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CRE and SRE mediate LPA-induced CCN1 transcription in mouse aortic smooth muscle cells

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Abstract

Lysophosphatidic acid (LPA), one component of oxidized low-density lipoprotein (ox-LDL), is a potent bioactive phospholipid. Our recent data reveal that LPA induces matricellular protein CCN1 (also known as Cyr61) expression in aortic smooth muscle cells (SMC) and that CCN1 bridges LPA and integrin signaling pathways leading to SMC migration. Whether and how LPA regulates the transcriptional machinery of the CCN1 gene are unknown. In this study, we found that LPA markedly induces CCN1 mRNA expression in SMCs. Using deleting mutation/reporter gene strategies, we demonstrated regions from -2038 to -1787 and from -101 to +63 of the CCN1 promoter contain the essential regulatory elements. The serum response element (SRE) and cyclic AMP-response element (CRE) are located in these regions. LPA induced time dependent phosphorylation of serum response factor (SRF) and CRE-binding protein (CREB) in mouse SMCs. Luciferase assays of a series of deleted, mutated CCN1 promoter-reporter gene constructs and dominant negative construct revealed the distal SRE and the proximal CRE in the CCN1 promoter are required for LPA-induced CCN1 gene expression. Our results imply that elevated LPA levels may trigger SMC migration and exacerbate restenosis and atherosclerotic lesions through the induced CCN1, which communicates with a set of plasma membrane proteins/intracellular kinases.

Keywords

lysophosphatidic acid; CCN1; aortic smooth muscle cells; gene regulation; transcription

Introduction

Lysophosphatidic acid (LPA) is a potent bioactive lipid, which is formed in oxidized low-density lipoprotein (ox-LDL) (Siess et al. 1999) and produced by activated platelets (Eichholtz et al. 1993; Gerrard and Robinson 1989; Mauco et al. 1978). LPA regulates

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expression of numerous cytokines and inflammatory factors such as early growth response protein (Egr1), tissue factor (Cui et al. 2003), IL-6 (Hao et al. 2010). LPA also mediates multiple cellular events such as SMC migration (Cui 2011) and proliferation (Tokumura et al. 1994), which are important processes in the vascular lesion formation involved in atherosclerosis and restenosis.

CCN1 is a cysteine-rich matricellular protein. Chicken CCN1/Cef10 and mouse CCN1 were originally identified as growth factor-inducible genes (O'Brien et al. 1990; Simmons et al. 1989). Exogenous recombinant CCN1 has been reported to induce angiogenesis (Babic et al. 1998) and promote cell proliferation, migration, adhesion, and differentiation (Kireeva et al. 1996; Wong et al. 1997). In a previous study, we found that LPA-induced CCN1 protein mediates LPA signaling, leading to SMC migration. However, whether LPA mediates CCN1 gene expression has not been experimentally demonstrated, and if so, whether the LPA regulation occurred at the transcriptional level has not been illustrated.

In the present study, we aimed to address how the bioactive phospholipid LPA regulates the expression of CCN1 gene expression in SMCs. We presented experimental evidence that LPA markedly and rapidly induces CCN1 mRNA expression in SMCs. Our data also demonstrated that the transcriptional regulation contributes to the LPA-induced CCN1 gene expression. Our results indicate that both serum response element (SRE) and cAMP response element (CRE) motifs of the CCN1 promoter are required for LPA-induced CCN1 promoter activation.

Material and Methods

Materials

LPA (1-oleoyl-2-hydroxy-sn-glycero-3-phosphate) was purchased from Avanti Polar Lipids; DMEM (with 4.5g/L glucose) was from Corning. TRIzol reagent and ThermoScript RT-PCR system were from Invitrogen; GeneAmp PCR core reagents were from Applied Biosystems; antibodies against mouse phospho-CREB, CREB and phospho-SRF were from Cell Signaling, goat anti-rabbit HRP secondary antibody was from Invitrogen.; pGL2 plasmid luciferase reporter kit was from Promega; QuikChange Mutagenesis kit was from Stratagene; and CMV500A-CREB negative mutation plasmid was from Addgene.

