cAMP Response Element–Binding Protein Mediates Thrombin-Induced Proliferation of Vascular Smooth Muscle Cells

Tomotake Tokunou, Toshihiro Ichiki, Kotaro Takeda, Yuko Funakoshi, Naoko Iino, Akira Takeshita

Abstract—Thrombin is a potent mitogen for vascular smooth muscle cells (VSMCs) and plays an important role in the progression of atherosclerosis. Although recent reports have suggested that cAMP response element–binding protein (CREB) is necessary for the survival of neuronal cells, the role of CREB in VSMC proliferation is not determined. We examined the role of CREB in thrombin-induced VSMC proliferation and the effect of thrombin on phosphorylation of CREB at Ser133, which is a critical marker for activation by Western blot analysis. Thrombin induced phosphorylation of CREB in a dose-dependent manner. An oligopeptide, SFLLRN, which activates the thrombin receptor, also induced the phosphorylation of CREB. Inhibition of extracellular signal–regulated protein kinase or inhibition of p38 mitogen-activated protein kinase suppressed the thrombin-induced CREB phosphorylation. Inhibition of the epidermal growth factor receptor by AG1478 also inhibited the thrombin-induced CREB phosphorylation. Overexpression of the dominant-negative form of CREB inhibited thrombin-induced c-fos mRNA expression and incorporation of [3 H]thymidine and [3 H]leucine. These results suggest that CREB-dependent gene transcription plays a critical role in thrombin-induced proliferation and hypertrophy of VSMCs. Transactivation of the epidermal growth factor receptor and 2 mitogen-activated protein kinase pathways are involved in this process. CREB may be a novel transcription factor mediating the vascular remodeling process induced by thrombin. (Arterioscler Thromb Vasc Biol. 2001;21:1764-1769.)

Key Words: thrombin ■ cAMP response element–binding protein ■ epidermal growth factor receptor ■ mitogen-activated protein kinase

Thrombin belongs to the multifunctional serine protease family and plays an important role in the blood coagulation cascade through the cleavage of fibrinogen to fibrin. Thrombin also activates intracellular signaling pathways through the thrombin receptor (protease-activated receptor-1 [PAR-1]). Thrombin activates PAR-1 by cleaving its amino-terminal exodomain to unmask a new receptor aminoterminal beginning with the sequence of SFLLRN, which functions as a tethered ligand. The synthetic oligopeptide SFLLRN activates PAR-1 independently of proteolysis. PAR-1 is a member of the 7–transmembrane domain receptor family. PAR-1 couples with Gβγ, which activates phospholipase Cβ, and Gαi, which inhibits adenylate cyclase. Recently, it has been reported that transactivation of the epidermal growth factor receptor (EGF-R) played an important role in thrombin signaling.

Thrombin is a potent mitogen for vascular smooth muscle cells (VSMCs), and PAR-1 is widely expressed in the atherosclerotic lesion, suggesting that thrombin may contribute to inflammatory and proliferative changes of the vascular wall, which are believed to be crucial for atherogenesis. Indeed, inhibition of thrombin by heparin or hirudin prevented neointimal formation after balloon angioplasty.

cAMP response element (CRE)-binding protein (CREB) is a 43-kDa nuclear transcription factor that was originally found to be activated by cAMP-dependent protein kinase (protein kinase A). Phosphorylation of serine residue at 133 (Ser133) is necessary for transcriptional activation. Recent studies have shown that phosphorylation of Ser133 is also mediated by extracellular signal–regulated protein kinase (ERK), p38 mitogen-activated protein kinase (MAPK), calmodulin-dependent kinase (CaMK), and Akt protein kinase pathways. Phosphorylation of CREB at Ser133 permits an access of the transcriptional coactivator, designated the CREB-binding protein.

