Lysophosphatidic Acid Induces Early Growth Response Gene 1 Expression in Vascular Smooth Muscle Cells
CRE and SRE Mediate the Transcription

Mei-Zhen Cui, Essam Laag, Longsheng Sun, Mingqi Tan, Guojun Zhao, Xuemin Xu

Objective—Lysophosphatidic acid (LPA), one component of oxidized low-density lipoprotein, is a potent bioactive phospholipid. Early growth response gene-1 (Egr-1), an important transcription factor, regulates expression of an array of genes involved in vascular diseases. Whether and how LPA regulates the transcriptional machinery of Egr-1 gene is unknown and is addressed in this study.

Method and Results—We found that LPA markedly induces Egr-1 mRNA and protein in aortic smooth muscle cells (SMCs). RNA stability and nuclear run-on assays reveal that LPA-induced Egr-1 gene expression is controlled at the transcriptional level. Reporter gene analyses have shown that the −141 to +20 nt region of the Egr-1 promoter contains regulatory elements. Electrophoretic mobility shift assays reveal that the DNA-binding activities of both CREB and SRF to the CRE and SRE motifs of the Egr-1 promoter are markedly elevated in response to LPA. The increased binding activity depends on the phosphorylation of CREB and SRF. Luciferase assays of a series of deleted or mutated Egr-1 promoter-reporter gene constructs, along with dominant negative CREB transfection analysis revealed that the 2 CRE sites and the 2 proximal SRE sites in the Egr-1 promoter are required for maximal LPA-induced Egr-1 gene expression.

Conclusions—Our data reveal that LPA regulates Egr-1 expression via transcription factors CREB and SRF. These results establish a novel role for CREB in mediating LPA-induced gene expression. Our results imply that elevated LPA levels may, through activation of Egr-1, which regulates an array of atherogenic genes, exacerbate atheromatous lesions. (Arterioscler Thromb Vasc Biol. 2006;26:0000-0000.)

Key Words: aorta smooth muscle cells ■ early growth response gene 1 ■ gene regulation ■ lysophosphatidic acid ■ phospholipids

A ccumulating evidence from recent in vitro and in vivo experiments has shown that early growth response factor-1 (Egr-1), a zinc finger transcription factor, activates a set of genes implicated in the pathogenesis of atherosclerosis with subsequent thrombosis and restenosis. The products of these genes include proinflammatory cytokines, chemokines, adhesion molecules, growth factors, coagulation factors, matricellular modulators, such as tumor necrosis factor-α, IL-2, monocyte chemoattractant protein-1, intercellular adhesion molecule-1, CD44, platelet-derived growth factor A-chain and B-chain, fibroblast growth factor-2, transforming growth factor-β 1, tissue factor, plasminogen activator inhibitor-1, urokinase-type plasminogen activator, S-lipoxygenase, thrombospondin, and metalloproteinases.1 Thus, Egr-1 plays an important role in the regulation of vascular disease-related gene expression. Understanding how transcription factor Egr-1 gene expression is controlled in vascular cells is an important step toward an understanding of the mechanism of vascular diseases.

Phospholipid lysophosphatidic acid (LPA) is a potent bioactive lipid with specific and multiple effects on vessel wall cells and blood platelets. LPA is released from activated platelets and many other cells in response to a wide array of inflammatory stimuli.2 LPA has recently been identified as a biologically active lipid derived from oxidized low density lipoproteins and has been shown to accumulate in high concentrations in atherosclerotic lesions.3 LPA has recently been proposed as a villain provoking cardiovascular disease4 and has attracted increased attention in vascular disease research.5

In the present study, we aimed to address whether and how the bioactive phospholipid LPA regulates the expression of the key transcription factor Egr-1 in vascular smooth muscle cells (SMCs). We present evidence that LPA markedly and rapidly induces Egr-1 mRNA and protein expression in SMC. The data demonstrate that the transcriptional regulation controls the LPA-induced Egr-1 gene expression and that both the cAMP response element (CRE) and the serum response element (SRE) motifs of the Egr-1 promoter are required for LPA-induced Egr-1 promoter activation.

