**Lysophosphatidic Acid Induction of Tissue Factor Expression in Aortic Smooth Muscle Cells**

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**Objective**—Tissue factor (TF), the initiator of the coagulation cascade, is expressed by cells in atherosclerotic lesions. Lysophosphatidic acid (LPA) is a component of oxidized lipoproteins and an agent released by activated platelets. The present study investigated whether and how TF expression is regulated by LPA.

**Methods and Results**—Northern blotting, Western blotting, and TF activity assays demonstrated that LPA markedly induced TF mRNA, protein, and activity in vascular smooth muscle cells. LPA-induced TF expression is primarily controlled at the transcriptional level. Phosphorylation of mitogen-activated protein kinase kinase (MEK) and extracellular signaling–regulated kinases (ERK1/2) was rapidly and markedly induced by LPA. MEK inhibitors U0126 and PD98059 blocked both ERK activation and the increase in TF mRNA. In contrast, the specific p38 MAP kinase inhibitor SB203580 had no effect on LPA-induced TF mRNA increase. The G protein inhibitor, pertussis toxin, abolished LPA-induced phosphorylation of MEKs and ERKs, as well as the induction of TF mRNA.

**Conclusions**—Our data demonstrate that a G protein and activation of MEKs and ERKs mediate LPA-induced TF expression. Our data suggest that elevated LPA could be a thrombogenic risk factor by upregulating TF expression. These results may have important implications in vascular remodeling and vascular diseases. (Arterioscler Thromb Vasc Biol. 2003;23:224-230.)

**Key Words:** atherosclerosis ■ arterial thrombosis ■ lipid/lipoprotein metabolism ■ gene expression
induced TF gene induction in SMCs depends on the activation of a GI protein and the subsequent phosphorylation of mitogen-activated protein kinase kinases (MEKs) and extracellular signaling-regulated kinases (ERKs) but not p38 mitogen-activated protein kinase (MAPK). Our data suggest that induction of TF expression by LPA may accelerate atherogenesis and worsen atherosclerotic lesion vulnerability to thrombotic complications.

Methods

Tissue Culture
SMCs were prepared from explants of excised aortas of rats as previously described.27 SMCs between passages 5 and 17 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 hours as previously described.28,29 LPA used in this study was (16:0) palmitoyl LPA from Avanti Polar Lipids, Inc. SMCs from human arterial tissue were obtained as previously described.27 Human SMCs were used only for TF protein detection in Western blot analysis, because antibody against rat TF is not available.

TF Surface Activity
Cell-surface TF activity was measured with the Actochrome TF activity assay kit purchased from American Diagnostica Inc. TF activity was determined as the peptideyl activity for TF complex formation with recombinant factor VIIa and factor X. The complex converts factor X to factor Xa. The amount of factor Xa generated was measured by its ability to cleave Spectrozyme Xa, a highly specific chromogenic substrate for factor Xa. Rat aortic SMCs were seeded in 12-well plates at a density of 3×10⁴. SMCs were stimulated with LPA (25 μmol/L) for the indicated times. The cells were washed twice with phosphate-buffered saline (PBS). Assay buffer (300 μL, pH 8.4), 25 μL of factor VIIa, and 25 μL of factor X were added, and the plates were stirred on an orbital rotator for 15 minutes at 37°C. Then 25 μL of Spectrozyme factor Xa substrate was added and incubated at 37°C for 20 minutes with constant stirring. Aliquots of the reaction mixture were pipetted into 96-well plates and read along with the standards provided by American Diagnostica on a Universal microplate reader ELX 800 (Bio-TEK Instruments Inc) at 405 nm. TF activity was measured against the linear range of a standard curve. The TF standard curve was established by following the instructions from the vendor. The initial reaction rate was in the linear range of the curve. One milliunit was defined as the change in OD₅₄₀nm by 100 pg TF in 10 minutes at 37°C.