Cell Culture

Mouse aortic SMCs were prepared from explants of excised mice aortas as described previously (Brock et al. 1985). Cells were maintained in DMEM (4.5 g/L glucose) containing 10% fetal bovine serum and the cells of passage 7-8 were used for experiment. Cells were made quiescent by incubation in serum-free DMEM for 48 h. LPA was dissolved in PBS and a final concentration of 5 μ mol/L was used in this study.

RNA isolation and Northern Blot Analysis

Total cellular RNA was isolated using TRIzol reagent according to the manufacturer's instructions. Total RNA (8-10 μ g) was subjected to denaturing electrophoresis on formaldehydeagarose gels. RNA was blotted onto Nytran membranes (Schleicher & Schüll)

and hybridized with radiolabeled CCN1 cDNA probes. Ribosomal RNA 28 S and 18 S (amount ratio is about 1.0) were used as internal controls.

Polymerase Chain Reaction Amplification of Fragments of the mouse CCN1 Promoter, Cloning, Mutagenesis and Plasmid Preparation

Based on the published sequence of the 5'-flanking region of the mouse CCN1 gene (Latinkic et al. 1991), we synthesized the following forward primers. F-2038: 5'-CGG GGT ACC TCC TGC TCT GGG AAC AGA AGT-3', F-1787: 5'-CGG GGT ACC TTC TAC CCT GTT CCT TGC CTT-3', F-1497: 5'-CGG GGT ACC TGT CTT CAC TGG AGT GTG CG-3', F-967: 5'-CGG GGT ACC ATC TGT TTT GTC CCG GTA GCT TT-3', F-422: 5'-CGG GGT ACC CAA ACA GCT CGC TGC CTT TC-3', F-173: 5'-GAT GGT ACC GAG AAG AGG GGG GAA AAG GT-3', F-101: 5'-CAA GGT ACC TGA GAT GTT TGA GAA TTC TA-3', with the same reverse primer R: 5'-GGA TCA AAT GCG CCC GGA GT-3'. These primers were used for amplification of specific regions of the CCN1 promoter. The sequential shorter fragments were amplified and cloned into the promoterless luciferase reporter plasmid pGL2 (Promega) to generate pGL2 (-2038/+63), pGL2 (-1787/+63), pGL2 (-1497/+63), pGL2 (-967/+63), pGL2 (-422/+63), pGL2 (-173/+63) and pGL2 (-101/+63). Plasmid mSRE-pGL2 (-2038/+63) containing mutations in the SRE sequence at position -1922 were created using a QuikChange Mutagenesis kit from Stratagene. The mutation site and mutated sequence are as follows, -1922-CCAAATATGG -1913 to -CCGGATATGG-. The plasmid was sequenced to confirm base pair substitutions. Dominant negative mutation plasmid pcDNA3CMV500A-CREB (Addgene) and pGL2(-101 /+63) were co-transfected into cells to examine the function of CRE.

Western Blot Analysis

Cultured mouse aortic SMCs were rinsed with cold PBS and lysed in Western blot lysis buffer (50 mM Tris-HCl, pH 6.8, 8 M urea, 5% mercaptoethanol, 2% SDS, and protease/phosphatase inhibitors) with sonication for 30 s on ice. Cellular proteins were separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore). Membranes were then probed with the specific anti-phospho-SRF, anti-phospho-CREB and CREB antibodies (1:1000), followed by incubation of secondary goat anti-rabbit HRP antibody (1:2000), and specific protein bands were viewed using ECL Plus (GE Healthcare).

Transient Transfection Assay

Plasmid transfection was performed by using Superfect reagent as described previously (Cui et al. 1999). Transfected cells were incubated in serum-free medium for 48 h before a 3-h induction with 5 µmol/L LPA. Luciferase activity was determined using the Luciferase Assay System (Promega) and D20/20 luminometer (Turner Design).