Methods
Tissue Culture
SMCs were prepared from explants of excised aortas of rats as previously described.6 SMCs between passages 5 and 17 were used...
in this study. Cell culture condition was described previously. LPA (1-oleoyl-2-hydroxy-sn-glycerol-3-phosphate) was purchased from Avanti Polar Lipids (Alabaster, Ala.). LPA was dissolved in phosphate-buffered saline (PBS), and a final concentration of 25 μM was used in this study.

Northern Analysis
Total cellular RNA was isolated by using TRRizol Reagent (Invitrogen). RNA was blotted as described in our previous study. A 1507-bp fragment of mouse Egr-1 cDNA was amplified by polymerase chain reaction from the cDNA clone (Genbank M22326). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as an internal control.

Nuclear Transcription Assay
 Cultures of 5 × 10⁷ cells were treated as indicated in the text, and nuclei were isolated as described previously. 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 as described previously.

Western Blot Analysis for Egr-1
Cellular proteins were obtained from rat aortic SMCs grown in 60-mm dishes. Protein separation by SDS-PAGE and antibody detection were performed as described previously. The Egr-1 antibody was from Santa Cruz Biotechnology.

Immunocytochemistry
Cells grown in 24-well chamber coverglass slides were fixed in 4% paraformaldehyde solution for 30 minutes, permeabilized with 0.3% triton X-100 in PBS for 5 minutes at room temperature, blocked with 5% goat serum (Sigma) plus 0.1% Tween-20 in PBS for 1 hour, and then incubated with Egr-1 antibody (Santa Cruz biotechnology) in 1/100 dilution for 2 hours at room temperature. After being washed with PBS 3 times (5 minutes for each), the cells were incubated with the secondary antibody, goat anti-rabbit IgG Alexa Fluor 488 for 30 minutes at room temperature, then washed with PBS 3 times at room temperature. Subsequently, the coverslips were mounted on slides with permanent aqueous mounting medium (Biogenex), and the labeled cells were analyzed by fluorescence microscopy with a Nikon Eclipse E600 microscope.

Polymerase Chain Reaction Amplification of Fragments of the Rat Egr-1 Promoter, Cloning, Mutagenesis and Plasmid Preparation
Based on the published sequence of the 5'-flanking region of the mouse Egr-1 gene, we synthesized several primers, which were used for amplification of regions of the Egr-1 promoter. We cloned the promoterless luciferase reporter plasmid pGL2 (Promega) to generate p(-611 Egr), p(-447 Egr), p(-328 Egr), p(-141 Egr), and p(-92 Egr). Plasmid p(-141 Egr) containing mutations in the CRE and/or SRE sequences were created using a QuikChange Mutagenesis kit from Stratagene. The following shows the mutation of CRE/SRE in p(-141 Egr).

<table>
<thead>
<tr>
<th>CRE Mutations (for CRE, AC → TG; for SRE, GG → CC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>141 mCRE: CTT GTC GCA CCT CCG GGT CTC CCG TGG CTG GGG GAC TGT GCA CAG</td>
</tr>
<tr>
<td>141 dmSRE (double mutations in SREs): CTT GTC GCA CCT CCG GGT CTC CCG TGG CTG GGG GAC TGT GCA CAG</td>
</tr>
<tr>
<td>141 mSRE: CTT GTC GCA CCT CCG GGT CTC CCG TGG CTG GGG GAC TGT GCA CAG</td>
</tr>
<tr>
<td>141 dmCRE/dmSRE (double mutations in both CREs and SREs): CTT GTC GCA CCT CCG GGT CTC CCG TGG CTG GGG GAC TGT GCA CAG</td>
</tr>
</tbody>
</table>

Results
LPA Induces Rapid and Transient Increase in Egr-1 mRNA in SMCs
Rat aortic SMCs were first starved in serum-free DMEM for 48 hours and then treated with various doses of LPA for 35 minutes. Egr-1 mRNA levels were determined by Northern blotting. As shown in Figure 1A, Egr-1 mRNA expression was induced in a dose-dependent manner; 25 μM LPA was used in the present study because it is in the range of pathological concentrations found in atherosclerotic plaques, and it markedly induced Egr-1 expression. LPA also induced Egr-1 mRNA in a time-dependent fashion (Figure 1B). The Egr-1 mRNA accumulation reached its highest level at ~35 minutes after LPA stimulation and then declined to the basal level within 3.5 hours. To access full quantitative data illustrations, visit http://atvb.ahajournals.org.

