Native and Oxidized Low Density Lipoprotein Induction of Tissue Factor Gene Expression in Smooth Muscle Cells Is Mediated by Both Egr-1 and Sp1*

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Tissue factor, in association with factor VIIa, initiates the coagulation cascade. We studied the influences of two pathophysiological stimuli, native (unmodified) and oxidized low density lipoprotein, on tissue factor gene expression in a cell important in vascular remodeling and vascular diseases, the smooth muscle cell. Our results demonstrated that both lipoproteins significantly induced tissue factor gene expression in rat aortic smooth muscle cells; oxidized low density lipoprotein was slightly more potent. Both lipoproteins increased tissue factor mRNA in a concentration- and time-dependent manner. Results from nuclear run-on assays and mRNA stability experiments indicated that increased tissue factor mRNA accumulation in response to the lipoproteins was principally controlled at the transcriptional level. By using lipid extracts of low density lipoprotein or methylation of the intact lipoprotein to block receptor recognition, we showed that this lipoprotein induced tissue factor mRNA via both receptor-independent and receptor-activated pathways. Transfection studies using a series of deleted tissue factor promoters revealed that a 143- to +106-base pair region of the rat tissue factor promoter contained regulatory elements required for lipoprotein-mediated induction. Electrophoretic mobility shift assays showed that the binding activities of the transcription factor Egr-1, but not Sp1, were markedly elevated in response to these lipoproteins. Transfection of site-directed mutants of the tissue factor (TF) promoter demonstrated that not only Egr-1 but also Sp1 cis-acting elements in the TF (−143) promoter construct were necessary for optimal TF gene induction. Our data show for the first time that both low density lipoprotein and oxidized low density lipoprotein induce tissue factor gene expression in smooth muscle cells and that this tissue factor gene expression is mediated by both Egr-1 and Sp1 transcription factors.

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The abbreviations used are: TF, tissue factor; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LDL, low density lipoprotein; LPS, lipopolysaccharide; oxLDL, oxidized LDL; SMC, smooth muscle cell; TNF-α, tumor necrosis factor-α; bp, base pair; SRE, serum response element; PCR, polymerase chain reaction.

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(24). To inhibit oxidative modification, 0.5 mM EDTA was present throughout the isolation procedure. LDL preparations were stored in 0.5 mM EDTA at 4 °C in the dark prior to use in experiments. LDL preparations were used less than 2 weeks after isolation. LDL was oxidized by dialysis against 5 μM ferrous sulfate in 0.15 M NaCl for 23–24 h at room temperature. After oxidation, preparations were dialyzed against 0.15 M NaCl, 0.5 mM EDTA, pH 8.5, to remove Fe2+. The quality of all preparations of LDL and selected preparations of oxLDL was checked for endotoxin level using BioWhittaker kit (QCL 1000). The average endotoxin level for all LDL preparations was 0.00174 ± 0.00236 pg/μg LDL protein (n = 43). The range of the endotoxin level of all preparations was 0.000 to 0.013 pg/μg. Electro- phoretic mobility shift assay (Corning) and thiorbituric acid reactivity (25, 26) were tested in all lipoprotein preparations. Thioarbituric acid reactivities for LDL were 0.1 to 0.9 nmol of malondialdehyde (as standard)/mg LDL cholesterol, and for oxLDL were 3.9 to 8.1. Commercial lipopolysaccharide (LPS, Escherichia coli 0111 B4 from Calbiobech) was used as a positive control in the LPS assay and was also used in experiments testing the possible role of LPS in lipoprotein induction of TF. Liprotein preparations were analyzed for protein (27) and cholesterol (Sigma cholesterol assay kit catalog 352-20, using Sigma cholesterol standards catalog number C0534); LDL and oxLDL concentrations are expressed as μg (protein)/ml, except where specifically indicated. Extraction of LDL lipids was by methods used by us previously (28), and the lipid concentration of the extract was indexed by total cholesterol content.

