Thrombin Induces Interleukin-6 Expression Through the cAMP Response Element in Vascular Smooth Muscle Cells

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Abstract—The plasma level of interleukin-6 (IL-6) is elevated in patients with acute coronary syndromes and has prognostic value. Thrombin is a potent mitogen for vascular smooth muscle cells (VSMCs) and plays an important role in the progression of atherosclerosis. We examined the mechanism of thrombin-induced IL-6 expression in VSMCs. Thrombin induced IL-6 mRNA and protein expression in a dose-dependent manner. Pharmacological inhibition of extracellular signal-regulated protein kinase (ERK), p38 mitogen-activated protein kinase (MAPK), or epidermal growth factor receptor (EGF-R) suppressed the thrombin-induced IL-6 expression. Deletion and mutation analysis of the promoter region of the IL-6 gene by using luciferase as a reporter showed that the DNA segment between −228 and −150 bp containing the cAMP response element (CRE) site played a critical role. Thrombin also induced phosphorylation of CRE binding protein (CREB) in an ERK- and a p38 MAPK–dependent manner. Overexpression of the dominant-negative form of CREB inhibited thrombin-induced IL-6 mRNA expression. These results suggest that the CRE site and CREB play an important role in thrombin-induced IL-6 gene expression in VSMCs. Transactivation of EGF-R and activation of ERK and p38 MAPK are involved in this process. CREB may be a novel transcription factor that regulates thrombin-induced gene expression. (Arterioscler Thromb Vasc Biol. 2001;21:1759-1763.)

Key Words: interleukin-6 • thrombin • vascular smooth muscle cells • cAMP response element • epidermal growth factor receptor

It has been suggested that the inflammatory reaction characterizes the process of atherogenesis. Cytokines such as interleukin-6 (IL-6) and monocyte chemotactic protein-1 secreted from activated macrophages, lymphocytes, endothelial cells, and vascular smooth muscle cells (VSMCs) are critical mediators of inflammation in blood vessels. In a substantial proportion of patients with acute coronary syndromes, plasma levels of C-reactive protein and IL-6 are elevated. In addition, IL-6 has both short- and long-term prognostic value in patients with or without overt plaque rupture. It was also reported that the IL-6 concentration in coronary sinus blood was correlated with the rate of late restenosis after balloon angioplasty. These results suggest that IL-6 plays an important role in atherogenesis.

Thrombin belongs to the multifunctional serine protease family and converts fibrinogen to fibrin, which is a final reaction of the coagulation cascade. Thrombin also activates intracellular signaling pathways through the thrombin receptor (protease-activated receptor-1: PAR-1) by cleaving the amino-terminal exodomain of PAR-1 to “unmask” a new receptor amino terminus beginning with the sequence SFLLRN. Thrombin is a potent mitogen for 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. PAR-1 is a member of the 7-transmembrane-domain receptor family and couples to Gβγ, which activates phospholipase Cβ and Goi, which inhibits adenylate cyclase. Recently, it was reported that transactivation of the epidermal growth factor receptor (EGF-R) played an important role in thrombin signaling. The inhibitory effect of heparin on thrombin-induced VSMC migration was partially due to a blockade of heparin-binding EGF-like growth factor–mediated EGF-R transactivation.

Induction of IL-6 by thrombin is reported in monocytes and VSMCs. However, the precise mechanism and signaling pathway for this induction have not been determined. We show in the present study that the cAMP response element (CRE) is critical for thrombin-induced IL-6 expression.

Methods

Reagents

Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum were purchased from GIBCO BRL. Thrombin was purchased from Ito Ham Co. One unit per milliliter thrombin is approximately equivalent to 20 nmol/L. PAR-1 agonist, a 6–amino acid peptide (SFLLRN), was purchased from Bachem. PD98059, an extracellular...
signal–regulated protein kinase (ERK) kinase inhibitor, was obtained from Research Biochemicals International. SB203580, a p38 mitogen–activated protein kinase (MAPK) inhibitor, was a generous gift from SmithKline Beecham Pharmaceuticals (UK). AG1478, an EGF-R inhibitor, was obtained from Sigma. All antibodies used in the experiments were obtained from New England Biolabs except for an antibody against activating transcription factor-2 (ATF-2; Santa Cruz Biotechnology, Inc) and the horseradish peroxidase–conjugated second antibodies (anti-rabbit or anti-mouse IgG, Vector Laboratories Inc). A recombinant adenovirus vector expressing a mutant of CRE binding protein (CREB) (AdCREBM1),17 in which the phosphorylation site at Ser133 is 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 aortas of Sprague-Dawley rats and maintained as described previously.18 Passages between 5 and 15 were used. VSMCs were grown to confluence, growth-arrested in DMEM with 0.1% bovine serum albumin for 2 days, and used for the experiments.

