LDL Induces Transcription Factor Activator Protein-1 in Human Endothelial Cells

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Abstract—Low density lipoprotein (LDL) has been shown to perturb endothelial cells, with manifestations ranging from alterations in free radicals and arachidonate metabolism to stress fiber formation and monocyte recruitment. Some of these changes are regulated by LDL at the transcriptional level. Using mobility shift assays with consensus sequences for various transcription factors, we have detected an increase in activator protein 1 (AP-1), but not nuclear factor-κB (NF-κB), binding in human umbilical vein endothelial cells exposed to LDL. Following transfection, AP-1–driven chloramphenicol acetyltransferase and AP-1–driven luciferase are upregulated by LDL. In contrast, there is no effect on NF-κB–driven chloramphenicol acetyltransferase. AP-1 increases in a biphasic fashion, with the first peak occurring 6 hours after and the second 48 hours after exposure to LDL. This AP-1 binding increase involves c-Jun, but not c-Fos, as shown by gel supershift, Northern hybridization, and Western blotting analyses. c-Jun mRNA levels are elevated by 9 hours after and remain so until at least 24 hours after exposure to LDL. c-Jun protein levels increase at 12 hours and continue to rise for 24 hours after exposure to LDL. Moreover, this LDL-increased AP-1 binding is suppressed by several protein kinase (PK) inhibitors: the PKC inhibitor calphostin C, the cAMP–dependent PK inhibitor H89, and the tyrosine PK inhibitors genistein and lavendustin A. This study demonstrates that (1) LDL is an endothelial agonist distinct from other cell stimulators, such as cytokines, endotoxin, and phorbol 12-myristate 13-acetate, because LDL appears to activate human umbilical vein endothelial cells predominantly through the transcription factor AP-1 and not NF-κB; and (2) LDL increases AP-1 via mechanisms involving multiple kinase activities and c-Jun transcription. (Arterioscler Thromb Vasc Biol. 1998;18:473–480.)

Key Words: LDL ▪ activator protein 1 ▪ c-Jun ▪ c-Fos ▪ human umbilical vein endothelial cells

Low density lipoprotein is a well-established risk factor for atherosclerosis.1–4 When ECs are incubated with LDL in concentrations associated with premature coronary artery diseases, the cells become activated.5–11 Manifestation of this activation ranges from an alteration in free radicals5 and arachidonate metabolism6,7 to stress fiber formation8 and monocyte recruitment9 via ICAM-110 and VCAM-111 induction.11 Many inducible molecules in ECs, eg, VCAM-1, ICAM-1, and monocyte-specific chemoattractant protein-1 (MCP-1), contain a number of AP-1–like and NF-κB–like binding motifs within their gene promoter regions.12–15 Both AP-1 and NF-κB transcription factors have been reported to be involved in the regulation of these molecules.11,12,15–17 Recently, when the effects of LDL on VCAM-1 promoter activation were studied, we found LDL activation of AP-1 but not NF-κB.11 Thus, LDL-activated AP-1 may play an important role in the perturbation of ECs.

AP-1 is a key transcription factor that translates external stimuli into both short- and long-term changes of gene expression.18,19 AP-1 proteins consist of a variety of homodimers and heterodimers, including members of the Fos, Jun, and CREB/ATF families. These dimers are generated through the interactions between the leucine zipper motifs on each monomer.20 Different members of the Fos, Jun, and CREB/ATF families exhibit different structural features. Such features lead to subtle differences in their DNA binding and transcriptional activation properties, and in turn, suggest specific functions in gene regulation for individual dimers.19,20 The activity of AP-1 is regulated by complex mechanisms. This regulation occurs through interactions with specific PKs and a variety of transcriptional coactivators. These mechanisms include posttranslational events acting on preexisting AP-1 proteins and transcriptional activation leading to increased amounts of AP-1 proteins.

In this study, the goal of which was to identify the transcription factor(s) activated by LDL in human ECs, LDL is shown to be an endothelial agonist distinct from other cell stimulators. Specifically, in cell culture medium containing

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20% FBS, LDL activates HUVECs predominately through AP-1, whereas other cell stimulators activate these cells primarily through the transcription factor NF-kB. On the basis of these results, which are the first to document such an effect of LDL in EC modulation, we propose a pathway for LDL activation of ECs that is distinct from other endothelial agonists.