LPA Markedly Induces Expression and Nuclear Localization of Egr-1 Protein
To determine whether the increased Egr-1 mRNA levels by LPA leads to the increase in Egr-1 protein expression, cells were lysed at indicated time points and subjected to 10% SDS-PAGE analysis, followed by Western blotting using Egr-1-specific antibody. As shown in Figure 1C, robust expression of Egr-1 protein was observed after 30 minutes of treatment, and the expression peak of LPA was induced over background between 30 to 60 minutes. Next, we determined the subcellular localization of the LPA-induced Egr-1 protein. Quiescent SMC were stimulated with LPA for 1 hour followed by immunofluorescence labeling of the intact cells. As shown in Figure 1D, Egr-1 immunofluorescence was observed predominantly in the nuclei of SMC after 1 hour of LPA treatment. This result is in agreement with the fact that Egr-1 is a nuclear transcription factor. These data clearly indicate that LPA induces a rapid increase in Egr-1 mRNA expression.
expression and the subsequent Egr-1 protein expression and nuclear localization in SMCs.

**LPA Controls Egr-1 Expression at the Transcriptional Level**

To determine whether transcriptional regulation contributes to LPA-induced Egr-1 expression, we performed nuclear run-on assays. In unstimulated cells, a low basal rate of transcription of the Egr-1 gene was observed (Figure 2A, lane 1), which is consistent with the low levels of Egr-1 mRNA observed in quiescent cells (Figure 1A, lane 1). The rate of transcription increased 6.3-fold after 30 minutes of exposure to LPA (Figure 2A). To access full quantitative data illustrations, visit http://atvb.ahajournals.org. This result indicates that the LPA-induced increase in Egr-1 mRNA occurs at the transcriptional level.

Using the transcription factor-SEARCH program,12 sequence analysis of the Egr-1 promoter reveals that the region (−611 to +20) contains 2 NF-κB sites, 6 SRE sites, 1 c-Ets site, 1 Sp1 site, and 2 CRE sites (Figure 3). To further determine which of these transcription factor’s binding sites in the Egr-1 promoter is responsible for mediating LPA-induced Egr-1 gene expression, we performed luciferase reporter assays. As shown in Figure 2A, lane 2 (25 μmol/L LPA for 3 hours), the luciferase activity of transfected cells stimulated with LPA is increased compared with unstimulated controls. The number “n” indicates the number of independent experiments.
transcription, we constructed a series of 5' deletion mutants of the Egr-1 promoter and examined the responsiveness of these constructs to LPA stimulation. As shown in Figure 3, deletion of the 2 upstream NF-κB binding sites from −611 to −447 had no effect on LPA-induced Egr-1 promoter activity, indicating that NF-κB does not contribute to the LPA-induced Egr-1 expression. Further deletion from −447 to −141, which eliminates the 4 distal SRE binding sites as well as the c-Ets and Sp1 binding sites, also had no effect on LPA-induced Egr-1 promoter activity, indicating that these 4 distal SRE binding sites contribute to Egr-1 transcription in response to LPA. Interestingly, further deletion from −141 to −92, which eliminates 1 CRE site and 1 SRE site, resulted in a significant reduction of LPA-induced Egr-1 promoter activity. As shown in Figure 3, the LPA-induced promoter activity decreased to one-third of the activity induced by LPA in the full length promoter. This result clearly indicates that the region from −141 to +20 contains the fully responsive elements that mediate LPA-induced Egr-1 transcription.

