Factor Xa induces mitogenesis of coronary artery smooth muscle cell via activation of PAR-2

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Abstract Factor Xa-induced stimulation of coronary artery smooth muscle cells (CASMC) was investigated by analyzing [^{3}H]thymidine incorporation, cell proliferation, and ERK-1/2 activation. Exposure of the cells to factor Xa evoked a time-dependent activation of ERK-1/2 with increased [^{3}H]thymidine incorporation and cell proliferation. The factor Xa-induced ERK-1/2 activation was not desensitized by preincubation of the cells with thrombin. However, ERK-1/2 activation was markedly attenuated by prior exposure of the cells to protease-activated receptor-2 (PAR-2) activating peptide, SLIGKV. The mitogenic effect of factor Xa was significantly reduced in the presence of anti-PAR-2 monoclonal antibody. Several lines of experimental evidence indicate that factor Xa-induced mitogenesis of CASMC is a cellular process mediated by PAR-2 activation.

Key words: Coronary artery smooth muscle cell; Factor Xa; Protease-activated receptor-2

1. Introduction

While percutaneous transluminal coronary angioplasty has become an important tool for the management of coronary artery disease, restenosis in at least 30–50% of cases remains the major limiting factor of long-term efficacy [1]. Neointimal hyperplasia is an important mechanism of restenosis after percutaneous transluminal coronary angioplasty [2]. Vascular smooth muscle cell (SMC) migration and proliferation are both necessary events that contribute to the formation of neointimal plaque [3]. After arterial injury, SMC mitogenesis is triggered by a large number of growth factors, cytokines, and vasoregulatory molecules that participate in the pathogenic process [4]. Furthermore, SMC provides a source of tissue factor (TF) following arterial injury [5] and TF forms a high-affinity complex with factor VII/VIIa and thereby initiates extrinsic blood coagulation, leading to the formation of factor Xa and thrombin [6]. It has been well established that thrombin acts as a mitogen for SMC [7,8], as well as a coagulant. There are several reports suggesting that the mitogenic effect of thrombin is due to the expression and release of bFGF [9] or at least in part, PDGF [10,11]. In analogy to the functions of thrombin, it has also been suggested that factor Xa acts as a potent mitogen for aortic SMC [12–14] and SMC from human saphenous vein [15]. Furthermore, it has been confirmed that factor Xa stimulates the release of PDGF from rat and human aortic SMC [13,14]. Thus, factor Xa-stimulated mitogenic effect on aortic SMC appears to be mediated via release of PDGF from SMC, whereas factor Xa-induced signaling and mitogenic activity in human venous SMC are independent of PDGF [15]. However, detailed cellular mechanism of factor Xa-induced SMC proliferation has not been elucidated yet. In this work, we have demonstrated that factor Xa-induced mitogenesis of human coronary artery SMC undergoes via signalling pathway triggered by protease-activated receptor-2 (PAR-2) activation.

2. Materials and methods

2.1. Materials

Human coronary artery smooth muscle cells (CASMC) and Clonetics SmGM®-2 BulletKit (CC-3182) were purchased from BioWhittaker (USA) and [^{3}H]thymidine from NEN Life Science Products (USA). Clonetics SmGM®-2 BulletKit (CC-3182) contains SMC basal medium (modified MCDB 131) and some growth supplements. Human factor Xa was purchased from Haematologic Technologies (USA) and Pefabloc Xa from PentaPharm (Switzerland). Recombinant tick anticoagulant protein (TAP) was kindly gifted by Dr. Y. Jang (Cardiovascular Research Institute, Yonsei University College of Medicine, Seoul, Korea). NuPAGE™ 4–12% Bis-Tris gel was from Invitrogen (USA). DC protein assay reagent was purchased from Bio-Rad (USA). Phospho-ERK-1/2 antibody from Cell Signaling Technology (USA), peroxidase labeled goat anti-rabbit IgG from KPL (USA), and ECL kit from Amersham Pharmacia Biotech (Sweden). The PAR-2 agonist peptide SLIGKV was synthesized by Peptron (Korea) and monoclonal anti-PAR-2 antibody (SAM11) was purchased from Santa Cruz Biotechnology (UK). All other reagents were of the highest commercial purity.