Overexpression of the dominant-negative CREB transgene induced apoptosis in T cells in response to activation signals, and inhibition of CREB function induced neuronal cell death. These results suggest that CREB is critical for the survival of these cell types. However, these changes were not observed in mice with targeted deletion of the CREB gene. This discrepancy is explained by upregulation of other
CREB family genes in CREB knockout mice. Therefore, overexpression of the dominant-negative form of CREB has an advantage over gene deletion for the functional analysis of CREB. In the present study, we examined the role of CREB in thrombin-stimulated VSMCs by adeno-virus-mediated overexpression of dominant-negative CREB.

Methods

Reagents

DMEM and FBS were purchased from Gibco-BRL. Thrombin was purchased from Ito Ham Co. One unit per milliliter of thrombin is approximately equivalent to 20 nmol/L. 6-Amino acid peptide (SFLLRN), a PAR-1 agonist, was purchased from Bachem. PD98059 and U0126, inhibitors of ERK kinase, were purchased from Research Biochemicals International and Promega Co, respectively. SB203580 and FR167653, inhibitors of p38 MAPK, were generous gifts from SmithKline Beecham Pharmaceuticals and Fujisawa Pharmaceutical Co, Ltd, respectively. AG1478, an inhibitor of EGFR, and [Tyr(SO3 H)63]-hirudin fragment (54-65) were obtained from Sigma Chemical Co. All antibodies used in the experiments were obtained from New England Biolabs except for the horseradish peroxidase–conjugated second antibodies (anti-rabbit or anti-mouse IgG, Vector Laboratories Inc). A recombinant adeno-virus vector expressing a mutant of CREB (AdCREBM1)20 in which the phosphorylation site at Ser133 was changed to alanine was a gift from Dr Anthony J. Zeleznik (University of Pittsburgh, Pittsburgh, Pa). Unless mentioned otherwise, other chemical reagents were purchased from Wako Pure Chemicals.

Cell Culture

VSMCs were isolated from the thoracic aorta of Sprague-Dawley rats and maintained as described previously.21 Passages between 5 and 15 were used. VSMCs were grown to confluence, growth-arrested in DMEM with 0.1% BSA for 2 days, and used for the experiments.

Western Blot Analysis

VSMCs were lysed in sample buffer (5 mmol/L EDTA, 10 mmol/L Tris-HCl, pH 7.6, 1% Triton X-100, 50 mmol/L NaCl, 30 mmol/L sodium phosphate, 50 mmol/L NaF, 1% aprotinin, 0.5% pepstatin A, 2 mmol/L phenylmethylsulfonyl fluoride, and 5 mmol/L leupeptin). Protein concentrations were determined with the bicinchoninic acid protein assay kit (Pierce Chemical Co). Cell lysates (20 μg) were heated at 95°C for 5 minutes, electrophoresed on 12% SDS-PAGE, and transferred to polyvinylidene difluoride membrane (Millipore). The blots were blocked with TBS-T (20 mmol/L Tris-HCl, pH 7.6, 137 mmol/L NaCl, and 0.1% Tween 20) containing 10% nonfat dry milk at room temperature for 1 hour. Phosphorylated CREB at Ser133 was detected by a phospho-CREB antibody (recognizes only the phosphorylated form) by enhanced chemiluminescence (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. The membranes were exposed to x-ray film. The membranes were probed with hirudin for 5 minutes failed to induce CREB phosphorylation. Preincubation with PD98059 (30 μmol/L), also significantly suppressed the thrombin-induced CREB phosphorylation (Figure 2A). A combination of PD98059 and PD11021 partially suppressed the thrombin-induced CREB phosphorylation (Figure 2A). PD11021 (10 μmol/L) also significantly suppressed the thrombin-induced CREB phosphorylation (Figure 2A). PD11021 (10 μmol/L) also significantly suppressed the thrombin-induced CREB phosphorylation (Figure 2A).

Northern Blot Analysis

Total RNA was prepared according to an acid guanidinium–phenol–chloroform extraction method.22 Northern blot analysis of c-fos mRNA was performed as described previously.23 The hybridized membrane was stripped by boiling in 0.5% SDS solution and hybridized to a 32P-labeled 18S rRNA probe to obtain a reference for the amount of applied RNA. The radioactivity of hybridized bands of c-fos or 18S rRNA was quantified with a MacBAS bioimaging analyzer (Fujiﬁlm).