**Methods**

**Reagents**

All PK inhibitors were from LC Laboratories. TNF-α was a generous gift from Knoll Pharmaceutical, Whippany, NJ. Nonfrozen human plasma was obtained from the San Bernadino Blood Center, San Bernadino, Calif. Collagen was from Collaborative Biomedical Products. Collagenase was from Worthington Biochemical Corp. Recombinant human fibroblast growth factor was a generous gift from Dr J.A. Thompson, University of Alabama, Birmingham. [α-32P]dCTP (3000 Ci/mmole) and [γ-32P]ATP (3000 Ci/mmole) at 10 μCi/mL were from ICN Biomedicals. The DECApriming II DNA labeling kit was from Amersham. Antibodies for Western blotting and the ECL system were from Amersham Inc. In brief, cells were allowed to swell on ice in a hypotonic buffer, their nuclei were pelleted, and nuclear proteins were extracted in a high-salt buffer. To minimize proteinysis, all buffers contained freshly added 0.2 mmol/L PMSF, 0.5 mmol/L DTT, 5 μg/mL aprotinin, 5 μg/mL leupeptin, and 5 μg/mL antipain. The nuclear extract was snap-frozen in liquid N2 and stored at −80°C. Protein concentrations were determined by the BCA method of Pierce.

**Electrophoretic Mobility Shift Assay**

The consensus sequences for transcription factors AP-1 and NF-kB were end-labeled with [γ-32P]ATP using T4 DNA kinase. Unincorporated [32P]ATP was removed via a Sephadex G-25 spin column. Binding of the labeled oligonucleotides to their corresponding factors was performed according to the method described with modifications. In brief, a specific amount of nuclear protein (6 μg for NF-kB and 3 μg for AP-1) was incubated with labeled oligonucleotide at room temperature for 20 minutes in a buffer containing 20 mmol/L HEPESE (pH 7.9), 90 mmol/L KCl, 1 mmol/L MgCl2, 1 mmol/L EDTA, 0.5 mmol/L DTT, 0.1 mg/mL poly(dI-dC) · (dI-dC), and 4% glycerol. Reaction mixtures were resolved on 5% nondenaturing polyacrylamide gels, and the binding patterns were detected by autoradiography. To test the specificity of binding, a 100-fold molar excess of unlabeled competing (or irrelevant) oligonucleotide was used for competition experiments. To identify the components involved in the LDL-increased AP-1 binding, a supershift assay was performed. The nuclear extracts were incubated with antibodies for 3 hours on ice before addition of the labeled probes.

**Western Blotting Analysis**

Protein analysis was performed according to the manufacturer’s protocol using the ECL system from Amersham Inc. In brief, 25 μg of nuclear extract mixed in sample buffer was boiled for 5 minutes. Proteins were separated on a 10% SDS polyacrylamide resolving gel with a 4% stacking gel and then transferred from the gel to a nitrocellulose membrane. After being blocked in 5% blocking reagent in Tris-buffered saline–TWEEN 20 (TBS–TWEEN), the membrane was incubated with primary antibodies against c-Jun or c-Fos for 1 hour at room temperature. After three washes with TBS–TWEEN, the membrane was incubated with a secondary antibody conjugated with horseradish peroxidase for another hour at room temperature, followed by three washes in TBS–TWEEN. The proteins were detected using ECL detecting reagents and autoradiography. Nuclear extracts from PMA-treated cells served as a positive control.