2.2. Cell culture

Human CASMC were cultured in Clonetics SmGM®-2 medium supplemented with 10% fetal bovine serum (FBS). Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. All SMC used in these studies were from passages 4–9.

2.3. [^{3}H]Thymidine incorporation

CASMC (4×10^4) were seeded into a 24-well plate and incubated for 24 h in Clonetics SmGM®-2 medium. After further incubation of the cells in SMC basal medium (modified MCDB 131) containing 0.5% FBS for 48 h, they were treated with inhibitors or antibodies for 30 min before stimulation. Following an additional 24-h incubation, cells were then pulsed with 2 μCi of [^{3}H]thymidine for 8 h at 37°C and washed twice with phosphate-buffered saline (PBS). Cells were fixed in methanol at 4°C for 5 min and washed twice in 5% TCA. The acid-insoluble material was dissolved in 0.3 M NaOH at room temperature followed by radioactivity measurement with liquid scintillation counter.

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2.4. Cell proliferation assay

CASMC (1.25×10^4) were plated onto 24-well culture plates and incubated at 37°C for 24 h. After cells were rendered quiescent in SMC basal medium (modified MCDB 131) containing 0.5% FBS for 48 h, they were treated for 30 min with competitive factor Xa inhibitors. The cells were then stimulated with 50 nM factor Xa for 72 h. The medium was removed and cell number/well was counted using a hemocytometer.

2.5. Immunoblotting

CASMC were allowed to grow to confluence in 6-well plates and made quiescent in SMC basal medium (modified MCDB 131) containing 0.2% FBS for 48 h. After stimulation with 50 nM factor Xa, in the presence or absence of inhibitors and antibodies for the times indicated, cells were rinsed in ice-cold PBS and treated with lysis buffer (1% Triton X-100, 0.1% β-mercaptoethanol, 1 mM EDTA, 1 mM EGTA, 50 mM Tris–HCl (pH 7.0), 1 mM PMSF) for 20 min on ice. Cell lysates were collected into microcentrifuge tubes, vortexed, and centrifuged at 12 000 rpm for 20 min. Protein concentration was measured in the supernatant using a DC protein assay reagent according to the manufacturer's instructions and equalized for all samples. Reduced samples (30 μg) were subjected to SDS–PAGE (NuPAGE™ 4–12% Bis-Tris gel) and then electrotransferred to nitrocellulose membrane. For detection of phosphorylated ERK-1/2, membranes were incubated with antibody directed against a phospho-specific ERK-1/2 followed by incubation with goat anti-rabbit IgG conjugated to horseradish peroxidase. ECL detection method was employed for color development.

3. Results and discussion

In this study, we have demonstrated that factor Xa induces mitogenesis of CASMC via activation of PAR-2. It has been well established that factor Xa promotes aortic SMC [12,14,16] and saphenous vein SMC [15] mitogenesis. Likewise, stimulation of CASMC by factor Xa caused an increase in both [^{3}H]thymidine incorporation and cell number as compared to unstimulated CASMC (Fig. 1). The factor Xa-induced DNA synthesis was markedly suppressed by the competitive factor Xa inhibitors such as TAP [17] and Pefabloc Xa [18] (Fig. 1A). Similar inhibitory effect on factor Xa-induced cell proliferation was also obtained with these inhibitors (Fig. 1B). Factor Xa inhibitors did not influence [^{3}H]thymidine incorporation and cell proliferation on their own (data not shown). Exposure of CASMC to factor Xa evoked a time-dependent activation of ERK-1/2 (Fig. 2A). Maximal kinase activation was observed in 5 min after stimulation of the cell, and then returned to its basal level in 40 min. The factor Xa-induced activation of ERK-1/2 was completely suppressed by concomitant treatment of CASMC with factor Xa inhibitors (Fig. 2B). Factor Xa inhibitors did not affect the activation of ERK-1/2 on their own (data not shown). These results are correlated to the findings of Gasic et al. [12] who first observed that nanomolar concentrations of factor Xa promote cultured rat aortic SMC mitogenesis. Herbert et al. [14] subsequently demonstrated that factor Xa increased phosphoinositide turnover as a potent mitogen in human aortic SMC. Moreover, these effects were markedly reduced by DX9065a, a specific competitive inhibitor of factor Xa [19]. In SMC from human saphenous vein, the mitogenic effect of factor Xa was also significantly suppressed by factor Xa inhibitors [15]. These results indicate that factor Xa-induced mitogenic effect on SMC is closely associated with the proteolytic enzyme activity of the factor. Furthermore, it has been shown that factor Xa exerts its mitogenic effect via stimulating the release of PDGF in human umbilical vein endothelial cells [20] and in cultured vascular SMC of rat