Data analysis

All results presented in this study are representative of at least 3 experiments, data were analyzed with Microsoft Excel and shown as mean $\pm$ SD. Single comparison was performed using *student's t test*. A *P* value of 0.05 was considered statistically significant.

Results

LPA induces rapid and transient increase in CCN1 mRNA in SMCs

Mouse aortic SMCs were first starved in serum-free DMEM for 48 h and were then treated with 5 μ mol/L LPA for various times. CCN1 mRNA levels were determined by Northern blotting. As shown in Figure 1A and 1B, LPA rapidly and markedly induced CCN1 mRNA expression in SMCs and the LPA induction of CCN1 mRNA was in a time-dependent fashion. The CCN1 mRNA accumulation reached its highest level at ~1h after LPA stimulation and then declined to the basal level within 6 h.

The regions from -2038 to -1787 and from -101 to +63 have response elements regulating LPA-induced CCN1 transcription

The binding elements of the following transcription factors are present in the mouse CCN1 promoter according to a previously published program (Heinemeyer et al. 1998): SRF, Sp1, Oct-1, CREB, NF- κ B and C/EBP (Fig. 2). To determine whether LPA-induced CCN1 gene expression is mediated by transcriptional mechanism, we cloned CCN1 promoter region (-2038/+63) into the promoterless luciferase reporter plasmid pGL2 (Promega) to generate pGL2(-2038/+63). The transfection results showed that LPA induced CCN1 promoter activity about 4-fold of the control (Fig. 3), indicating that transcriptional mechanism contributes LPA-induced CCN1 gene expression. We then determined which of these transcription factor's binding sites in the CCN1 promoter is responsible for mediating LPA-induced CCN1 transcription. We tested a series of 5' deletion mutants of the CCN1 promoter and examined the responsiveness of these constructs to LPA stimulation. The sequential shorter fragments of CCN1 promoter were amplified and cloned into the promoterless luciferase reporter plasmid pGL2 (Promega) to generate pGL2(-1787/+63), pGL2(-1497/+63), pGL2(-967/+63), pGL2(-422/+63), pGL2(-173/+63) and pGL2(-101/+63). These plasmids were transferred into SMCs and the LPA-stimulated promoter luciferase activity was examined. As shown in Figure 3, the LPA-induced luciferase activities of plasmids pGL2(-1787/+63), pGL2(-1497/+63), pGL2(-967/+63), pGL2(-422/+63), pGL2(-173/+63) and pGL2(-101/+63) were significantly reduced to 50% of luciferase activity of the full-length promoter pGL2 (-2038/+63), indicating that the region (-2038 to -1787) contains the essential response site(s). These results suggest that SRE site may be accountable for partial CCN1 promoter activity. We also observed that deletion from -1787 to -101, which eliminates 7 SP1 sites, 1 SRE site, 2 Oct-1 sites, 1 NF- κ B site and 1 C/EBP site, did not result in significant reduction of LPA-induced CCN1 promoter activity, suggesting that these binding sites are not responsible for LPA-induced CCN1 promoter activation. The luciferase activity of pGL2 (-101/+63) still maintains 1.8-fold of the control's, suggesting that the region from -101 to +63 contains response element(s) that may also contribute to LPA-induced CCN1 transcription. A CRE site in this region was likely a candidate.

LPA induces phosphorylation of SRF and CREB

To determine the functional roles of SRE site in the region from -2038 to -1787 and CRE site in the region from -101 to +63 contribute to the regulation of LPA-induced CCN1 expression, we first examined whether LPA stimulates phosphorylation of SRF and CREB in mouse aortic SMCs. We performed Western blot analysis using specific antibodies against phospho-CREB and phospho-SRF. As shown in Figure 4A, 4B and 4C, upon LPA stimulation, CREB phosphorylation was rapidly and dramatically induced and the phosphorylation reached its maximum at 10 min. Similarly, SRF was also transiently phosphorylated and reached the peak at 30 min. In contrast, the level of total CREB remained unchanged throughout the time course. This data provides further evidence that LPA induced phosphorylation of both transcription factors CREB and SRF in mouse aortic SMCs. These data strongly suggest that CREB and SRF contribute to LPA-induced CCN1 transcription.