DNA and Protein Synthesis

After infection with AdCREBM1 or AdLacZ, VSMCs were cultured in DMEM with 0.1% BSA for 2 days. Then the cells were stimulated with thrombin for an additional 48 hours. VSMCs were labeled with [3H]thymidine or [3H]leucine during the last 24 hours of stimulation. The cells were washed with PBS, fixed in 10% trichloroacetic acid, and then washed with a mixture of ethanol and ether (2:1). The cells were lysed in 0.5N NaOH, and the radioactivity of incorporated [3H]thymidine or [3H]leucine was measured with a liquid scintillation counter.

Statistical Analysis

Statistical analyses were performed by 1-way ANOVA and multiple comparison (Fisher) tests if appropriate. A value of P<0.05 was considered significant. Data were expressed as mean±SE.

Results

Phosphorylation of CREB at Ser133 by Thrombin

To investigate whether CREB is activated by thrombin, we performed Western blot analysis by using an antibody that recognizes the phosphorylated form of CREB at Ser133. Phosphorylation of CREB was significantly increased in thrombin-stimulated VSMCs compared with unstimulated cells (Figure 1A). Figure 1B indicates that thrombin dose-dependently increased CREB phosphorylation. Figure 1C shows that an oligopeptide, SFLLRN (10 μmol/L), also induced phosphorylation of CREB at 10 minutes after stimulation, suggesting that the thrombin-induced phosphorylation of CREB is mediated by PAR-1. Hirudin binds to thrombin and inhibits thrombin function. Thrombin preincubated with hirudin for 5 minutes failed to induce CREB phosphorylation (Figure 1D).

MAPKs Are Important for Thrombin-Induced CREB Phosphorylation

We examined the pathways responsible for thrombin-induced phosphorylation of CREB. Preincubation with PD98059 (30 μmol/L) partially inhibited the thrombin-induced CREB phosphorylation (Figure 2A). SB203580 (10 μmol/L) also partially suppressed the thrombin-induced CREB phosphorylation (Figure 2A). A combination of PD98059 and...
SB203580 additively suppressed the thrombin-induced CREB phosphorylation. Figure 2C and 2D indicates that PD98059 and SB203580 almost completely suppressed thrombin-induced the activation of ERK and p38 MAPK, respectively, suggesting that the concentrations of these inhibitors were sufficient. To confirm the role of MAPKs, we used other MAPK inhibitors. U0126 (50 μmol/L) and FR167653 (1 μmol/L) also inhibited the thrombin-induced CREB phosphorylation (Figure 2B). Inhibition of EGF-R by AG1478 (2.5 μmol/L) also inhibited the thrombin-induced CREB phosphorylation (Figure 2E). AG1478 almost completely inhibited thrombin-induced activation of ERK and p38 MAPK (Figure 2E).

**Activation of CRE-Dependent Transcription by Thrombin**

We investigated whether thrombin activated CRE-dependent gene transcription by using a CRE/luciferase reporter construct. As shown in Figure 3, thrombin (1 U/mL) increased luciferase activity by 2.1-fold compared with that of unstimulated cells (P<0.01). The enhancement of luciferase activity by thrombin was significantly (P<0.01) suppressed by PD98059 and/or SB203580.

**Inhibition of Thrombin-Induced c-fos mRNA Expression by Overexpression of Dominant-Negative Form of CREB**

To clarify the role of CREB in thrombin signaling, we overexpressed the dominant-negative form of CREB by an adenovirus vector (AdCREBM1), which inhibits CREB function by replacing endogenous CREB with the overexpressed mutant CREB rather than by inhibition of phosphorylation. Although immunoreactivity of CREB was increased in an MOI-dependent manner, the expression of c-fos mRNA was significantly (P<0.01) suppressed by AdCREBM1.
manner by AdCREBM1 (Figure 4A), thrombin-induced CREB phosphorylation was not increased. CRE is one of the important cis DNA elements in the c-fos gene promoter.23 Infection of AdCREBM1 strongly inhibited thrombin-induced c-fos mRNA expression in VSMCs (Figure 4B).