**LPA Increases Binding Activity of CREB to the CRE Site of the Egr-1 Promoter, and the Increased Binding Activity Is Caused by the Increased Phosphorylation of CREB**

As shown in Figure 3, there are 2 CRE sites and 2 SRE sites in the region from −141 to +20, suggesting a potential role of CREB and/or SRF in mediating LPA-induced Egr-1 transcription. To determine whether both or only one of these transcription factors are indeed involved, we first performed EMSA experiments to determine the effect of LPA on the binding activity of CREB to the CRE site of the Egr-1 promoter. A 32P dCTP-labeled DNA fragment that contains the CRE site of Egr-1 (see Methods) was incubated with the nuclear proteins extracted from LPA treated or untreated cells. As shown in Figure 4A, on LPA treatment, the density of the protein-DNA complex was increased in a time-dependent manner. A remarkable increase in the protein-DNA complex formation was observed after 10 minutes of stimulation with LPA. The density of the complex at 35 minutes decreased to the same level as that at 5 minutes. This result suggests that LPA induces transient DNA binding activity of CREB to the CRE site of the Egr-1 promoter in SMCs.

To determine the specificity of this complex formed between CRE-containing DNA fragment and nuclear proteins, we performed an oligonucleotide competition experiment and antibody-supershift assay. First, nuclear proteins from cells stimulated or unstimulated by LPA were incubated with a 50-fold molar excess of cold consensus CRE DNA fragment before incubation with 32P labeled CRE oligonucleotides of the Egr-1 promoter. As shown in Figure 4B, pre-incubation with the cold consensus CRE DNA fragment completely prevented the complex formation (lanes 1 and 2), indicating a specific binding to CRE. Second, when the nuclear proteins were incubated with a 32P labeled mutant CRE probe, no protein-DNA complex was detected (lanes 5 and 6). This result provides further evidence that the complexes detected in lanes 3 and 4 are CRE-specific.

Figure 4. LPA induction of CREB binding activity to the CRE site of the Egr-1 promoter. A, The kinetics of the complex formed on LPA stimulation of CREB. B, Identification of proteins that bind to the CRE motif. Complexes formed between oligonucleotides containing CRE site and nuclear extracts from quiescent or LPA-stimulated cells were analyzed by competition with a 50-fold molar excess of unlabeled oligonucleotides containing prototypic CRE site. Oligonucleotides containing a CRE site were incubated with nuclear extracts from LPA-stimulated (10 minutes) SMCs were analyzed by competition with an antibody specific for phosphorylated CREB. As shown in lanes 7 and 8, Figure 4B, incubation of the nuclear proteins with a CREB-specific antibody caused a shift of the complex to a higher position, indicating that the complex detected is a CREB-specific complex. Furthermore, when the nuclear proteins were pre-incubated with an antibody specific for phosphorylated CREB, the shift of the CREB-CRE complex was detected only in the LPA-treated sample (lane 10), and the complex band in the untreated sample remained unmoved (lane 9). These results indicate that the basal level of the CREB-CRE complex is formed between CRE and unphosphorylated CREB, and the increased binding activity of CREB to CRE on LPA stimulation is caused by LPA-induced phosphorylation of CREB.

To further characterize the complex formed on LPA treatment, the nuclear proteins were pre-incubated with either a CREB-specific antibody or an antibody specific for phosphorylated CREB. As shown in lanes 7 and 8, Figure 4B, incubation of the nuclear proteins with a CREB-specific antibody caused a shift of the complex to a higher position, indicating that the complex detected is a CREB-specific complex. Furthermore, when the nuclear proteins were pre-incubated with an antibody specific for phosphorylated CREB, the shift of the CREB-CRE complex was detected only in the LPA-treated sample (lane 10), and the complex band in the untreated sample remained unmoved (lane 9). These results indicate that the basal level of the CREB-CRE complex is formed between CRE and unphosphorylated CREB, and the increased binding activity of CREB to CRE on LPA stimulation is caused by LPA-induced phosphorylation of CREB.