Methylation—Methylation of LDL has been well documented to block LDL receptor recognition (29). LDL (2–10 mg of protein/ml) in 30 mM sodium borate buffer, pH 8.5–9.0, was methylated by adding formaldehyde (1 μl of 37% w/v formaldehyde per 10 mg of protein LDL every 6 min for 30 min at 4 °C) as described previously (30). Methyl- ation was terminated by dialysis at 4 °C against 0.15 M NaCl, 0.5 mM EDTA. Methylation level was measured using trinitrobenzene-sulfonic acid to quantify alteration in methylation of LDL as reported (17, 33).

Tissue Culture—SMC were prepared from explants of excised aortas of rats as described previously (32). SMC between passages 4 and 14 were used in these studies. Cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Cells were made quiescent by incubation in serum-free Dulbecco’s modified Eagle’s medium for 48 h prior to the addition of LDL, oxLDL, or other agonists as described previously (17, 33).

Nuclear Analysis—Total cellular RNA was isolated by using TRIzol reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. Total RNA (6–8 μg) was subjected to denaturing electrophoresis in formaldehyde/agarose gels. RNA was blotted onto GeneScreen (NEN Life Science Products) membranes and hybridized with radiolabeled cDNA probes (34). A 685-bp EcoRI fragment of rat TF cDNA (GenBank Accession number M70781) was a gift from Dr. Mark B. Taubman (Mount Sinai School of Medicine, New York) and was used to detect TF mRNA, whereas glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as an internal control.

Nuclear Transcription Assay— Cultures of 5 × 103 cells were used as indicated in the text and nuclei isolated as described previously (35). Transcription initiated in intact cells was allowed to proceed to completion in the presence of α[32P]UTP, and the RNA was isolated and hybridized to slot-blotted plasmids containing specific cDNA inserts (7 μg/slot), as described previously (36). The α-tubulin gene was used as an internal control, and β-ActinII SK (Stratagene) was used to assess transcript background since rat TF CDNA was inserted in this vector.

TF mRNA Stability—Following a 1 h of lipoprotein stimulation or control (untreated) incubation, actinomycin D (Sigma) was added to a concentration of 10 μg/ml. Cells were washed once with phosphate-buffered saline at the stated times and immediately lysed with TRIzol reagent (Life Technologies, Inc.) for RNA isolation. After Northern analysis, densitometric measurements were made, and the relative density (normalized by the density at the time of actinomycin D addition and the amount of GAPDH) was fit to a single exponential, reflecting first-order kinetics for mRNA degradation. Half-lives for the relative density of the nuclear extracts were calculated from the best fit equation for each treatment.

Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared from 1 × 107 cells as described previously (37). Protein concentrations of the nuclear extracts were 0.5–4 μg/μl, as measured using a protein assay dye reagent (Bio-Rad). Oligonucleotides containing four rat TF promoter regions (regions 1–4) were chosen and obtained from Operon Technologies (Alameda, CA). Electrophoretic mobility shift as-

says (EMSA) were performed as described previously (14). Oligonucleo-
tides of consensus or mutant Egr-1, Sp1, and a serum response element (SRE) obtained from Santa Cruz Biotechnology were used in the competition assays in EMSA. Consensus Egr-1, Sp1, or SRE sites are bold, and the underlined regions indicate the mutant bases (for Egr-1, GG → TA, for Sp1 GG → TT; 5′-GGA TCC AGCGGGGCCGAGGCGG-3′ (Egr-1), 5′-ATT CGA TCGGGGCGGGG GAG C-3′ (Sp1) and 5′-GAGA TGT CCATATGAGG ACT-3′ (SRE). For antibody supershift experiments, 2 μg of a rabbit antipeptide antibody to Egr-1 or Sp1 was incubated with the nuclear extracts in the binding mixture for 20 min at room temperature prior to the addition of the radiolabeled oligonucleotide. Anti-Egr-1 (se-110x) and anti-Sp1 (se-59x) rabbit polyclonal antibodies were obtained from Santa Cruz Biotechnology.