Western Blot Analysis for TF
Total cellular proteins were obtained from human arterial SMCs grown in 100-mm dishes. At the end of each incubation, cell layers were washed twice with PBS, and the protein was extracted in ice-cold RIPA buffer (50 mmol/L Tris-Cl [pH 7.5], 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors (leupeptin, PMSF, and pepstatin; all from Sigma). Cellular DNA was removed by collecting the supernatant after centrifugation at 10 000 rpm in a microcentrifuge for 10 minutes. The same amounts of protein from the lysates of unstimulated cells or cells stimulated with 25 μmol/L LPA were loaded, separated by SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to Hybond-enhanced chemiluminescence membranes (Amersham Pharmacia Biotech). TF was visualized by using a human TF antibody (final concentration, 0.2 μg/mL; American Diagnostica) followed by a 1:5000 dilution of a peroxidase-labeled secondary antibody (final concentration, 0.08 μg/mL). The signal was developed by exposure to film for 1 to 3 minutes with enhanced chemiluminescence (Amersham).

Results

Northern Blot Analysis
Total cellular RNA was isolated by using TRizol reagent (Gibco BRL) according to the manufacturer’s instructions. Total RNA (6 to 8 μg) was subjected to denaturing electrophoresis in formaldehyde/agarose gels. RNA was blotted onto Nytran membranes (Schleicher & Schuell Inc) and hybridized with radiolabeled cDNA probes.30 A 685-bp EcoRI fragment of rat TF cDNA (Genebank accession No. U07619) was a gift from Dr Mark B. Taubman (Mount Sinai School of Medicine, New York, NY) and was used to detect TF mRNA; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as an internal control.

TF mRNA Stability
After 1 hour of LPA stimulation or control (untreated) incubation, actinomycin D (Sigma) was added to achieve a concentration of 10 μg/mL to stop transcription. At the times indicated, cells were washed once with PBS and immediately lysed with TRizol reagent (Gibco) for RNA isolation. After Northern blot analysis, densitometric measurements were made, and the relative density was calculated and normalized to GAPDH. Half-lives for the relative mRNA degradation were calculated from the best-fit equation for the untreated or treated groups.

Nuclear Transcription Assay
Cultures of 5×10⁴ cells were treated as indicated in the text, and nuclei were isolated as described previously.31 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.32 The α-tubulin gene was used as an internal control, and pBluescript II SK (Stratagene) was used to assess transcript background because rat TF cDNA was inserted into this vector.

Measurement of MEK, ERK, and p38 MAPK Activation
To monitor MEK1/2, ERK1/2, and p38 MAPK activation, SDS-PAGE was performed, followed by Western blotting with specific antibodies against phosphorylated MEK1/2, MEK1/2, phosphorylated ERK1/2, ERK1/2, and phosphorylated p38 MAPK, following the manufacturer’s instructions (Cell Signaling Technology).

LPA Induced Cell-Surface TF Pathway Activity
TF pathway activity (factor Xa activity) was measured in intact SMC monolayers incubated with purified clotting factors from America Diagnostica. Quiescent rat aortic SMCs contained low levels of TF activity. We determined the dynamics of TF surface activity by exposure of these SMCs to 25 μmol/L LPA for various times. As shown in Figure 1A, LPA significantly increased TF activity on the surface of SMCs. Maximal levels of TF activity (5.3-fold) were observed 5 hours after LPA addition compared with the basal activity of quiescent SMCs.

LPA Increased TF Protein
To test whether increased TF activity by LPA was correlated with an increase in newly synthesized TF protein, Western blot analysis was performed after a 4-hour LPA stimulation in human SMCs. (Antibody to rat TF was not available; however, we found that human and rat SMCs responded similarly in our system: LPA also enhanced cell-surface TF activity in human SMCs; data not shown). As shown in Figure 1B, TF protein expression was increased by 25 μmol/L LPA.
LPA Induced TF mRNA

Northern blot analysis revealed that TF mRNA accumulation by LPA was concentration dependent, with maximal induction at 25 to 50 μmol/L LPA (Figure 2A). LPA (25 μmol/L) induced TF mRNA accumulation transiently. Maximal induction reached 7.8-fold at 1.5 hours and decreased to basal levels 7 hours after LPA addition (Figure 2B). The time to peak TF mRNA accumulation was similar to that described earlier for SMCs exposed to other stimuli.12,33