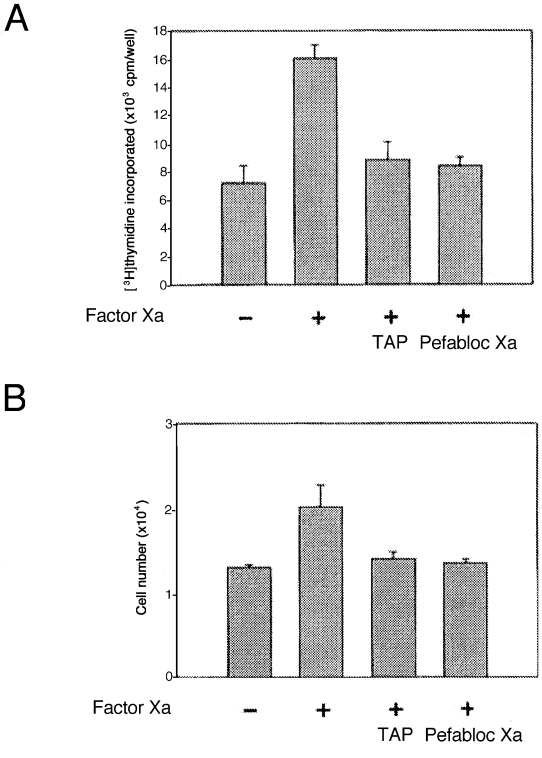

Fig. 1. Factor Xa-induced mitogenesis of CASMC. Quiescent CASMC were stimulated with 50 nM factor Xa in the presence or absence of competitive factor Xa inhibitors, 2 μM TAP and 2 μM Pefabloc Xa, respectively. CASMC mitogenesis was estimated by measuring (A) [^{3}H]thymidine incorporation into DNA, and (B) number of cells as described in Section 2. Error bars represent the S.E.M. ($n=4$).

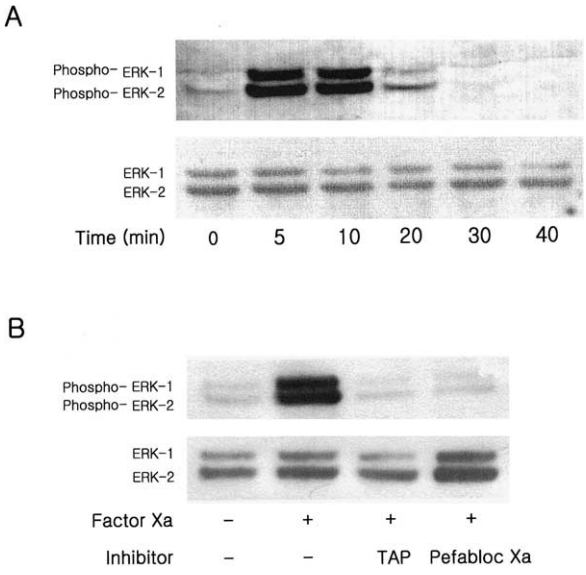

Fig. 2. Factor Xa-induced ERK-1/2 activation in CASMC. A: Time-dependent activation of ERK-1/2 by factor Xa. Cells were exposed to 50 nM factor Xa for the indicated times. B: Effect of factor Xa inhibitors on factor Xa-stimulated ERK-1/2 activation. Cells were treated with 2 μM TAP or Pefabloc Xa for 30 min before exposure to 50 nM factor Xa for 10 min. Cell lysates were assayed for the phosphorylation of ERK-1/2 ($n=3$; representative experiment).