PCR Amplification of Fragments of the Rat TF Promoter, Cloning, and Plasmid Preparation—Based on the published sequence of the 5′-flanking region of the rat TF gene (38), we synthesized several primers, which were used for amplification of regions of the promoter. A 660-bp region upstream of the transcription site, as well as several shorter fragments were amplified using rat genomic DNA and cloned into the luciferase reporter plasmid pXP2 (39). Constructs TF1–5, TF1–182, and TF1–79 were generously provided by Dr. Taubman. Mutations in the Egr-1 and/or Sp1 sites were generated by PCR. The sequences of wild-type TF (134) and the four plasmids containing mutations in the Egr-1 and/or Sp1 sequences are shown in “Results.” All plasmids were sequenced to confirm base pair substitutions. (Our sequence data revealed a G instead of an A at –74 bp in the TF promoter as reported (38)).

Transient Transfection Assay—SMC were plated at 3 × 105 cells/60-mm dish 24 h prior to transfections. Cells were transfected with 3.5 μg of TF reporter construct using the SuperFect reagent from Qiagen in accordance with the manufacturer’s recommendations. Transfected cells were incubated in serum-free medium for 48 h before a 4-h induction with either LDL or oxLDL. Luciferase activity was determined using the Luciferase Assay System (Promega) and ML2250 luminometer (Dynatech). pSV-β-Gal DNA (0.4 μg) (Promega) was used as an internal control to assess transfection efficiencies. β-Galactosidase activity was measured using LumiGlo 530 Assay Reagent (Lumigen).

RESULTS

Induction of TF mRNA by LDL and OxLDL—Previously we reported that in SMC, cell surface TF activity was dramatically increased by oxLDL but not appreciably increased by LDL (23); however, LDL increased TF mRNA (17). In the present study, we asked whether increased TF activity by oxLDL and increased mRNA by LDL were related to the induction of TF gene expression. Quiescent rat aortic SMC contained low levels of TF mRNA. We found both LDL and oxLDL significantly increased TF mRNA accumulation. The dependence of the enhanced mRNA levels on oxLDL concentration is shown in Fig. 1. For both LDL and oxLDL, maximum responses were observed at about 200 μg/ml. We have tested over 30 different isolates of LDL and all significantly increased TF mRNA levels. Whereas both LDL and oxLDL are capable of inducing TF gene
expression, when matched for lipoprotein (protein) concentration, oxLDL was generally capable of inducing TF gene expression more strongly. (From densitometric assays of Northern analyses, oxLDL stimulation exceeded that of LDL by an average of 38%, n = 8 pairs of LDL and oxLDL data; p < 0.003 by paired t test.) When human aortic SMC were used, the induction of TF mRNA by LDL or oxLDL was also observed (data not shown), suggesting that the response applies to multiple species.

**Rapid TF Messenger RNA Accumulation Was Observed in Response to LDL and OxLDL Stimulation**—Maximum levels of TF mRNA were observed about 90 min after either LDL or oxLDL stimulation (Fig. 2). The increased accumulation of TF mRNA induced by LDL or oxLDL was transient and declined significantly between 2 and 7 h. The time courses of the TF mRNA inductions are similar to those induced by serum and growth factors in HeLa cells and SMC (13, 40); however, the magnitude of the response to lipoproteins was less than that to serum (data not shown).

**Endotoxin (LPS) Is Not Responsible for the Increased TF Gene Expression of LDL**—We and others (41, 42) have reported that LPS can bind to LDL and be delivered to cells via LDL. As a result, we exercise special precautions during the preparation of LDL and oxLDL to avoid LPS contamination. We examined whether the trace amounts of LPS typically measurable on LDL could be contributing to the induction of TF by LDL in SMC. We tested whether these trace amounts of LPS alone can induce TF mRNA, and we tested whether LPS could augment LDL induction of TF gene expression. One pg/ml of LPS alone, which is 2.9-fold above the average amount present in 200 μg/ml LDL, did not increase the TF mRNA level. At LDL levels above 10 ng/ml (29,000 times the average amount present in 200 μg/ml LDL), the induction of TF mRNA was detectable. Importantly, even very high levels of LPS (1 μg/ml) did not synergistically enhance LDL induction of TF mRNA (data not shown), indicating that trace contamination by LPS did not contribute to the TF gene regulation by LDL and suggesting that the signaling pathways of induction of TF gene expression by lipoproteins and LPS are distinct in SMC.