The distal SRE site and the proximal CRE Site of CCN1 promoter are the major regulatory elements responsible for LPA induction

To further determine whether the distal SRE site (between -2038 and -1787) in the CCN1 promoter is functionally required for mediating LPA induction of CCN1 gene, we generated mutations in the CCN1 promoter to abolish the distal SRE site of the CCN1 promoter. We then compared luciferase activity of the mutant SRE CCN1 promoter pGL2(-2038/+63) with that of the wild type SRE CCN1 promoter pGL2(-2038/+63) in response to LPA stimulation. As shown in Fig. 5A, we observed that mutations in SRE of the CCN1 promoter markedly reduced LPA-induced luciferase activity from 4.2-fold to 2.8-fold, indicating that the distal SRE contributes to CCN1 transcription. We next assessed the regulatory effect of CRE within region -101/+63. We employed a dominant negative approach by examining whether overexpression of mutant CMV500A-CREB inhibits luciferase activity of pGL2(-101/+63). As shown in Fig. 5B, compared to control plasmid pcDNA3, overexpression of dominant negative mutant CMV500ACREB abolished the LPA-induced luciferase activity (from 1.8-fold to 1-fold), indicating that proximal CRE contributes to LPA-induced CCN1 gene expression. This result indicates that the distal SRE and proximal CRE in the CCN1 promoter are necessary regulatory elements that mediate LPA induction of CCN1 gene expression.

Taken together, our data reveal that LPA induces CCN1 gene expression. We demonstrated that the distal SRE and the proximal CRE in the CCN1 promoter mediate the CCN1 transcription in response to LPA in SMCs.

Discussion

LPA, as a potent biolipid component of oxLDL, exerts its functions through LPA receptors which couple with G-proteins, such as G0/i, G12/13, and Gq, to activate a variety of kinases (Anliker and Chun 2004; Moolenaar et al. 2004). These signaling cascades lead to the regulation of various downstream cellular functions such as migration, proliferation, differentiation, and contraction. The downstream mechanism of how LPA regulates gene expression in SMCs is less understood. In the present study, we investigated the

transcriptional regulatory mechanism of the CCN1 gene by LPA. Our data demonstrated that the distal SRE and proximal CRE of CCN1 promoter are responsible for LPA-induced CCN1 gene expression.

Matricellular protein CCN1 regulates vascular SMC migration, which is a crucial step in the development and progression of restenosis and atherosclerosis. As one of the potent bioactive components of oxLDL, LPA concentration is 0.6 to 0.7 $\mu\text{mol/L}$ in normal human plasma (Baker et al. 2001), while the amount of LPA in atherosclerotic lesions increased 13-fold compared with the level in the wall of normal arteries (Siess et al. 1999). 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 CCN1 expression may contribute to understanding the development and progression of vascular disease.

In a previous study, we demonstrated that LPA induces Cyr61 protein expression. In the current study, our data reveal that LPA induces CCN1 gene expression. It has been shown that LPA via SRF and Sp1 regulates c-fos gene expression (Spencer and Misra 1999), via NF- κ B and AP-1 regulates IL-8 gene expression (Fang et al. 2004), and via NF- κ B activates the urokinase plasminogen activator (uPA) gene (Li et al. 2005). As to CCN1 regulation, it has been reported CREB and AP1 regulates S1P-induced CCN1 expression in calf SMCs (Han et al. 2003) and MRTF-A/SRF mediates CCN1 gene activity in response to mechanical strain in human bladder SMCs (Hanna et al. 2009). However, the regulation of CCN1 expression induced by LPA at transcriptional level has not been revealed. Sequence analysis of the mouse CCN1 promoter revealed that the promoter region contains various transcription response elements (Fig. 2). However, deletion mutagenesis analysis excluded the contribution of the upstream 1 proximal SRE, 2 Oct-1 site, 1 NF- κ B site, 1 C/EBP site, as well as 7 Sp1 sites. Our previous study showed that 3'-SRE and 3'-CRE sites mediate immediate early gene Egr-1 expression (Cui et al. 2006). Interestingly, the present study suggested that LPA-induced rapid phosphorylation of CREB and SRF contribute to CCN1 gene expression. Our data revealed that the transcriptional regulatory mechanism mediates CCN1 gene expression. Both the distal SRE and the proximal CRE in the CCN1 promoter are required for the activation of the CCN1 promoter in response to LPA stimulation.