Quantification of Rat IL-6 by Sandwich ELISA
An ELISA for rat IL-6 was performed with a Cytoscreen ELISA kit (BioSource International) according to the manufacturer’s instructions, as described previously.18

Northern Blot Analysis
Total RNA was prepared according to an acid guanidinium–phenol–chloroform extraction method.19 Northern blot analysis of IL-6 mRNA was performed as described previously.18 The hybridized membrane was stripped by boiling in a 0.5% SDS solution and hybridized to a 32P-labeled 18S ribosomal RNA probe to obtain a reference for the amount of applied RNA. The radioactivity of hybridized bands of IL-6 or 18S ribosomal RNA was quantified with a MacBAS bioimaging analyzer (FUJIFILM).

Mutations of the IL-6 Gene Promoter Region and Transfection to VSMCs
Deletion mutants of the IL-6 gene promoter fused with the luciferase gene and of the IL-6 gene promoter construct with mutation at the CRE site were described previously18 and introduced into VSMCs with the DEAE dextran method according to the manufacturer’s instruction’s (Promega Corp) with the LacZ gene driven by an SV40 promoter-enhancer sequence. After transfection, the cells were cultured in DMEM with 10% fetal bovine serum for 48 hours, washed twice with PBS, and stimulated with 1 U/mL thrombin for 24 hours in DMEM with 0.1% bovine serum albumin. Then the luciferase activity was measured and normalized by β-galactosidase activity, as described previously.18

Western Blot Analysis
VSMCs were lysed in sample buffer (5 mmol/L EDTA, 10 mmol/L Tris-HCl [pH 7.6], 1% TritonX-100, 50 mmol/L NaCl, 30 mmol/L sodium phosphate, 50 mmol/L NaF, 1% aprotinin, 0.5% pepstatin A, 2 mmol/L PMSF, 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–polyacrylamide gel electrophoresis gels, and transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore). The blots were blocked with TBS-T (20 mmol/L Tris-HCl [pH 7.6], 137 mmol/L NaCl, 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 (which recognizes only the phosphorylated form) by ECL chemiluminescence (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. The membranes were exposed to x-ray film. The membranes were stripped by incubating them in buffer containing 62.5 mmol/L Tris-HCl, 2% SDS, and 100 mmol/L 2-mercaptoethanol at 50°C for 1 hour and reprobed with an antibody against CREB (which recognizes both phosphorylated and nonphosphorylated forms) by the same procedure. Activation of ERK, p38 MAPK, and ATF-2, which is downstream from p38 MAPK, was examined by the same method.

Infection by AdCREBM1 and AdLacZ
Confluent VSMCs were washed with PBS 3 times and incubated with AdCREBM1 or adenovirus vector expressing LacZ (AdLacZ) for 2 hours at room temperature in PBS under gentle agitation. Then the cells were washed 3 times with PBS, cultured in DMEM with 0.1% bovine serum albumin for 2 days, and used for the experiments. Multiplicity of infection indicates the number of viruses per cell added to the culture dish. The infection efficiency of adenovirus is almost 100%, as determined by staining for β-galactosidase activity expressed by AdLacZ (data not shown).

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

Results

Thrombin-Induced IL-6 Production
VSMCs were stimulated with thrombin, and the protein level of IL-6 in the supernatant was measured by sandwich ELISA. As shown in Figure 1A, the protein level of IL-6 in the tissue culture medium of thrombin-stimulated VSMCs was increased significantly in a time-dependent manner. A small amount of IL-6 was detected in the supernatant of unstimulated VSMCs. Figure 1B shows that thrombin dose-dependently increased IL-6 production after 24 hours of stimulation.

Expression of IL-6 mRNA by Thrombin
We examined the expression of IL-6 mRNA by Northern blot analysis in thrombin-stimulated VSMCs. Two species of IL-6 gene transcript were detected.18 For quantification of IL-6 mRNA, the radioactivities of both mRNA species were taken into account. As shown in Figure 2A, thrombin induced IL-6 mRNA expression with a peak at 30 minutes of stimulation. VSMCs were incubated with varying concentrations of thrombin for 30 minutes (Figure 2B). Thrombin dose-dependently increased IL-6 mRNA expression. Figure 2C shows that an oligopeptide, SFLLRN, which activates PAR-1 independently of proteolysis, also induced expression of IL-6 mRNA at 30 minutes after stimulation, suggesting that the thrombin-induced IL-6 mRNA expression is mediated by PAR-1.
Figure 2. Expression of IL-6 mRNA by thrombin. Thrombin-induced IL-6 mRNA expression was detected by Northern blot analysis, and the radioactivities of the bands were measured with an imaging analyzer. The radioactivity of IL-6 mRNA was standardized with that of ribosomal RNA (rRNA). Results are expressed as mean ± SE. *P < 0.01 vs without stimulation; NS, not significant. (A) VSMCs were stimulated with thrombin (1 U/mL) for the indicated periods (n = 4). (B) VSMCs were stimulated with varying concentrations of thrombin for 30 minutes (n = 3). (C) VSMCs were stimulated with thrombin (10^2 U/mL), an oligopeptide SFLRRN (10 μmol/L) for 30 minutes (n = 3). A representative autoradiograph of IL-6 mRNA and rRNA is shown in upper panel. Lower bar graph shows relative IL-6 mRNA expression normalized to rRNA expression. The ratio in unstimulated cells was designated as 1.0.