**Effect of LDL and OxLDL on the Stability of TF mRNA**—An increase in TF mRNA level detected by Northern blot analysis can be due to an increase in the rate of transcription, stabilization of previously transcribed mRNAs, or a combination of both mechanisms. TF mRNA is known to have a destabilizing region containing four copies of an AUUUA motif (43). To investigate whether LDL and oxLDL stimulation can modify post-transcriptional mechanisms to stabilize TF mRNA and thus enhance message levels in SMC, we examined TF mRNA stability in cells that were untreated or treated for 1 h with LDL or oxLDL. The cells received 10 μg/ml actinomycin D to stop transcription. We separately determined that TF transcription was completely arrested at this concentration (data not shown). As expected, compared with the untreated group, a 1-h stimulation with LDL or oxLDL significantly increased TF mRNA levels (6- and 10-fold, respectively); however, the treatment with LDL or oxLDL did not markedly affect the TF mRNA degradation rate after arresting transcription, as shown in Fig. 3 for one experiment. The half-lives of the TF mRNA in untreated, LDL-treated or oxLDL-treated cells, calculated using data from two experiments, were 107, 88, and 88 min, respectively. Therefore, treatment of cells with LDL or oxLDL did not stabilize TF mRNA.

**Transcriptional Regulation Controls TF Gene Expression in Response to LDL and oxLDL**—The fact that the LDL and oxLDL could markedly increase TF mRNA without a stabilizing effect on TF mRNA suggested that LDL and oxLDL regulate TF gene expression at the transcriptional level. To assess this possibility further, nuclear transcription run-on assays were performed at 40 and 60 min following LDL and oxLDL stimulation. In unstimulated cells, there was a low basal rate of transcription of the TF gene, consistent with the low levels of TF mRNA observed in Fig. 1. This basal rate of transcription was increased 7.6- and 11.4-fold after 40 min of exposure to LDL and oxLDL, respectively, or 6.2- and 13.4-fold after 1 h exposure to LDL and oxLDL, respectively (Fig. 4). These data, together with the mRNA stability results, demonstrated that the LDL- and oxLDL-induced increases in TF mRNA were controlled at the transcriptional level.

**Receptor Recognition Enhances, but Is Not Required for, LDL Induction of TF Gene Expression**—We tested whether LDL lipids in the absence of the apolipoprotein B (apoB) moiety could induce TF mRNA levels. As shown in Fig. 5, 200 μg of cholesterol/ml of the total lipids extracted from LDL was able to increase the TF mRNA level, although only to about half the level induced by intact LDL at 200 μg of cholesterol/ml. In another approach, LDL was methylated to the extent that 38% of the lysine residues of apoB were blocked, a level shown by others to be sufficient to prevent LDL receptor recognition. (Methylation of 20% or more lysine residues of LDL will reduce binding to the receptor to negligible levels (29, 30).) Methylated LDL also significantly increased TF mRNA accumulation but
The lipid extract of LDL was dissolved in ethanol, and the final ethanol concentration was less than 0.4%.

Nuclei were isolated from SMC untreated or treated with either LDL (200 μg/ml) or oxLDL (200 μg/ml) for the indicated periods, and the rate of transcription of TF gene was examined. The rate of transcription of the tubulin gene was unaltered in this experiment and was used to normalize signals from individual hybridizations. Vector pBlueScript II SK was used as a negative control. Run-on reactions and hybridizations of the purified run-on RNA to the indicated DNA probes were performed as described under “Experimental Procedures.” Input hybridizations of the purified run-on RNA to the indicated DNA probes were performed as described under “Experimental Procedures.” Input counts per min were equalized to 4 × 10⁶ per hybridization. The autoradiograph was exposed for 15 h, and the results were quantitated by scanning densitometry. The nuclear run-on experiment was repeated with similar results to these shown.