**Northern Hybridization**

Total RNA was isolated by the guanidinium isothiocyanate/CsCl ultracentrifugation method and subjected to Northern analysis for c-Jun and c-Fos expression. The c-Jun cDNA was from Dr L. Ransone, Salk Institute, La Jolla, Calif, and the c-Fos cDNA from Dr T. Curran, St Jude Children’s Research Hospital, Memphis, Tenn. The probes were labeled with [α-32P]dCTP by the DECApriming II method of Amersham.
Construction of pTATA-luc and p3xAP-1–TATA–luc Plasmids

A DNA fragment containing the TATA box of the herpes simplex virus thymidine kinase gene (HSV-TK) was cloned into pGL2-Basic (Promega) upstream from the luc gene to generate pTATA-luc. A double-stranded oligonucleotide containing a general AP-1 consensus binding site (Promega) was phosphorylated for ligation with T4 polynucleotide. It was ligated into the EcoRV site of pBluescript (SK+), by using a 120:1 oligonucleotide-to-vector molar ratio. After transformation into bacteria, a plasmid containing the vector with an insert consisting of three copies of the oligonucleotide was identified by DNA sequencing. The trimeric oligonucleotide was then removed from pBluescript (SK+) by using Kpnl and SalI and cloned upstream from the TATA box in TATA-luc to generate p(AP-1)3–TATA–luc.

Transfection

The promoter reporter constructs for assessing AP-1 and NF-κB activities, p3xAP1–37TKcat and p2xB-B-37TKcat, and their vector p–37TKcat were from Dr. M. Meyer, European Molecular Biology Laboratory, Heidelberg, Germany.21 Plasmid pRSV–β-gal, included in all transfection experiments for determining the relative transfection efficiency, was the generous gift of Dr. T. Parks, Boehringer Ingelheim Pharmaceuticals Inc, Ridgefield, Conn. All plasmid DNAs were purified through two rounds of CsCl/ethidium bromide equilibrium centrifugation.21

HUVECs were seeded on 100-mm dishes and incubated overnight in EC medium. The next morning, the medium was switched to Dulbecco's modified Eagle's medium with 5% FBS for 2 hours before the transfection was performed. Superocharged DNA was precipitated in calcium phosphate in the HEPES-buffered saline solution, pH 7.08 to 7.12, and then added dropwise to the dishes. After a 3 to 4-hour incubation with the calcium phosphate/plasmid mixture, the cells were washed with PBS and switched back to the regular EC medium. Each of the transfectants was cotransfected with pKS–β-gal to normalize the transfection efficiency and standardize the comparison. The posttransfected cells were then incubated for 48 hours with or without 220 mg/dL LDL. A parallel transfected group was treated with 100 ng/mL PMA for 16 hours before the samples were collected as a positive control. The promoter activities were measured by their reporter luc. Transfection results were expressed as luc activity normalized against β-gal.

Transfection of COS-7 cells was performed in six-well plates by using the DEAE-dextran method with the ProFection kit from Promega. The cells were transfected with AP-1 (p3xAP1–37TKcat) or NF-κB (p2xB-B-37TKcat) reporter gene constructs and pRSV–β-gal at a 5:1 ratio. The posttransfected cells were then incubated for 48 hours with or without 220 mg/dL LDL. A parallel transfected group was treated with 100 ng/mL PMA for 16 hours before the samples were collected as a positive control. The promoter activities were monitored by their reporter CAT, and the amount of CAT protein in the cell lysate was quantified by a CAT ELISA kit. Transfection results were expressed as CAT protein normalized against β-gal.

Results

LDL Increases AP-1 Binding Activity

Confluent HUVECs were incubated in EC medium with LDL for 6 hours or with 10 ng/mL TNF-α or 10 ng/mL PMA for 2 hours. Binding of AP-1 was increased by LDL, TNF-α, or PMA as shown in Fig 1a. LDL had a greater effect than TNF-α in elevating AP-1 binding. In contrast, NF-κB, which was markedly activated by TNF-α and PMA, was virtually absent in LDL-exposed HUVECs. LDL increased AP-1 binding in a dose-dependent fashion beginning at 160 mg/dL. This effect was maximal at 240 mg/dL (Fig 1b). The increase in AP-1 binding in HUVECs is biphasic as revealed in a time-course study: the first peak appeared at 6 hours and the second after 48 hours of exposure to LDL (Fig 2). Basal AP-1 binding was not changed as observed at all time points (data for only two time points shown). There was no AP-1 change relative to control cells incubated with BHT (1 to 4 μmol/L) or EDTA (0.001% to 0.005%); these are the amounts of BHT or EDTA contained in the LDL incubation medium. Also, no effect was observed in cells exposed to M199 used in the last LDL dialysis. With a gel shift assay, neither AP-1 nor NF-κB binding was increased in any of the above controls after a 6-hour incubation (data not shown).