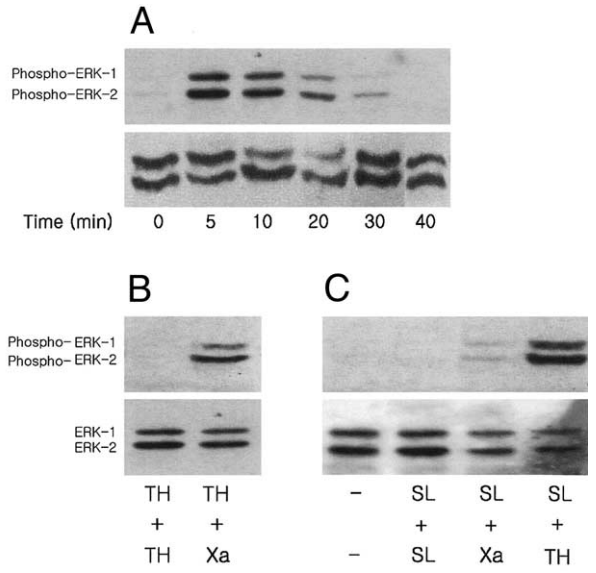

Fig. 3. Desensitization of agonist-stimulated ERK-1/2 activation by thrombin or PAR-2 agonist peptide pretreatment. A: Time-dependent activation of ERK-1/2 by SLIGKV. Cells were exposed to 200 μM SLIGKV for the indicated times. B: Cells were treated with 50 nM thrombin (TH) for 40 min prior to the second exposure to 50 nM thrombin (TH) or 50 nM factor Xa (Xa) for 10 min. C: Cells were stimulated with 200 μM SLIGKV (SL) for 40 min prior to the second exposure to 200 μM SLIGKV, 50 nM factor Xa, or 50 nM thrombin for 10 min. Cell lysates were then assayed for the phosphorylation of ERK-1/2 ($n=4$; representative experiment).

[13]. Ko et al. [13] suggested that factor Xa, via its serine protease activity, is responsible for the release of preexisting PDGF from vascular SMC, and the PDGF in turn activates ERK-1/2 through the receptor tyrosine kinase. There has been no clear evidence, however, related to the specific receptor that is activated by factor Xa leading to the release of PDGF and mitogenesis of SMC.

Assuming that the mitogenic effect is evoked by the proteolytic activity of factor Xa, it was possible to postulate that the signaling events might be induced via one of the PARs which

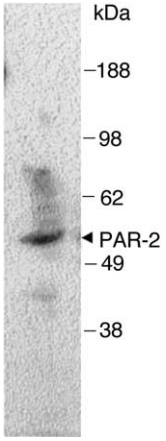

Fig. 4. Expression of PAR-2 in human CASMC. Cultured cells were sonicated in PBS for 30 s and then centrifuged at 100 000 × g for 60 min. Precipitates were solubilized in lysis buffer and subjected to SDS–PAGE (50 μg protein/lane). After transfer to nitrocellulose membrane, it was reacted with monoclonal anti-PAR-2 antibody (SAM11). The protein was visualized using anti-mouse IgG conjugated to horseradish peroxidase and ECL detection reagent ($n=3$; representative experiment).