Fig. 4. Effects of LDL and oxLDL on the rate of transcription of TF. Nuclei were isolated from SMC untreated or treated with either LDL (200 μg/ml) or oxLDL (200 μg/ml) for 1 h, and the rate of transcription of TF gene was examined. The rate of transcription of the tubulin gene was unaltered in this experiment and was used to normalize signals from individual hybridizations. Vector pBlueScript II SK was used as a negative control. Run-on reactions and hybridizations of the purified run-on RNA to the indicated DNA probes were performed as described under “Experimental Procedures.” Input counts per min were equalized to 4 × 10⁶ per hybridization. The autoradiograph was exposed for 15 h, and the results were quantitated by scanning densitometry. The nuclear run-on experiment was repeated with similar results to these shown.

Further Definitions of cis-Acting Elements and Functional Involvement of Transcription Factors—To define which transcription factors regulate TF gene expression and the precise cis-acting elements in the TF (−143) promoter region mediating the lipoprotein induction, we first examined whether binding activities of transcription factors to the −143-bp TF promoter were induced in response to LDL and oxLDL by performing EMSA. Protein-DNA complexes were identified by incubating nuclear extracts from untreated and LDL- or oxLDL-stimulated SMC (1 h) with radiolabeled oligonucleotides (regions 1–4, defined in Fig. 7A). As shown in Fig. 7B, upon LDL or oxLDL stimulation (1 h), an inducible complex (II) was formed when region 3 was used as a radiolabeled probe. Complexes I and III were constitutively present in untreated and treated cells. There were no detectable changes induced by LDL or oxLDL compared with the untreated cells when radiolabeled regions 1, 2, and 4 were used as probes (Fig. 7B). Therefore, we chose radiolabeled region 3 as a probe in the following EMSA experiments to determine whether the binding of nuclear proteins to the TF promoter region was specific and to elucidate which transcription factor binding activity was increased in response to lipoproteins. A 50-fold molar excess of unlabeled region 3 completely blocked the formation of complexes I, II, and III (lanes 4–6 of Fig. 7C), indicating that the binding is specific. We also employed consensus and mutated Egr-1 and Sp1 sites as competitors, since Egr-1- and Sp1-binding sites were observed in region 3 as shown in Fig. 7A. When 50-fold molar excess of the unlabeled consensus site or the mutant oligonucleotide Egr-1 was used as a competitor, we observed that the consensus oligonucleotide Egr-1 completely blocked the induced complex II (lanes 7 and 8 compared with lanes 2 and 3 in Fig. 7C), whereas the mutant Egr-1 had no effect on the induced complex II (lanes 9 and 10 compared with lanes 2 and 3). This suggested that the induced complex was an Egr-1 complex. When 50-fold molar excess of the unlabeled wild-type Sp1 site was used as a competitor, we observed that the bands of complex I and complex III were removed (lanes 11 and 12 compared with lanes 2 and 3), with no effect on complex II. Moreover, when the unlabeled mutant Sp1 site was used as a competitor, the formations of complexes I, II, and III were not affected (compared lanes 13 and 14 to lanes 2 and 3), suggesting that the constitutive binding activities of complex I and III represented the transcription factor, Sp1. As a negative control, 50-fold excess of the unlabeled commercial consensus site SRE was also used as a competitor. The complexes were unaffected by the competitor SRE site (lanes 15 and 16 in Fig. 7C), again confirming that the formation of complexes I, II, and III was specific.

We then examined the kinetics of complex formation. As shown in Fig. 8A, EMSA revealed that the oxLDL-induced binding activity (complex II) transiently appeared in nuclear but not cytosolic proteins. The induced complex II formed within 30 min, reached a maximum at 1 h, and disappeared after 3.5 h of oxLDL treatment (Fig. 8A). The same result was also observed with LDL (data not shown).