LDL Functionally Increases AP-1–Dependent Protein Expression

LDL increased AP-1 but not NF-κB binding as demonstrated by gel shift assays. To further determine whether LDL could activate an AP-1–driven-promoter, we examined the effect of LDL by transfecting COS-7 cells with the following constructs: p3xAP1–37TKcat, p2xB-B-37TKcat, and the enhancer-less p–37TKcat. Fig 3a shows the averaged results of five independent experiments. p3xAP1–37TKcat was more efficient and produced a higher basic level of CAT protein than did p–37TKcat and p2xB–37TKcat. LDL doubled the pro-
moter activities driven only by the AP-1 motif (P<.01) but not the activity of the promoter driven by the NF-κB motif. PMA, on the other hand, significantly activated both AP-1– and NF-κB–dependent reporter CAT activities (P<.001). Thus, LDL specifically regulates genes driven by the AP-1 motif and has no effect on the NF-κB–dependent reporter, which is highly responsive to PMA. To confirm this finding in HUVECs, the TATA box of the HSV-TK promoter with or without the trimeric AP-1 binding sequences was constructed into a more sensitive reporter, the luc vector. These constructs, pTATA-luc and p3xAP-1–3TATA–luc, were transfected into HUVECs. After exposure to LDL, the samples were collected and assayed for luc expression. As shown in Fig 3b, similar results were observed in HUVECs and COS-7 cells. LDL consistently induced AP-1–driven luc activity 2.13-fold compared with basal controls (25.60±8.12 versus 11.99±5.82, P<.05). Therefore, in both cell types, LDL was equally effective in activating AP-1–driven gene expression.

LDL-Induced AP-1 Binding Does Not Involve c-Fos

To further address the question of which members of the AP-1 family are present in nuclear extracts from LDL-stimulated ECs, supershift experiments were implemented. As shown in Fig 4, binding of AP-1 from LDL-treated cells’ nuclear extracts could be supershifted by antibodies against c-Jun and JunD, but not by antibodies against c-Fos and p65, a component of NF-κB. The results suggest that the LDL-increased AP-1 dimers in the nuclear extract largely contain c-Jun and JunD. Two different antibodies against c-Fos were tested in this study: one antibody (catalog No. sc-52, Santa Cruz Biotechnology Inc.) that reacts specifically with c-Fos and the other (catalog No sc-413, Santa Cruz Biotechnology Inc.) that reacts broadly with all Fos family members. Neither antibody could supershift the AP-1 band. The same antibodies could supershift the increased AP-1 band in a nuclear extract treated by PMA. Furthermore, since the AP-1 band could be completely blocked by unlabeled consensus AP-1 and partially decreased by unlabeled consensus CREB, but not by the NF-κB sequence, the involvement of CREB members cannot be ruled out.

LDL Induces c-Jun but Not c-Fos in mRNA and Protein Levels

To determine the effects of LDL on the upregulation of subunits of AP-1 proteins and to further study the mechanism of AP-1 increases by LDL, a time-course study by Northern blotting and Western blotting analyses was performed. As shown in Fig 5, incubation of HUVECs with 220 mg/dL LDL for as long as 24 hours increased the steady-state levels of c-Jun mRNA. This increase started at 9 hours and rose for at least
another 15 hours. In accordance with our findings in the supershift study, an increase of c-Fos was not observed by incubation with LDL at any time point (Fig 5a). As positive controls, these HUVECs could be induced to express both c-Jun and c-Fos by incubation with 50 ng/mL of PMA. In Northern analysis, PMA significantly increased both c-Jun and c-Fos mRNA within 15 minutes, reaching a peak at 30 minutes for c-Fos and at 45 minutes for c-Jun (Fig 5b). In Western blotting results, c-Jun protein, but not c-Fos, in nuclear extracts was also increased at 12 hours and remained at a high level until at least 24 hours after exposure to LDL (Fig 6).