The results of this study imply that elevated LPA levels through the activation of SRF and CREB contribute to CCN1 expression, which in turn mediates SMC migration, contributing to vascular lesion formation.

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Figure 1. LPA induction of CCN1 mRNA in mouse aortic SMCs. A. Time course of LPA induction of CCN1 mRNA accumulation. Visualized bands of 28S and 18S were used to assess RNA loading. B. Digitized bar graph of CCN1 mRNA expression.

-2038 TCCTGCTCTGGGAACAGAAGTAAGTCTATATTACAACTTTGAAGCTGTCAAGAATGCCTTGTGGTTG
 -1968 GATAACAGAGGGAGAAAAATGTTAAACGCAGGCTACTGCTATACCCAAATATGGAAATATGTTACGT
 -1898 CTGGTGTCTGATTACCATCTGGAAATACTTACAAACATGAAGTCAAATAGAAAAAGCCACAACAGAA
 -1828 GCCAGGAACCCCTCTCTCATCTGTTTCTCCAGCGCTCTTCTACCTGTCTTCTGCTTCTCCACCC
 -1758 TCACCCCTACCTCTCCGAGCTCCCTAAGATGGAAACAATGAACGTGCGCAGGAGGAACGTCTCTA
 -1688 ATAAATCGGCAGCCGAGGGACAGGCTAGGCTTGTCTTATTATTTGCATAACACTTGACAACCCA
 -1618 TTGCTTTAAAGTCCCAACCCAGAAAGCCTTGAGAACCTACCGCTCTAGCAAATCTCCAGGAC
 -1548 TTACGAAAGGGCGCTTTTGAATGAATGAACCTTCTTCCAGTAACAGTGTCTTCTAGGAGTGTG
 -1478 GCAACAGCCTCTCTCTCTACTGGAGTCAATATAGAACAGGCAACGAGGAGGCTCTTAAAGCAAGAT
 -1408 CCGCAAGCTGCAAGGCAGAGAGGCCCTAAATATTAATAAGAAAGCTATCCAGTTTATGAATGG
 -1338 CATTAATAACCCCAATAGGCTCTTGTGGGAGGGGTGTCTCAGTGTATGTTTAAAGATCTTAA
 -1268 ACCCAACTTCTCTATTACAGTGTGCTTCTAAATCCCTCATGTCTCGAACTTGGAGGGAGCCACTGC
 -1198 ACTACCTTGAAGAAAGAGGCTCTAAGTGATCCAAACAAGGAGTTAAACATGGACGGGGCCAGAC
 -1128 ACACACACACACACACACACACACACTCACACACACGGGGGGGGGCAACAAATACACACA
 -1058 GCGAGACATAGGGGGCGTGGGGAGGGTGTGCAACACCCCGAGTCTTTTCCAGCTATCCACA
 -988 GGGCACAATGGTCTGTAGAATCTGTTTGTCCGGTAGCTTTGCACCTGGTGATTCAGGACAAGTC
 -918 ACTCCACGGTGTCTCTCCGCTTCTGCTTTGGCTGCTCAGTTTCCACCGCCAGTCCAGGAGAGAG
 -848 GCCAGGAGGTTGACCCGGCTCCGGCATCCGTGAGGAAGTGACGTACAGGCTCCGGGAGAACTCTGCAC
 -778 TCGTTTCTCTTGGATTCCAATGCAGGAGTGGGCTGGAACCTAAAGTGGGAACCTCCAAAAACAACA
 -708 AGTACAATACCAAAAAAGGGGGAGGGATGAGGGAAGACCTTGGCTGGAATATGCCAGCGAAGAG
 -638 GGAAGCTTCCCTCCCCCCCCATTCATAAATGCCACTCCGGGTATTAATTGCAATACACTTCTCTTGG
 -568 CTAATAAACATATGCCAAAGCTTTGGGACTTGATCCGAGCATGCCTTTTGAAGTCCACAAATAGATCC
 -498 CTGGCTTAGAAACCAACTCCCCCTCCCTTTGGACTCTCAGTAGATAACTTACTCTCACCTTGGTTGA
 -428 AACAAGCAACAGCTCGCTGCTTTCCCGTAGGCATCACCAACAAACCACTTTTGTCTCTGTCTC
 -358 CCGCCCTCCCATGTCTAGGCAAGATCTGAAGGGTTCACACCAACATACACACGCCCCCCCCCCCA
 -288 TATACCTCTTCAATCAAAATCATCACCTCGCGCCCAAGCATCCCGCTCCCAAGGAAGTTGTTA
 -218 TTTAAAGTGAAAAACGAACCTTTTCTCTCTGATGGATCTGAGAAAGGGGGGAAAAGGTGCAAC
 -148 GGAGCCAGGGAGGTGCTTGGCAGCAGCCCGGCCAACCAATCTGAGATGTTGAGAAATCTAGAA
 -78 CGCGCCGACAGAGCTACGTCACTGCAACACGCGCGCTAGGCAGGCATATATAAGGGCGGGCTCCCGGC
 -8 GCTGGGGCAGACCGTGAGCGAGAGCGCCCAAGAGAGCGCTGCAATCTCTGCGCTCTCCCGCAGCAC
 +63 CTGAGAGAAGGACACCCGCGCTCGGCCCTCGCTCACCACCTCCGGCGCATTTGATCCCGCTGCTC
 +133 GCCGGCTGTGGTCTGTGTCGCCGCTCGCCCGGTTCTCTGCGGCCACAATGAGCTCCAGCACC
 Met