Further identification of the proteins present in these complexes was achieved using antibodies that specifically recog-
nize the transcription factors Egr-1 and Sp1. Other members of the Egr family can bind to the Egr-1 site (44, 45), but these transcription factors are not recognized by the Egr-1 antiserum used. Complex I observed in oxLDL-stimulated cells was abolished by preincubation with an Sp1-specific antibody but not the Egr-1 antibody. Conversely, induced complex II was abolished by preincubation with the Egr-1-specific antibody but not the Sp1 antibody (Fig. 8B). Therefore, we concluded that complexes I and II, formed using oligonucleotides containing the Sp1 site and the Egr-1 site (region 3), represented binding of Sp1 and Egr-1, respectively. In contrast to Sp1 binding, Egr-1 binding activity was not detected in nuclear extracts from quiescent cells but was rapidly induced by stimulation with LDL or oxLDL.

Finally, we conducted site-directed mutagenesis to define the role of cis-acting regulatory element(s) in the TF promoter in response to LDL and oxLDL induction. Our EMSA data in Figs. 7 and 8 showed that Egr-1 binding activity was induced in response to both LDL or oxLDL, suggesting that Egr-1 contributed to regulating TF gene expression. Therefore, we made and transfected TF (−143 mEgr-1), which is an Egr-1 site mutation of wild-type TF (−143) (Fig. 9A). The results revealed that this Egr-1 mutation reduced the LDL and oxLDL induction of TF promoter activity from 4.1- to 2.1-fold and 4.9- to 2.4-fold,
respectively (Fig. 9B). These data indicated that Egr-1 plays an important role in regulating TF gene expression in response to lipoproteins, but the transcription factor Egr-1 alone was not the only transactivator, since the mutant Egr-1 did not completely abolish induction. To determine if Sp1 might also contribute to the increased TF gene expression in response to lipoproteins, we mutated either one Sp1 site or all three Sp1 sites in the TF promoter (region 3) was incubated with either nuclear proteins or cytoplasmic proteins from cells untreated or treated with oxLDL for the times shown. B, identification of proteins present in the protein-DNA complexes using specific antibodies. Antibodies (2 μg) were incubated with nuclear extracts 20 min before addition of the radiolabeled probe (region 3). The Sp1 (complex I) and Egr-1 (complex II) complexes are indicated. Complexes were separated using 6% nondenaturing acrylamide gels.

FIG. 8. The kinetics of the complex induced by oxLDL and the identification of the complexes binding to TF promoter. A, comparison of the time course of EMSA patterns using nuclear proteins and cytoplasmic proteins from cells stimulated with oxLDL. A radiolabeled oligonucleotide containing the Egr-1 site overlapping with Sp1 site of the TF promoter (region 3) was incubated with either nuclear proteins or cytoplasmic proteins from cells untreated or treated with oxLDL for the times shown. B, identification of proteins present in the protein-DNA complexes using specific antibodies. Antibodies (2 μg) were incubated with nuclear extracts 20 min before addition of the radiolabeled probe (region 3). The Sp1 (complex I) and Egr-1 (complex II) complexes are indicated. Complexes were separated using 6% nondenaturing acrylamide gels.

DISCUSSION

The present study demonstrates that the lipids of LDL and oxLDL induce TF gene expression in SMC through a LDL receptor-independent pathway. Our data also revealed a part of the molecular mechanism by which LDL and oxLDL induce TF gene expression in SMC.

Our data show that both LDL and oxLDL dramatically increased TF mRNA in a time- and concentration-dependent fashion. Generally, oxLDL induced slightly higher levels of TF mRNA than LDL. We performed multiple ancillary experiments to examine whether the LDL effect was due to mild oxidation of the lipoprotein. We compared freshly isolated LDL, stored LDL and freshly dialyzed, stored LDL. (Stored LDL is LDL stored at 4 °C in the dark for up to 2 weeks.) These variants induced TF mRNA to the same level (data not shown). Moreover, neither preincubation of LDL with antioxidants (0.4 mM reduced glutathione or 10 μM ebselen for 2.5 h) before addition of LDL to SMC nor adding LDL to SMC in the presence of ebselen (5 μM), glutathione (1 mM), or N,N′-diphenyl-1,4-phenylenediamine (1 μM) had an effect on the LDL induction of TF mRNA (data not shown). These results suggested that the induction of TF gene expression by LDL was not due to small amounts of oxidation products formed during LDL preparation or incubation with cells.