LDL-Induced AP-1 Binding Is Suppressed by Inhibitors of Various PKs

Since we observed that LDL increased AP-1 binding earlier than c-Jun upregulation at the mRNA and protein levels, we reasoned that posttranslational activation of AP-1 might be occurring. To investigate the possible role of PK activity in the induction of AP-1 by LDL, the effects of a group of PKA, PKC, and PTK inhibitors were examined. A 30-minute treatment with the PKA inhibitor H89, the PKC inhibitor calphostin C, or the PTK inhibitors genistein or lavendustin A was applied prior to a 6-hour incubation of LDL in HUVECs. As shown in Fig 7, all of these inhibitors appreciably reduced the LDL-induced AP-1 binding. These reductions of AP-1 binding by PK inhibitors are not due to general cell toxicity, since no increase in lactate dehydrogenase leakage into the medium was detected in cells with PK inhibitor pretreatments, c-Fos mRNA within 15 minutes, reaching a peak at 30 minutes for c-Fos and at 45 minutes for c-Jun (Fig 5b). In Western blotting results, c-Jun protein, but not c-Fos, in nuclear extracts was also increased at 12 hours and remained at a high level until at least 24 hours after exposure to LDL (Fig 6).

Figure 4. Components involved in AP-1 binding increased by LDL. HUVEC nuclear extracts were prepared from cells incubated with EC medium in the presence of LDL (220 mg/dL) for 6 hours or with PMA (10 ng/mL) for 2 hours. A gel shift assay was performed as mentioned above. For supershift, nuclear extracts were incubated with antibodies for 3 hours on ice before adding the labeled AP-1 probe. Nuclear extracts from untreated (lane 1), LDL-exposed (lanes 2–10), and PMA-treated (lanes 11–13) HUVECs were analyzed for binding activity. A 100-fold molar excess of unlabeled AP-1 probe (lane 3), NF-κB probe (lane 4), or CREB probe (lane 5) was added as specific and nonspecific competitors. Antibodies against c-Jun, c-Fos, JunD, and NF-κB p65 were included in the protein-DNA complex as labeled. Data presented are representative of 3 independent experiments.

Figure 5. Effects of LDL on c-Jun and c-Fos mRNA levels. a, Confluent HUVECs were incubated in EC medium with LDL (220 mg/dL) for different periods of time as indicated. An untreated sample was collected at 0.5 hour (lane 1) as a control. b, Confluent HUVECs were incubated in EC medium with 50 ng/mL PMA at different periods of time as indicated. RNA was isolated by the guanidinium isothiocyanate/CsCl ultracentrifugation method. Samples of 15 μg of total RNA were resolved by gel electrophoresis and then hybridized with α-32P-labeled c-Jun and c-Fos cDNA, respectively, von Willebrand factor (vWF) mRNA levels from corresponding samples were also included to show equal loading. Resulting hybridization bands were quantified by densitometry and normalized against vWF. Relative density is expressed as a percentage of basic control (lane 1). Results shown are representative of 3 independent experiments.

Figure 6. Effects of LDL on c-Jun and c-Fos protein expression. Confluent HUVECs were incubated in EC medium with 220 mg/dL LDL for different periods of time as indicated, and nuclear proteins were extracted. Nuclear extract (25 μg) mixed in sample buffer was separated on a 10% SDS polyacrylamide resolving gel and then transferred to a nitrocellulose membrane. After being soaked in 5% blocking reagent, the membrane was incubated with primary antibodies against c-Jun or c-Fos, followed by incubation with a secondary antibody conjugated with horseradish peroxidase. The proteins were detected by autoradiography with ECL detecting reagents. Nuclear extracts from PMA-treated cells were used as a positive control. Results shown are representative of 3 independent experiments.

Figure 7. Effects of PK inhibitors on AP-1 binding induced by LDL. Before the 6-hour exposure to LDL, HUVECs were pretreated for 30 minutes with the PKA inhibitor H89 (5 μmol/L), the PKC inhibitor calphostin C (CPC, 100 nmol/L) or the PTK inhibitors genistein (GEN, 50 μmol/L) or lavendustin A (LAV, 1 μmol/L). Nuclear extracts were then prepared and assayed by gel shift. AP-1 bands were quantified by densitometry, and relative density was expressed as a percentage of basic control. Results shown are representative of 3 independent experiments. Within each experiment, 2 repetitive samples were run.
as assessed by an in vitro toxicology assay kit (Sigma; data not shown). This result suggests that multiple kinase activities, especially those of PKC and PTK, may be involved in the process of AP-1 activation by LDL.