Effect of LPA on the Stability of TF mRNA

An increase in TF mRNA levels as detected by Northern blot analysis can be due to an increase in the rate of transcription, stabilization of previously transcribed mRNA, or a combination of both mechanisms. We examined TF mRNA stability in cells that were untreated or treated for 1 hour with LPA. The cells received 10 μg/mL actinomycin D to stop transcription. We have determined that TF transcription is completely arrested at this concentration. As expected, 1 hour of stimulation with LPA significantly increased TF mRNA levels (6-fold) above control levels; however, the treatment with LPA did not markedly affect the TF mRNA degradation rate after transcription was arrested, as shown in Figure 3A. The half-lives of TF mRNA in untreated and LPA-treated cells, calculated by averaging data from 2 experiments, were 92 minutes and 87 minutes, respectively. Therefore, treatment of cells with LPA did not stabilize TF mRNA.

Transcriptional Regulation Controls TF Gene Expression in Response to LPA

The fact that LPA could markedly increase TF mRNA without stabilizing TF mRNA suggested that LPA regulates TF gene expression at the transcriptional level. Nuclear transcription run-on assays were performed at 60 minutes after LPA 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 Figures 2A and 2B. This basal rate of transcription was increased 6.8-fold after 1-hour exposure to LPA (Figure 3B). These data, together with the mRNA stability results, confirmed that the LPA-induced increases in TF mRNA were controlled at the transcriptional level.

Pertussis Toxin–Sensitive G Proteins Mediate LPA-Induced TF Gene Expression

We then took the beginning steps toward defining an intracellular sequence of events that mediated the LPA induction of TF. In the last few years, LPA receptors have been cloned from mice, humans, and frogs; all have been shown to be G-protein–coupled receptors.21–25 We therefore examined whether a G protein was involved in LPA-induced TF expression in SMCs. Our data showed that preincubation of cells for 16 hours with 100 ng/mL pertussis toxin, an inhibitor of the Gqi subfamily of G proteins, blocked TF mRNA induction by LPA (Figure 4A), indicating the involvement of a Gi protein in the signaling pathway.

Figure 1. LPA induces cell-surface TF pathway activity and TF protein in SMCs. A, Time course of LPA-induced TF surface activity. Quiescent rat aortic SMCs were treated with 25 μmol/L LPA for the indicated times. Cell-surface TF activity was assessed by using a TF activity assay kit from America Diagnostica. Results are means±SEM from 3 experiments. Probability value is <0.05, calculated by Student’s t test from KaleidaGraph/Synergy software. B, Western blot analysis of TF protein levels in total cell lysate with TF-specific antibody. Human SMCs were lysed after 4 hours of either no treatment or treatment with 25 μmol/L LPA. The result shown is representative of 2 experiments.

Figure 2. LPA induces TF mRNA in SMCs. A, Various concentrations of LPA were exposed to quiescent rat SMCs. Total RNA (8 μg per lane) was isolated from cells exposed to LPA for 1.5 hours, and TF mRNA levels were determined by Northern blot analysis. A rat TF cDNA fragment (685 bp) was used as a probe. 28S and 18S rRNAs were visualized by ethidium bromide staining. B, Time course of LPA induction of TF mRNA. LPA (25 μmol/L) was added to quiescent SMCs. At the times indicated, total RNA was isolated, and TF and GAPDH mRNA levels were determined. GAPDH mRNA levels were used to assess RNA loading.
MEK-ERK1/2 Pathway Activation, but Not p38 MAPK, Is Required in LPA-Induced TF Gene Expression in SMCs