Another potentially confounding factor examined was the possibility that the effects we observed were due to endotoxin contamination of the lipoprotein preparations. Our results indicate that this is unlikely since (a) even those LDL preparations with undetectable endotoxin were effective inducers; (b) endotoxin levels in our preparations were monitored carefully and were very low; (c) a commercial endotoxin, added at levels 2.9-fold above the average endotoxin in our preparations, did not affect TF mRNA levels; and (d) most importantly, there was no synergistic effect found on TF gene regulation when we added endotoxin with LDL.
Lipoproteins have been reported to alter TF expression in other cell types, but their modes of action are apparently distinct from the effects we observed in SMC. In endothelial cells, oxLDL but not LDL was shown to induce TF mRNA and procoagulant activities (18, 19). In pigeon monocyte-macrophages, oxLDL was reported to enhance TF activity (20), and in human monocyte-macrophages, very low density lipoprotein, LDL, and particularly oxLDL augmented TF expression (21). However, in another study of human adherent monocytes, oxLDL alone did not induce TF but enhanced TF expression by LPS (22).

Our nuclear run-on and mRNA stability experiments revealed that LDL and oxLDL elevation of TF mRNA in SMC was principally controlled at the transcriptional level. This result differs from LPS induction of TF mRNA in human umbilical vein endothelial cells and human monocytic THP-1 cells. In endothelial cells Crossman et al. (46) found that the accumulation of TF mRNA was largely dependent on increased mRNA stability. In THP-1 cells, Brand et al. (47) showed that both transcriptional and post-transcriptional regulation played roles in LPS induction of TF mRNA. Interestingly, the recent report of the induction of TF mRNA in human SMC by the platelet-derived growth factor BB (33) is similar to our observation, in that induction was transcriptionally regulated, consistent with a cell type-specific regulation of TF gene expression.

It has been reported that LPS and TNF-α induce TF gene expression in monocyctic cells and endothelial cells through activation of NF-κB and nuclear factor κB transcription factors (9, 10, 48). Serum stimulates TF gene expression in fibroblasts via AP-1 cis-acting element responding to FXa (30, 39). The transcription factor Fos is a component of the AP-1 complex, which is a homo- or heterodimer of the Fos, Jun, and JunB family of activator protein (2459–2465). Serum stimulates TF gene expression in fibroblasts via AP-1 (9, 10, 48). We observed that Egr-1 binding activity was dramatically and transiently induced in response to LDL or oxLDL (Figs. 7 and 8). In contrast, Sp1 binding activity was constitutively expressed in SMC. Sp1 has been reported to activate a set of genes, including the TF gene, in epithelial HeLa cells (14, 50, 51); however, Egr-1 has been shown both to activate and repress transcription in transfection assays (52–54). In the present study, our transfection results showed that the TF (−143) promoter construct responded to LDL and oxLDL induction, indicating that this region of the TF promoter contains at least one cis-acting element responding to LDL and oxLDL. There are three Sp1 sites and an Egr-1 site that overlaps one of the Sp1 sites in this region. Our transfection data, using deleted and mutated TF promoter constructs, revealed that not only the Egr-1 site but also the three Sp1 sites contribute to the optimal induction of TF gene expression in response to LDL and oxLDL.

Our results demonstrate for the first time TF gene regulation in SMC by lipoproteins and reveal a part of the mechanism by which the TF gene is regulated in SMC. The present findings have important implications for a connection between LDL, oxLDL, and TF in atherosclerotic lesions, where they are known to be elevated. TF could play a role as a procoagulant or a local stimulus of SMC proliferation. Understanding TF gene regulation in response to LDL and oxLDL in SMC may help to elucidate factors promoting the development of vascular diseases and suggest novel therapeutic approaches.

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