MAPKs and Transactivation of EGF-R Are Important for Thrombin-Induced IL-6 Expression

A recent study has suggested that ERK and p38 MAPK play an important role for the signaling of PAR-1, and that transactivation of EGF-R mediates thrombin-induced ERK activation. Therefore, we examined the effect of the ERK kinase inhibitor PD98059, the p38 MAPK inhibitor SB203580, and the EGF-R inhibitor AG1478 on thrombin-induced IL-6 mRNA expression. PD98059 or SB203580 dose-dependently inhibited thrombin-induced IL-6 mRNA expression (Figures 3A and 3B). AG1478 also inhibited the thrombin-induced IL-6 mRNA (Figure 3C). These data suggest that transactivation of ERK and p38 MAPK and transactivation of EGF-R are important for thrombin-induced IL-6 expression.

Activation of IL-6 Gene Promoter by Thrombin

To determine the promoter region responsible for the induction of IL-6 gene expression by thrombin, deletion analysis of the promoter region was performed. We constructed luciferase expression vectors with varying lengths of the promoter region of the IL-6 gene (Figure 4A). Luciferase activity was measured after 24 hours of thrombin stimulation. Thrombin increased luciferase activity (Figure 4A, constructs No. 1 through 4). The response to thrombin, however, was not observed in the construct that lacked the DNA segment between -228 and -150 bp (Figure 4A, construct No. 5). In the DNA segment between -228 and -150 bp, CRE is present. Therefore, we introduced a mutation into the CRE site. Figure 4B shows that the thrombin-induced luciferase activity expressed by the IL-6 gene promoter with a mutation in the CRE site was completely inhibited compared with that expressed by an intact promoter construct. These data suggest that the CRE site in the IL-6 gene promoter is important for thrombin-induced upregulation.

Thrombin-Induced Phosphorylation of CREB

Next, we examined whether thrombin activated CREB by Western blot analysis with an antibody that specifically recognizes the phosphorylated form of CREB, which is the active form. Thrombin indeed induced phosphorylation of CREB (online Figure I, which can be accessed at http://atvb.ahajournals.org). We examined the effect of PD98059, SB203580, and AG1478 on thrombin-induced CREB phosphorylation. PD98059 or SB203580 dose-dependently inhibited thrombin-induced CREB phosphorylation (online Figures IA and IB). We also examined whether PD98059 and SB203580 specifically inhibited ERK kinase and p38 MAPK, respectively. PD98059 dose-dependently inhibited thrombin-induced phosphorylation of CREB, ERK, and p38 MAPK (online Figure IC). These data suggest that transactivation of EGF-R by thrombin activates ERK and p38 MAPK, which is followed by CREB phosphorylation.

Inhibition of Thrombin-Induced IL-6 mRNA Expression by Overexpression of the Dominant-Negative Form of CREB

To confirm the role of CRE 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 molecule.\textsuperscript{17} Infection with AdCREBM1 strongly inhibited thrombin-induced IL-6 mRNA expression in VSMCs (Figure 4C), whereas infection with AdLacZ (used as a negative control) did not affect thrombin-induced IL-6 mRNA expression.

**Discussion**

We demonstrate in the present study that (1) thrombin-induced IL-6 expression is dependent on CRE in the promoter region of the IL-6 gene and (2) thrombin-induced EGF-R transactivation and activation of ERK and p38 MAPK play an important role for the induction of IL-6 expression. Induction of IL-6 by thrombin has been reported in epithelial cells\textsuperscript{4} and monocytes\textsuperscript{5} as well as VSMCs.\textsuperscript{3} This study, to the best of our knowledge, is the first report showing that EGF-R, MAPKs, and CRE are critical for thrombin-induced IL-6 expression in VSMCs.