Inhibition of Thrombin-Induced DNA and Protein Synthesis by AdCREBM1

We examined the role of CREB for thrombin-induced proliferation and hypertrophy of VSMCs. VSMCs were infected with AdCREBM1, and [3H]thymidine and [3H]leucine incorporation were measured. Incorporation of [3H]thymidine and [3H]leucine in thrombin-stimulated cells was increased significantly by 3.0-fold (P<0.01) and 2.3-fold (P<0.01), respectively, compared with the incorporation in unstimulated cells (Figure 5A and 5B). Infection of AdLacZ (30 MOI) did not affect the thrombin-induced incorporation of thymidine or leucine. However, infection of AdCREBM1 almost completely inhibited the thrombin-induced [3H]thymidine and [3H]leucine incorporation (Figure 5A and 5B). PD98059 and/or SB203580, which inhibited thrombin-induced phosphorylation of CREB, also inhibited the thrombin-induced [3H]thymidine and [3H]leucine incorporation (data not shown).

Discussion

We have found in the present study that (1) thrombin stimulated CREB phosphorylation and CRE-dependent gene transcription, (2) thrombin-induced phosphorylation of CREB was dependent on EGF-R transactivation and 2MAPK pathways, and (3) overexpression of the dominant-negative form of CREB inhibited thrombin-induced proliferation and hypertrophy of VSMCs. The present study, to the best of our knowledge, is the first report showing that CREB is a critical transcription factor for thrombin-induced proliferation of VSMCs.

Various protein kinases are reported to phosphorylate CREB at Ser133.11–15 We showed that ERK and p38 MAPK were involved in thrombin-induced CREB activation. We confirmed the role of ERK and p38 MAPK for thrombin-induced CREB phosphorylation by using second inhibitors for ERK (U0126) and p38 MAPK (FR167653). We also examined the effect of H89 (a protein kinase A inhibitor), KN93 (a CaMKII inhibitor), and wortmannin (an inhibitor of phosphatidylinositol 3-kinase that activates Akt/PKB) on thrombin-induced CREB phosphorylation. However, none of these inhibitors affected thrombin-induced CREB phosphorylation. The effect of these inhibitors on thrombin-induced CREB phosphorylation is summarized in the Table.

p90RSK-2,12 downstream from ERK, was reported to phosphorylate CREB, and MAPK-activated protein kinase-2,24 downstream from p38 MAPK, also phosphorylates CREB. Our data suggest that p90RSK-2 and MAPK-activated protein kinase-2 may phosphorylate CREB in response to thrombin. Furthermore, another protein kinase designated mitogen- and stress-activated protein kinase-1 (MSK1),25 which is activated by ERK and p38 MAPK, was reported. Activated MSK1 phosphorylates CREB at Ser133. Suppression of ERK and p38 MAPK is required to suppress the activation of MSK1. Therefore, it may be possible that MSK1 mediates thrombin-induced phosphorylation of CREB. Further study is necessary to determine the kinase that directly phosphorylates CREB downstream from ERK and p38 MAPK.

Transactivation of EGF-R is indispensable for thrombin-induced ERK activation.3 Because AG1478 suppressed
thrombin-induced ERK and p38 MAPK activation, inhibition of thrombin-induced CREB phosphorylation by AG1478 may be ascribed to the suppression of these MAPK pathways.

Although thrombin induced CREB phosphorylation by severalfold, CRE promoter activity was upregulated by 2.1-fold. The reason for this discrepancy is not clear. However, competition of phosphorylated CREB between CRE sites of endogenous genes and CRE-luciferase plasmid may occur. Most of the phosphorylated CREB may bind to and activate endogenous genes. Therefore, CRE-luciferase activity may not be upregulated to the same extent as the level of CREB phosphorylation.

