstrate that calphostin C totally abolished the stimulatory effect of LA on NF- $\kappa$ B activation and LOX-1 protein expression, support this hypothesis. Furthermore, our finding that incubation of the cells with GF109203X, a selective inhibitor of the conventional PKCs, exerts identical effects and that LA activates PKC- $\alpha$ , - $\beta$ <sub>I</sub>, - $\beta$ <sub>II</sub>, and - $\gamma$  further suggests a role for these isotype-specific PKC isoforms in the induction of

endothelial LOX-1 by LA. These results are in agreement with previous studies showing that fatty acids activate preferentially “soluble”  $\alpha$ ,  $\beta$ , and  $\gamma$  isoenzymes of PKC (42,43) and that LA activates PKC- $\alpha$  and - $\beta$  in mouse mammary epithelial cells (44) and PKC- $\alpha$  in rat pancreatic acinar cells (41).

Endothelial cells do express different types of scavenger receptors (SRs), including CD36, SR-A, SR-B, and LOX-1. Although the role of fatty acids on vascular cell SR expression has been poorly investigated, studies have shown that polyunsaturated fatty acids and myristic acid increase the expression of hepatic SR-B1 expression (45,46) and that LA enhances CD36 expression in human monocytic cells (47). To the best of our knowledge, the present study demonstrates for the first time a role for fatty acids in vascular LOX-1 regulation. In agreement with the notion that LOX-1 mediates the uptake of oxLDL by endothelial cells (5), we found that upregulation of LOX-1 by LA is associated with significant increased uptake of oxLDL by HAECs. Taken collectively, these data suggest a new mechanism by which LA may promote ED—that of triggering the toxic effects of oxLDL on vascular endothelium through LOX-1.

Multiple evidences have linked LA to atherosclerosis. First, it has been shown that adipose tissue levels of LA correlate with the degree of coronary artery disease (48) and with the appearance of new atherosclerotic lesions in coronary arteries (49). Second, evidence has been provided that concentrations of LA are increased in the phospholipidic fractions of human coronary arteries in cases of sudden cardiac death due to ischemic heart disease (50). Finally, a study in the Israeli population, which has one of the highest dietary polyunsaturated/saturated fat ratio in the world, concluded that diets rich in n-6 fatty acids may contribute to an increased incidence of atherosclerosis (51). Considering the potential key relationship between LA and atherogenesis, induction of endothelial LOX-1 expression by LA may represent a new mechanism by which this fatty acid may favor the development of atherosclerosis. Strategies aimed at reducing vascular LOX-1 expression could be beneficial in reducing atherosclerotic lesion development associated with human diabetes.

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