An association of plasma IL-6 level with atherosclerotic diseases has been reported. The plasma concentration of IL-6 is higher in patients with unstable angina than in those with stable angina\textsuperscript{21} and is an important factor for prognosis.\textsuperscript{7} In unstable angina and myocardial infarction, the hemostatic mechanism is activated,\textsuperscript{22} and thrombosis and fibrotic organization contribute to the progression of atherosclerosis after rupture of atherosclerotic plaques.\textsuperscript{23} PAR-1 is also widely expressed in the atherosclerotic lesion.\textsuperscript{11} These data suggest that inflammatory and coagulation systems are activated simultaneously in the coronary atherosclerotic region. The relationship and balance of these 2 systems are critical for the progression of the atherosclerotic region. IL-6 has been shown to be a potent stimulator of platelet production,\textsuperscript{24} and inhibition of IL-6 function by a neutralizing antibody was reported to attenuate endotoxin-induced blood coagulation.\textsuperscript{25} These results may suggest an important role for IL-6 in blood coagulation and the pathological significance of thrombin-induced IL-6 production.

We examined the role of EGF-R in thrombin-induced IL-6 expression. AG1478, an EGF-R inhibitor, inhibited thrombin-induced IL-6 expression. Transactivation of EGF-R is necessary for the activation of ERK by thrombin.\textsuperscript{14} PD98059, an inhibitor of ERK, also suppressed thrombin-induced IL-6 expression. SB203580, an inhibitor of p38 MAPK, also partially suppressed thrombin-induced IL-6 expression. We examined whether thrombin induced CREB phosphorylation by using a phospho-specific antibody against CREB. Thrombin induced CREB phosphorylation, and AG1478, PD98059, or SB203580 inhibited the thrombin-induced phosphorylation.\textsuperscript{9,25} which is a downstream kinase activated by ERK, is reported to phosphorylate CREB.\textsuperscript{26} and Tan et al\textsuperscript{27} showed that MAPK-activated protein kinase 2 in the region downstream from p38 MAPK also phosphorylated CREB. These reports and our data suggest that thrombin may upregulate IL-6 expression through transactivation of the EGF-R–ERK–p90RSK2–CREB pathway, and another pathway, EGF-R–p38 MAPK–MAPK-activated protein kinase 2-CREB may also be important. Further study is necessary to identify the kinase that directly phosphorylates CREB in response to thrombin.

Although AG1478 almost completely inhibited thrombin-induced IL-6 expression, overexpression of dominant-negative CREB, PD98059, or SB203580 partially suppressed it. These data suggest that another pathway activated by transactivation of EGF-R also plays a role in thrombin-induced IL-6 expression. We examined the effects of the protein kinase C inhibitor GF109203X and an extracellular Ca\textsuperscript{2+} chelator, EGTA, on thrombin-induced IL-6 mRNA expression. These inhibitors did not affect thrombin-induced IL-6 mRNA expression (data not shown). Further study is necessary to determine this additional pathway of PAR-1.

Results of the luciferase assay indicated that the DNA segment between −228 and −150 bp of the IL-6 gene promoter was critical for thrombin-induced IL-6 expression.
In this DNA segment, CRE (−156 to −149 bp) is present. Introduction of a mutation into the CRE site of the IL-6 promoter completely inhibited thrombin-induced luciferase activity. Overexpression of dominant-negative CREB also inhibited thrombin-induced IL-6 mRNA expression. These data indicate that CRE is one of the critical cis-DNA elements for thrombin-induced IL-6 gene expression. The IL-6 promoter has other cis-DNA elements, such as nuclear factor IL-6 (NF-IL-6, −147 to −137 bp) and NF-κB (−62 to −44 bp). NF-IL-6 and NF-κB may also contribute to thrombin-induced IL-6 expression, because these elements are located downstream from CRE. Deletion of the DNA segment containing NF-IL-6 and NF-κB reduced basal luciferase activity (Figure 4A, construct No. 6), and luciferase activity was not upregulated by thrombin in this construct. Therefore, these cis-DNA elements may be important for basal transcriptional activity of IL-6 gene. Further study is necessary to determine the role of these downstream cis-DNA elements.

We previously reported that angiotensin II induced IL-6 mRNA expression in VSMCs. We found that the CRE site is also important for angiotensin II–induced IL-6 expression. PD98059 completely inhibited angiotensin II–induced IL-6 mRNA expression, whereas thrombin-induced IL-6 expression was partially inhibited by the same concentration of PD98059. These data suggest that the main pathway of angiotensin II–induced IL-6 mRNA expression is the MAPK kinase/ERK pathway, and thrombin uses both MAPK kinase/ERK and p38 MAPK pathways. Eguchi et al reported that angiotensin II–induced ERK activity was inhibited by the EGF-R inhibitor AG1478, suggesting that EGF-R is also important for angiotensin II–induced IL-6 mRNA expression as well as that of thrombin.

In summary, we have shown in this article that CRE is a crucial cis-DNA element for thrombin-induced IL-6 expression and that EGF-R plays an important role in thrombin-induced IL-6 expression.

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