**Discussion**

The present report has examined the hypothesis that human native LDL activates human ECs by way of the transcription factor AP-1. This lipoprotein molecule regulates transcription factor AP-1–binding protein in ECs. Our findings demonstrate for the first time that LDL can increase AP-1 binding biphascially in human ECs. Biphasic AP-1 binding, albeit that induced by shea stress, has also been previously observed in ECs, but these were of bovine origin. Analysis of this latter result, however, was limited by the use of gel shift assays only. In the present study, the LDL–increased c-Jun gene expression was seen at the mRNA and protein levels. c-Jun mRNA and protein were increased after the first peak of AP-1 binding. Thus, LDL appears to regulate AP-1 activity in two steps: (1) a posttranslational event acting on preexisting c-Jun molecules, leading to the first peak of increased AP-1 binding, and (2) induction of c-Jun gene expression, leading to an increase in the total amount of c-Jun, resulting in a second peak of AP-1 binding. These findings may be explained by positive autoregulation of AP-1 on the c-Jun promoter. first demonstrated this autoregulatory loop in PMA-treated HeLa TK− and HepG2 cells. The variant AP-1–like sequence 5′-GT GACATCAT-3′ in the c-Jun promoter region seems to be the key element responsible for this response. Since this site is different from typical AP-1 sites, it may be recognized only by a subset of the complexes that bind to the consensus TPA response element (TRE) sequence. c-Jun homodimers and c-Jun-ATF-2 heterodimers are the most likely candidates. Our present results may represent an example of this model in a paradigm simulating vascular injury.

The positive autoregulatory loop of AP-1 on the c-Jun promoter is an attractive model for signal amplification and conversion of transient early events into long-term effects on gene expression. Positive autoregulation, however, may occur in only some cells under certain conditions. Fos proteins can associate with any of theJun proteins to generate stable heterodimers with higher DNA-binding activity than that of Jun homodimers. In most tissues c-Fos expression is highly regulated, and its mRNA is expressed at only relatively low levels; however, it can be rapidly and transiently induced as early as 10 to 15 minutes after the addition of growth factors, PMA, cytokines, or serum. Following a brief peak of expression, c-Fos mRNA levels are substantially reduced and remain at basal levels in the absence of external stimuli. Similar patterns of both c-Jun and c-Fos expression were observed in HUVECs after the addition of PMA. However, LDL did not induce c-Fos at either the mRNA or protein level in cell culture medium containing 20% FBS; this may explain why LDL–induced c-Jun proteins form homodimers and initiate a positive autoregulatory loop. Since the anti-Fos antibody used in this study was broadly cross-reactive with other members of the Fos family, it is unlikely that a member of the Fos family is involved in LDL–induced AP-1 binding. Moreover, AP-1 may be only one of the transcription factors activated by LDL, or the c-Jun affected may only be within the subset of those AP-1 proteins specifically increased by LDL. In addition to increasing AP-1, LDL increases consensus CREB binding. Both AP-1 and CREB bands were blocked by the same unlabeled probe and partially cross-competed with each other by cold consensus oligonucleotides (data not shown). In vivo foot-printing analysis has revealed that c-Jun and ATF-2 heterodimers bind to both the proximal and distal AP-1 binding sites in the c-Jun promoter. The possible involvement of other subsets of AP-1 complexes, such as c-Jun with other Jun dimers and c-Jun/ATF-2 heterodimers, cannot be ruled out. All components of the AP-1 complex are yet to be examined.

An objective in understanding the role of LDL in atherogenesis is to determine how it alters endothelial function. The present report extends previous studies by examining the mechanism of LDL induction of transcription factors. NF-κB is believed to be a key oxidative-stress transcriptional regulation factor for cytokines, PMA, lipopolysaccharide, and oxidatively modified LDL. All of these agonists induce adhesion molecules and other gene products through NF-κB activation in ECs. NF-κB activation and gene induction by mildly oxidized LDL appear to be due to the appearance of oxidized phospholipids. Recently, we demonstrated that lyso phosphatidylcholine, a phospholipid product of LDL oxidation, can activate NF-κB and induce ICAM-1 expression through a PTK-dependent pathway in HUVECs. However, in the present study, activation of transcription factors other than NF-κB appears to be the major intracellular signaling mechanism of EC activation by LDL.