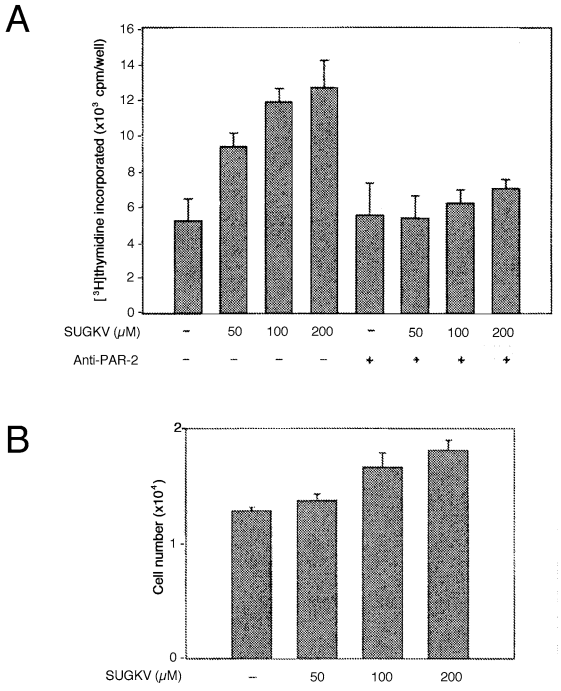

Fig. 5. PAR-2 agonist peptide-induced mitogenesis of CASMC. Quiescent CASMC were stimulated with the indicated concentrations of SLIGKV in the presence or absence of anti-PAR-2 monoclonal antibody (SAM11) (30 μg/ml). CASMC mitogenesis was estimated by measuring (A) [^{3}H]thymidine incorporation into DNA, and (B) number of cells. Error bars represent the S.E.M. ($n=5$).

have recently been indentified. PAR family currently consists of four related proteins: PAR-1 [21,22], which can be activated by thrombin or trypsin [21], PAR-2 by trypsin [23] or tryptase [24,25], PAR-3 by thrombin [26], and PAR-4 by thrombin or trypsin [27,28]. All of the four PAR family members are G protein-coupled receptors that are normally activated by proteolytic exposure of a new tethered ligand. There is experimental evidence demonstrating that thrombin stimulates SMC proliferation via proteolytic activation of a receptor [29,30], and tryptase- or trypsin-induced mitogenesis of SMC is mediated by the activation of PAR-2 [31–33]. In order to investigate whether factor Xa-induced signaling event is mediated by the activation of a PAR in CASMC, desensitization experiments were performed as described previously [34]. When the cells were treated with thrombin, it took 40 min for ERK-1/2 signal to return to its basal level (data not shown). However, it was able to induce ERK-1/2 activation again by immediately exposing the thrombin-desensitized cells to factor

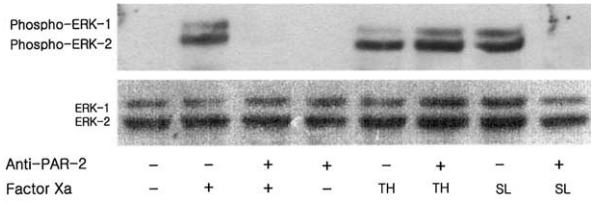

Fig. 6. Suppression of agonist-induced ERK-1/2 activation by anti-PAR-2 monoclonal antibody. Cells were stimulated with 50 nM factor Xa, 50 nM thrombin (TH), or 50 μM SLIGKV(SL) for 10 min in the presence or absence of anti-PAR-2 monoclonal antibody (SAM11) (30 μg/ml). Cell lysates were then assayed for the phosphorylation of ERK-1/2 ($n=4$; representative experiment).

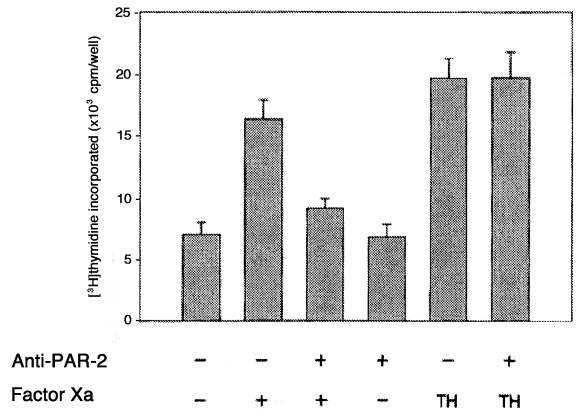

Fig. 7. Suppression of factor Xa-induced [^{3}H]thymidine incorporation by anti-PAR-2 monoclonal antibody. Quiescent CASMC were stimulated with 50 nM factor Xa or thrombin (TH) in the presence or absence of anti-PAR-2 monoclonal antibody (SAM11) (30 μg/ml). Error bars represent the S.E.M. ($n=4$).