Figure 2.
 Illustration of mouse CCN1 gene promoter sequence and transcription factor binding sites.

Figure 3. Localization of the LPA response region in the CCN1 promoter. Transfected cells were made quiescent for 48 h before the addition of 5 μ mol/L LPA for 3 h. Fold induction is the luciferase activity of transfected cells stimulated with LPA compared with unstimulated controls. The number “n” indicates the number of independent experiments.

Figure 4. LPA induces phosphorylation of SRF and CREB. A, Western blot analysis of CREB and SRF phosphorylation induced by LPA. B, Digitized bar graph of CREB phosphorylation. C, Digitized bar graph of SRF phosphorylation.

Figure 5.

The effect of mutations of distal SRE and dominant negative mutant CREB on CCN1 promoter activity in response to LPA stimulation. A, Luciferase assay of mutated distal SRE of CCN1 promoter. Data shown as mean $\pm$ SD, * P <0.05 vs pGL2 (-2038/+63) group. B, Luciferase assay of the effect of dominant negative mutant CREB on CCN1 promoter activity. Data shown as mean $\pm$ SD, * P <0.05 vs pcDNA3 group. SRE function examination: cells were transfected with wild type pGL2 (-2038/+63 CCN1) or mutated SRE construct mSRE-pGL2 (-2038/+63 CCN1), luciferase activities were compared between these two groups. CRE functional assay: pGL2 (-101/+63 CCN1) and dominant negative CMV500A-CREB were cotransfected into SMCs, pGL2 (-101/+63 CCN1) and pcDNA3 were cotransfected as controls. Transfected cells were stimulated with LPA for 3 hours and luciferase activities were measured. n indicates numbers of experiments; in each, transfections were performed in duplicate.