Thrombin rapidly induced c-fos mRNA expression as previously reported. CREB in the promoter region plays a critical role in the induction of c-fos gene expression in response to mitogens. We showed that overexpression of the dominant-negative form of CREB strongly suppressed thrombin-induced c-fos mRNA expression, confirming the previous results. Also, thrombin-induced incorporation of thyminidine and leucine was almost completely blocked by AdCREBM1. Because a number of genes are reported to have a CRE site in the promoter region, it is difficult to identify the target gene(s) that is critically involved in the suppression of VSMC growth by AdCREBM1. CRE may play a critical role in the expression of >1 gene and may be important in the thrombin-induced growth of VSMCs. Alternatively, accumulation of partial inhibition of CRE-dependent gene expression, such as the effect of AdCREBM1 that we observed on thrombin-induced c-fos mRNA expression, may cause strong growth inhibition. In addition to c-fos, 1 of the candidate genes is proliferating cell nuclear antigen (PCNA), an auxiliary factor of DNA polymerase δ. PCNA is necessary for DNA replication, and the promoter of the PCNA gene contains a CRE site. Further study is necessary to identify the critical gene(s) that is inhibited by the dominant-negative form of CREB.

Overexpression of wild-type CREB in VSMCs did not affect basal and thrombin-induced incorporation of thyminidine or leucine (data not shown). It was previously reported that the concentration of CREB in the nucleus of PC12 cells was so high that the high-affinity CREs were expected to be nearly saturated. CREB was readily detected by Western blot analysis in our VSMCs. Therefore, we assume that our VSMCs may also express a sufficient amount of CREB, and overexpression of CREB did not show any additional effect on basal and thrombin-induced incorporation of thyminidine or leucine.

Apart from the central role of the blood coagulation cascade, thrombin is a potent mitogen for VSMCs. Proliferation of VSMCs by thrombin requires reactive oxygen species (ROS), as recently reported in platelet-derived growth factor–induced and angiotensin II–induced mitogenesis. Diphenyleneiodonium, an inhibitor of NAD(P)H oxidase, inhibited thrombin-induced VSMC proliferation and ROS production, suggesting that PAR-1 activates NAD(P)H oxidase. We have not examined whether ROS is involved in CREB activation. Rao et al. reported that N-acetylcysteine, an antioxidant, inhibited thrombin-induced ERK and p38 MAPK activation. Therefore, thrombin-induced ROS may regulate CREB activation through MAPKs.

In normal arteries, the thrombin receptor is mainly expressed in the endothelial layer. In human atheroma, PAR-1 was widely expressed in the areas rich in macrophages and in VSMCs. PAR-1 expression was induced as early as 6 hours after balloon injury of the carotid artery, and the upregulation of PAR-1 expression continued throughout vascular lesion formation for up to 2 weeks. Gallo et al. reported that prolonged and continuous treatment with hirudin suppressed neointimal formation in the balloon injury model of porcine coronary arteries. It has also been reported that mice lacking PAR-1 suffer from less neointimal formation in response to balloon injury of the artery. These results suggest that thrombin and its receptor, PAR-1, are critically involved in the progression of atherosclerosis. We have shown in the present study that CREB is a crucial transcription factor for thrombin-induced mitogenesis and hypertrophy of VSMCs. CREB may be a novel target in the prevention of atherogenesis.

Acknowledgments

This study was supported in part by a grant from the Kaibara Morikazu Memorial Foundation, Fukuoka, Japan; by the Welfide Medicinal Research Foundation, Osaka, Japan; and by a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture (Nos. 1177035 and 12877113).

References


cAMP Response Element-Binding Protein Mediates Thrombin-Induced Proliferation of Vascular Smooth Muscle Cells
Tomohke Tokunou, Toshihiro Ichiki, Kotaro Takeda, Yuko Funakoshi, Naoko Iino and Akira Takeshita

doi: 10.1161/hq2112.098770

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/21/11/1764

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/