Xa, suggesting that the factor activates a receptor distinct from thrombin-activated ones in CASMC (Fig. 3B). Thus, we postulated that PAR-2 could be a strong candidate as a factor Xa-activated receptor on CASMC. Expression of PAR-2 was then identified in CASMC by Western blot analysis (Fig. 4). PAR-2 is expressed in many cell types including SMC [35], endothelial cells [36], keratinocytes [37], epithelial cells [38], fibroblasts [39], and mast cells [40]. Cellular function of the receptor is associated with proliferation of several cell lines [32,39,41], activation of blood coagulation in endothelial cell [42], SMC contraction [43], tissue repair [37], and inflammatory reactions [44]. PAR-2 is activated not only by trypsin [23] or tryptase [24,25], but also by synthetic peptide SLIGKV [45–47]. Involvement of PAR-2 in CASMC mitogenesis was verified by performing [^{3}H]thymidine incorporation and cell proliferation experiments with PAR-2 agonist peptide SLIGKV. As expected, the agonist peptide was able to induce cell proliferation as well as DNA synthesis in CASMC (Fig. 5). It has been reported that the agonist peptide stimulates mitogenesis in SMC [31–33]. The agonist peptide also evoked a time-dependent activation of ERK-1/2 with a maximum effect in 5 min, and then the kinase activation returned to its basal level in 40 min (Fig. 3A). After the first challenge with SLIGKV for 40 min, a second exposure of the cells to factor Xa failed to activate ERK-1/2 (Fig. 3C). These experimental results provide an evidence for the involvement of PAR-2 in factor Xa-induced cell signaling. In contrast, thrombin was able to elicit ERK-1/2 activation after initial exposure of the cells to SLIGKV. Similar findings were reported in human umbilical vein endothelial cells [48]. Factor Xa-induced Ca^{2+} signaling response was blocked by pretreatment of the endothelial cells with SLIGKV, whereas PAR-1-dependent response was unaffected. Enzymatic cleavage of PAR-2 in endothelial cells by factor Xa was also demonstrated by proteolytic digestion of a synthetic peptide duplicating the PAR-2 cleavage site [48].

To further investigate the requirement of PAR-2 for the cell activation, CASMC were stimulated by factor Xa in the presence of anti-PAR-2 monoclonal antibody raised against amino acid residues 37–50 of PAR-2 of human origin. As illustrated in Fig. 6, the particular monoclonal antibody was able to effectively suppress the factor Xa-induced activation of ERK-1/2, whereas thrombin-induced activation of the kinase remained intact. Similar inhibitory effect on factor Xa-induced [^{3}H]thymidine incorporation was also obtained with the monoclonal antibody (Fig. 7). In contrast, thrombin-activated DNA synthesis of CASMC was unaffected in the presence of anti-PAR-2 monoclonal antibody. The specificity of the anti-PAR-2 monoclonal antibody was demonstrated by effective suppression of SLIGKV-induced ERK-1/2 activation and [^{3}H]thymidine incorporation (Figs. 5 and 6). The monoclonal antibody itself did not influence the phosphorylation of ERK-1/2 and [^{3}H]thymidine incorporation (Figs. 5 and 6). Taken together the results obtained in Figs. 3–7, it appears to be clear that PAR-2 mediates factor Xa-induced cell signaling in CASMC.

In summary, we have demonstrated with experimental evidence in this communication that factor Xa promotes human CASMC mitogenesis which is mediated by PAR-2 activation. Further studies on factor Xa-induced signaling pathway of CASMC will provide useful information for understanding cellular function of factor Xa in detail which is associated with mitogenesis.

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