To further confirm LPA stimulation of CREB phosphorylation in SMCs, we performed Western blot analysis using a
specific antibody against phospho-CREB. As shown in Figure 4C, on LPA stimulation, CREB was transiently phosphorylated and the phosphorylation reached its maximum at 10 minutes. In contrast, the level of total CREB remained unchanged throughout the time course. This data provides further evidence that the LPA-induced increase in CREB binding activity is caused by the phosphorylation of CREB.

LPA Increases Binding Activity of SRF to the SRE Site of the Egr-1 Promoter and the Increased Binding Activity Is Caused by the Increased Phosphorylation of SRF

To evaluate the possibility that SRF may be involved in LPA-induced Egr-1 expression, the same approaches described above were used to determine whether LPA induces SRF binding activity to the Egr-1 promoter. As shown in Figure 5A, EMSA experiment revealed that LPA transiently increased the putative SRF binding activity, which reached its maximum between 10 and 35 minutes. To determine the specificity of the LPA-increased complex, we performed EMSA oligonucleotide competition and antibody supershift assays. As shown in Figure 5B, pre-incubation of the nuclear proteins with cold consensus SRE oligonucleotides completely prevented the formation of the complex (compare lanes 3 and 4 with lanes 1 and 2). When the nuclear proteins were incubated with radio-labeled mutant SRE, no complex formation was detected (lanes 5 and 6). These results indicate that the complexes detected in lanes 1 and 2 are SRE-specific. Furthermore, pre-incubation of nuclear proteins with a SRF-specific antibody caused a shift of the complex to a higher position (lanes 7 and 8), indicating that the complex is SRF-specific. When the nuclear proteins were pre-incubated with an antibody specific for phosphorylated SRF, we observed that the added phosphorylated SRF antibody completely erased the band of LPA-induced complex, but had no effect on the basal level of the SRF complex (compare lanes 9 and 10 with lanes 1 and 2). These results indicate that LPA-induced SRF binding activity is caused by the phosphorylation of SRF. Together, these data demonstrate that LPA induces a specific binding activity of SRF to the SRE site of the Egr-1 promoter, and the increased binding activity is caused by the increased phosphorylation of SRF protein. The rapid and transient phosphorylation of SRF in SMC by LPA was also detected by Western blot analysis (Figure 5C).

CRE Sites and the Proximal SRE Sites of Egr-1 Promoter Are Functionally Involved in LPA-Induced Egr-1 Gene Expression

The results from the EMSA experiments (Figures 4 and 5) indicate that LPA induces binding activities of CREB and SRF to the CRE and SRE motifs in the Egr-1 promoter, suggesting that CRE and the proximal SRE motifs of the Egr-1 promoter possibly contribute to LPA activation of Egr-1 transcription. To determine whether CRE sites and SRE sites in the Egr-1 promoter are functionally required for mediating LPA induction of the Egr-1 gene, we generated mutants in the Egr-1 promoter to sequentially knockdown CRE and SRE sites of the Egr-1 promoter.

These reporter gene constructs were transfected into SMCs, and the LPA-induced promoter activity of these constructs was determined. As shown in Figure 6A, we found that mutation in one CRE of the Egr-1 promoter reduced LPA-induced luciferase activity from 3.8-fold to 2.8-fold. Further mutation on both CRE sites nearly completely blocked LPA induction (reduced to 1.4-fold). This result indicates that the CRE sites in the Egr-1 promoter are necessary elements that mediate LPA induction of Egr-1 gene expression.