To further elucidate the molecular cascades involved in LPA-induced TF gene expression in SMCs, we tested whether MAPK regulated TF mRNA expression. First, we examined whether the MEK-ERK1/2 pathway was involved by testing whether the widely used, potent and selective MEK inhibitors, U0126 and PD98059, could block the induction of TF gene expression. Pretreatment with 3 μmol/L PD98059 or 10 μmol/L U0126 for 30 minutes before the addition of LPA nearly completely blocked LPA-induced TF mRNA accumulation (Figure 4B), suggesting that the activation of MEK and MAPK/ERK1/2 is required for LPA-induced TF expression. If the ERK pathway mediates LPA signaling, LPA would be expected to stimulate the MAPK cascade, leading to ERK activation. It is known that activation of ERK is a consequence of ERK phosphorylation at Thr-202 and Tyr-204 by its upstream kinase, MEK. Thus, we examined phosphorylation of MEK and ERK by using anti-MEK and ERK antibodies recognizing the phosphorylated Ser-217/221 of MEK1/2 and the phosphorylated Thr-202/Tyr-204 sites of ERK1/2 (Cell Signaling Technology). Phosphorylation of MEK, ERK1, or ERK2 was minimal in unstimulated SMCs (Figure 5A). Stimulation of SMCs with LPA rapidly and significantly increased phosphorylation of MEK, ERK1, and ERK2, reaching a peak at 2.5 minutes. Furthermore, phosphorylation of MEK, ERK1, and ERK2 was transient, as depicted in Figure 5A. We also observed that the specific MEK inhibitor U0126 completely inhibited activation of ERK1 and ERK2 (Figure 5B). Taken together, these results indicated that MEK-ERK1/2 pathway activation is required in LPA-induced TF gene expression in SMCs. In addition, our data also revealed that pertussis toxin nearly prevented the activation of MEK and ERK1/2 in response to LPA. (In Figure 5B, compare lanes 4 and 5 with lanes 2 and 3.) These data suggest that activation of a pertussis toxin–sensitive G protein links to the MEK-ERK1/2 pathway, leading to induction of the TF gene.

To address whether p38 MAPK participates in mediating LPA-induced TF gene expression, we examined whether LPA activates p38 MAPK and whether activation of p38 MAPK contributes to TF gene expression. As shown in Figure 6A, LPA rapidly and significantly activated p38 MAPK, as detected by measuring its phosphorylation at Thr180/Tyr182. However, pretreatment with the specific p38 MAPK inhibitor SB203580 at all concentrations tested (up to 5 μmol/L) failed to prevent LPA-induced TF mRNA expression (Figure 6B). Therefore, in contrast to the activation of ERK1 and ERK2, the activa-
tion of p38 MAPK is not required for LPA-induced TF gene expression.

**Discussion**

Evidence is mounting that the oxidation of LDL is a step in atherogenesis and that the resulting modified lipoprotein and its lipids are factors promoting lesion progression and plaque complications. Indeed, oxLDL has been detected in the plasma of atherosclerotic patients and in atherosclerotic lesions. Many of the atherosclerotic effects attributed to oxLDL by studies performed in tissue culture have been linked to specific oxLDL-borne lipids. Elucidating the mechanisms by which oxLDL and its lipids induce biologic events such as alterations in gene expression, cell migration, contractility, and cell proliferation are areas of very active research. The biologically active components of oxLDL, their receptors on cell surfaces, and their intracellular signal-transduction cascades are gradually being identified; many of the bioactive components are lysolipids and modified phospholipids. LPA has recently been reported to be among the phospholipid components of oxLDL, and it has been shown to accumulate in human atherosclerotic plaques in vivo. The mean level of LPA is increased 13-fold in atheromatous plaques above that found in normal arterial tissue. Our results demonstrated that LPA increased TF mRNA, TF protein, and TF pathway activity. Our data may thus have implications regarding ways in which oxLDL could contribute to atherosclerotic lesion development and plaque instability. Thrombosis plays an integral role in the development and progression of atherosclerosis, and enhanced TF expression within an atherosclerotic lesion is believed to play a critical role in determining its thrombogenicity. In addition, local generation of thrombin could play several other important atherogenic roles, including promoting SMC proliferation.

The present study demonstrates that LPA induction of TF transcription contributes to increased surface TF activity. The mechanism is, however, quite distinct from that which we reported for another oxLDL lipid, 7β-hydroperoxycholesterol. We previously showed that latent TF on the cell surface could be activated by 7β-hydroperoxycholesterol and other lipid hydroperoxides but without inducing the TF gene. The activity increase required a hydroperoxide-mediated oxidant stress and could also be observed after oxidant stimulation by exogenous hydrogen peroxide.