AP-1 is a key regulator for converting numerous signals into genetic responses. It is, therefore, highly regulated by both the levels of synthesis of c-Jun and c-Fos proteins and by posttranslational modification, principally phosphorylation, of its components. The direct phosphorylation of c-Jun can be catalyzed by members of the MAPK family, JNKs, also known as stress–activated protein kinases (SAPKs). The activities of JNKs/SAPKs, like those of other MAPK-related Pks, depend on the phosphorylation of specific threonine and tyrosine residues believed to be catalyzed by an upstream MAPKK-related PK. PKC activation appears to be a key regulator for AP-1 by PMA activation. On the other hand, TNF-α activates JNK–AP-1 via a PTK pathway. Both PKC and PTK may activate AP-1 through the MAPK-JNK pathway by phosphorylating and activating Ras, or c-Raf kinase. To further understand the pathway leading to activation of AP-1 by LDL in ECs, we have explored the potential role of Pks in AP-1 binding activity. All PKC, PKA, and PTK inhibitors examined in this study partially inhibited LDL–induced AP-1 binding in cultured HUVECs. Thus, multiple kinase activities are likely involved in the mechanism of LDL–induced AP-1 activation. These inhibitory effects by PK inhibitors on LDL–elevated AP-1 are not due to general cytotoxicity, as neither morphological changes nor an increase of lactate dehydrogenase in the medium was observed in the cells treated with any of the inhibitors at the indicated concentrations. HUVECs receiving the same concentration of these inhibitors were used in another experiment. Specifically, PKA and PKC inhibitors did not affect either NF-κB activation or ICAM-1 induction by lyso phosphatidylcholine, but
genistein almost abolished them.36 PKC inhibitors could not prevent the NF-κB increase induced by TNF-α, but they were effective in preventing the NF-κB increase induced by PMA.36 Thus, multiple PK activities may be involved in downstream signaling pathways in EC activation by LDL.

Exposure of mammalian cells to UV light or high-osmolarity solutions strongly induces clustering and internalization of cell surface receptors for epidermal growth factor, TNF-α, and interleukin-1, which results in activation of the JNK cascade.44 Likewise, exposure of ECs to LDL may perturb the cell surface or alter receptor conformation, thereby subverting signal pathways normally used by growth factors or cytokines. Prolonged incubation of ECs with LDL increased cellular cholesterol content and cholesterol phospholipid molar ratios of the EC membrane, resulting in a reduction in the relative EC plasma membrane fluidity.29 LDL-pretreated ECs were hyperresponsive to interleukin-1α or TNF-α induction of adhesion molecules (Zhu et al, unpublished observations 1997). Such enhancing effects may be explained by the results of synergistic effects between transcriptional factors induced by LDL or cytokines or by the results of LDL-changed membrane fluidity and altered cytokine receptors. Thus, LDL may initiate a variety of signal transduction pathways through affecting signaling at the level of the cell surface.

Collectively, the results of this and previous studies allow us to propose a pathway for EC activation by LDL, as shown in Fig 8. Exposure to LDL perturbs the cell membrane or alters receptor conformation and activates multiple kinase activities, such as the MAPK family and JNKs. Activation of JNK or other pathways activates preexisting c-Jun, which forms c-Jun homodimers to induce its own expression via the AP-1 sites on the promoter. This positive autoregulation of c-Jun can increase c-Jun protein and stimulating c-Jun transcription. The increase in AP-1 may be due to the formation of c-Jun homodimers or heterodimers of Jun and transcription factors other than c-Fos. Finally, multiple kinase activities are involved in the mechanism of LDL-induced AP-1 activation in HUVECs. The c-Jun positive autoregulation initiated by LDL may be an important mechanism helping to explain LDL’s chronic and long-lasting effects in EC activation and atherogenesis.

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