To investigate whether SRE sites are also required for LPA induction of Egr-1 promoter activity, we examined the effect of mutations of one or two SRE sites of the Egr-1 promoter on LPA-induced luciferase activity. Our results showed that mutation on one SRE site reduced luciferase activity from 3.8-fold to 2.8-fold. Further mutations on both SRE sites near completely blocked LPA induction (reduced to 1.4-fold). This result indicates that the SRE sites in the Egr-1 promoter are necessary elements that mediate LPA induction of Egr-1 gene expression. Mutations on both the 2 CRE sites and the 2 SRE sites completely abolished LPA-induced Egr-1 transcription (Figure 6A).
In the present study, we investigated the transcriptional regulatory gene expression in nuclei is less well understood. In the pathway, the downstream mechanism of how LPA regulates proliferation, migration, differentiation, and contraction. In these genes, in turn, modulate vascular cell functions, such as the immediate early gene Egr-1, which regulates lead to the regulation of a number of transcription factors, activates a variety of kinases. These signaling cascades G-proteins, such as G0/i, G12/13, and Gq, LPA signaling receptors, eg, LPA1, LPA2, and LPA3, which couple with the upstream components of the LPA signaling pathway have been well studied. It has been shown that through LPA have shown that Egr-1 regulates a variety of genes that contribute to the development of vascular diseases. Our previous studies have shown that Egr-1 is a key transcription factor in regulating oxidized low-density lipoprotein–inds. Egr-1 expression may contribute to understanding the progression of atherosclerosis.

Regarding the effect of LPA on Egr-1 gene activation, our data show that treatment with LPA at 25 μmol/L, which is within the range of pathological levels in atherosclerotic lesions, significantly increases Egr-1 mRNA in rat aortic SMC. In addition, Western blot and immunohistochemical analysis reveal that LPA markedly induces Egr-1 protein expression and that the increased Egr-1 proteins are primarily located in the nuclei of SMC, suggesting a role of Egr-1 in mediating LPA-induced transactivation of genes in their nuclei.

Using pharmacological inhibitors, a previous study suggested that MAPK and Rho signaling pathways were involved in LPA-induced Egr-1 expression in mesangial cells, but the downstream mechanism by which LPA signals transactivate Egr-1 transcription was unknown. It has been shown that LPA via SRF regulates c-fos gene expression, via SRF and Sp1 regulates SRF gene expression, via NF-kB and AP-1 regulates IL-8 gene expression, and via NF-kB activates the urokinase plasminogen activator (uPA) gene. Sequence analysis of the Egr-1 promoter revealed that the region (−611 to +20) contains most of the transcription response elements mentioned above (Figure 3). However, deletion mutagenesis analysis excluded the contribution of the 2 upstream NF-kB sites and the 4 distal SRE sites, as well as the c-Ets and Sp1 sites. Several previous studies have demonstrated that 5′-SRE is critical for Egr-1 gene expression in U-937 cells and in NFS60 cells in response to okadaic acid and the granulocyte-macrophage colony-stimulating factor. Others have suggested that 3′-SRE mediates Egr-1 expression.

**Figure 6.** The effect of mutations of CRE site(s) and/or SRE site(s) of the egr-1 promoter, and the dominant negative CREB on LPA-induced Egr-1 promoter activation. A, Luciferase assay of mutated CRE site(s) and SRE site(s) of Egr-1 promoters. B, Effect of expression of the dominant negative CREB on Egr-1 promoter activation induced by LPA. Cells were cotransfected with either p(−141 Egr-1) and pcMV-CREB, or p(−141 Egr-1) and pCMV-CREB133 for 24 hours before a 3-hour LPA stimulation. Cells transfected with p(−141 Egr-1) and pcDNA were served as controls; n indicates numbers of experiments; in each, transfections were performed in duplicate.

The upstream components of the LPA signaling pathway have been well studied. It has been shown that LPA receptors, eg, LPA1, LPA2, and LPA3, which couple with G-proteins, such as G0/i, G12/13, and Gq, LPA signaling activates a variety of kinases. These signaling cascades lead to the regulation of a number of transcription factors, including the immediate early gene Egr-1, which regulates expression of various genes in vascular cells. The products of these genes, in turn, modulate vascular cell functions, such as proliferation, migration, differentiation, and contraction. In comparison with the understanding of upstream signaling pathway, the downstream mechanism of how LPA regulates gene expression in nuclei is less well understood. In the present study, we investigated the transcriptional regulatory mechanism of the Egr-1 gene by LPA and found that a region of the Egr-1 promoter, which contains 2 CRE sites and 2 SRE sites, is responsible for LPA-induced Egr-1 expression. Furthermore, our data demonstrate that the 2 CRE sites and the 2 SRE sites are both required for full activation of Egr-1 promoter on LPA induction. These findings established for the first time a role of CRE and SRE in mediating LPA-induced Egr-1 expression.