**Figure 4.** LPA induction of TF mRNA is blunted by inhibitors of Gi protein and MEK. A, Northern blot analysis shows that pretreatment of SMCs with pertussis toxin (PTX) blocks the effect of LPA on TF mRNA induction. Quiescent rat SMCs were stimulated with LPA for 1.5 hours alone or after a 16-hour preincubation with PTX (100 ng/mL). The result shown in Figure 4A is representative of 5 experiments. B, Pretreatment with PD98059 (3 μmol/L) or U0126 (10 μmol/L) for 30 minutes completely blocked LPA-induced TF mRNA accumulation. The result in Figure 4B is representative of 4 experiments.

**Figure 5.** LPA activates MEK and ERK1/2; PTX and U0126 inhibit LPA-induced activation of MEK1/2 and ERK1/2. A, Time course of activation of MEK1/2 and ERK1/2 by LPA. Rat SMCs were stimulated with 25 μmol/L LPA, and at the times indicated, cell lysates were analyzed by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Phosphorylated MEK1/2 and ERK1/2 were detected with antibodies against phospho-MEK1/2 (P-MEK1/2, Ser217/221) and phospho-ERK1/2 (P-ERK1/2, Thr-202/Tyr-204), respectively (Cell Signaling Technology). The double bands indicate that both isoforms of MEK (MEK1 and MEK2) as well as ERK1 and ERK2 were phosphorylated. The membranes were stripped and reprobed with anti-MEK1/2 or anti-ERK1/2 antibody to detect inequalities in loading. Immunoblots shown are representative of results obtained in 4 separate experiments. B, Effect of pertussis toxin (PTX) and U0126 on the activation of MEK1/2 and ERK1/2. Rat SMCs were pretreated with 100 ng/mL PTX for 16 hours or pretreated with 10 μmol/L U0126 for 30 minutes before stimulation with LPA (25 μmol/L) for either 2.5 or 5 minutes. The cell lysates were analyzed as described in Figure 5A. Immunoblots shown are representative of 3 experiments.
We have taken some early steps in defining signaling pathways relevant to LPA-mediated stimulation of TF. We found, for example, that activation of a pertussis toxin–sensitive G protein, MEK and ERK1/ERK2 are required for TF gene expression in response to LPA but not p38 MAPK. It has been reported that LPA exerts many of its actions through G-protein–coupled receptors. Results from several studies indicate that the LPA receptors are coupled to any of at least 3 distinct G proteins: Gq, which links the receptor to phospholipase C; G12/13, which mediates Rho activation; and Gi, which triggers Ras-GTP accumulation and inhibition of adenyl cyclase. Our results reveal that pertussis toxin–sensitive G proteins are essential for LPA-mediated induction of TF gene expression in SMCs. Others have shown that TF expression is mediated by G proteins. For example, lipopoly-saccharide (LPS) and monocyte chemoattractant protein (MCP)-1 were reported to induce TF gene expression through pertussis toxin–sensitive Gi pathways in monocytes and SMCs. However, LPS-induced TF expression was dependent on LPS-binding protein and the CD14 receptor. In addition, MCP-1 induced TF by way of a Gi-coupled, unidentified MCP-1 receptor in SMCs. We speculate that LPA induction of TF in SMCs may be dependent on Gi-protein–coupled Edg-2 or Edg-7, because these 2 receptors have recently been detected in vascular SMCs. Experimental data from us and others suggest that various agonists regulate TF gene expression in a variety of cells by activating specific G-protein–coupled receptors.

In summary, the present study, to our knowledge, reveals for the first time gene induction by LPA in SMCs. Our data demonstrate that LPA markedly induces TF expression in SMCs. TF expression by SMCs may be an important influence on the outcome of vascular remodeling after balloon injury and, more generally, on the progression of vascular diseases. Our data implicate LPA as a thrombogenic risk factor owing to its ability to upregulate TF expression in cells present in atherosclerotic lesions.

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