Egr-1, an immediate early gene, is an important transcription factor regulating an array of genes. Specifically, studies have shown that Egr-1 regulates a variety of genes that contribute to the development of vascular diseases. Our previous studies have shown that Egr-1 is a key transcription factor in regulating oxidized low-density lipoprotein–induced expression of tissue factor, which is a prothrombotic molecule. LPA is one of the potent bioactive components of oxidized low-density lipoprotein and represents one of the important bioactive lipids produced during inflammation. In normal human blood plasma, the LPA concentration is 0.6 to 0.7 μmol/L. It has been shown that the amount of LPA in atherosclerotic lesions increased 13-fold compared with the level in the wall of normal arteries. The locally elevated LPA levels could be a result of the oxidation of low-density lipoprotein or could be produced by activated platelets. The presence of the high concentration of LPA in the atherosclerotic lesions has been implicated in their progression and thrombus formation. Thus, understanding the mechanism of how LPA regulates the key transcription factor Egr-1 expression may contribute to understanding the progression of atherosclerosis.
expression in response to the epidermal growth factor. Sakamoto et al has shown that one 3′-SRE and one 3′-CRE contribute to both granulocyte-macrophage colony-stimulating factor–induced and IL-3-induced Egr-1 expression in tissue factor-1 cells. Interestingly, the present study reveals that both of the 2 CRE sites and the 2 SRE sites in the 3′ promoter are required for the full activation of the Egr-1 promoter on LPA stimulation. Our results revealed the transcriptional regulatory mechanism by which LPA activates Egr-1 gene expression. To our knowledge, the finding that CRE is required for LPA-induced Egr-1 expression also established for the first time, a novel role for CREB in mediating LPA-induced gene expression. In addition, our data demonstrate that LPA-induced protein-DNA binding activity is caused by LPA-induced rapid phosphorylation of CREB and SRF. In support of our findings, a recent study showed that LPA stimulates phosphorylation of CREB in fibroblast cells. The rapid phosphorylation of these transcription factors makes it possible to promptly activate the immediate early gene Egr-1 in response to LPA stimulation.

In summary, our data revealed that CREB is an important transcription factor acting downstream of the LPA signaling pathway to mediate gene expression in SMCs. The results of this study provide the first evidence to our knowledge that the transcription factor CREB links the LPA signal with cellular gene expression. We have further shown that phosphorylation of both CREB and SRF contributes to the binding activity of both CREB and SRF to their cis-acting elements of the Egr-1 promoter. Our data demonstrate the importance of both CREB and SRF in the LPA-activation of Egr-1 gene expression. The results of this study imply that elevated LPA levels through activation of Egr-1, which regulates an array of atherogenic genes, may worsen atheromatous lesions.

Acknowledgments

This work was supported by National Institutes of Health grants (HL074341 to M.-Z. C. and NS42314 to X. X.) and an ARA Award (to M.-Z. C.) sponsored by Pfizer Inc. The authors thank Drs M. Donald McGavin and Robert L. Donnell for careful reading of the manuscript.
Lysophosphatidic Acid Induces Early Growth Response Gene 1 Expression in Vascular Smooth Muscle Cells. CRE and SRE Mediate the Transcription

Mei-Zhen Cui, Essam Laag, Longsheng Sun, Mingqi Tan, Guojun Zhao and Xuemin Xu

Arterioscler Thromb Vasc Biol. published online February 23, 2006;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2006/02/23/01.ATV.0000214980.90567.b5.citation

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2006/02/24/01.ATV.0000214980.90567.b5.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Fig. Ia, Bar-graph illustration: dose dependence of LPA induction of Egr-1 mRNA.

Fig. Ib, Bar-graph illustration: time course of LPA stimulation of Egr-1 mRNA.
Fig. IC, Bar-graph illustration: time course of LPA stimulation of Egr-1 protein.

Fig IIb, Bar-graph illustration of nuclear run-on results.