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Research Article

The agonist of the protease-activated receptor-1 (PAR1) but not PAR3 mimics thrombin-induced vascular endothelial growth factor release in human vascular smooth muscle cells

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Abstract. Thrombin, a serine protease generated by the activation of the blood coagulation cascade following vessel injury, induces vascular endothelial growth factor (VEGF) release. However, the molecular mechanism of thrombin-induced VEGF release is largely unknown. An agonist of protease-activated receptor-1 (PAR1), SFLLRNPNDKYEPF, mimicked thrombin-induced VEGF release in human vascular smooth muscle (HVSM) cells, as determined by enzyme-linked immunosorbent assay, reverse transcriptase-polymerase chain reaction, and Northern blotting. In contrast, the agonist of PAR3, TFRGAP, did not affect VEGF release or expression. SFLLRNPNDKYEPF, but not TFRGAP, up-regulated $[Ca^{2+}]_i$. Moreover, the calcium ionophone A23187 was found to trigger VEGF release in HVSM cells. Thrombin-induced VEGF release was blocked by anti-thrombin, heparin, a synthetic thrombin receptor inhibitor E5510, the calcium chelator BAPTA, the protein kinase C inhibitor calphostin C, and the MEK1/2 inhibitor U0126. Thus, our data show that thrombin caused VEGF release via PAR1 activation in a manner dependent on $[Ca^{2+}]_i$ and p44/42 downstream from the receptor activation.

Key words. Thrombin; HVSM cell; PAR1; Ca^{2+}; p44/42; VEGF.

Thrombin is a multifunctional serine protease, which is generated proteolytically from prothrombin by factor Xa in the final step of the blood coagulation cascade following vessel injury. Thrombin converts fibrinogen to fibrin, activates platelets and several coagulation factors, and plays a crucial role in thrombosis and hemostasis [1, 2]. These effects of thrombin are mediated, at least in part, through the thrombin receptor that is coupled to the G protein [3, 4]. There are at least three protease-activated receptors (PARs) that have been identified as thrombin receptors, PAR1, 3, and 4 [3, 5, 6]. Like other G-protein-coupled receptors, the three PAR family members are comprised of a single polypeptide with seven membrane-spanning domains and an extended extracellular N terminus. However, unlike the receptors for most cellular growth factors, PAR1 does not require the traditional ligand-receptor complex formation for activation. Instead, the receptor serves as a substrate for proteolytic

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digestion, yielding an irreversibly activated form on the cell surface to convey additional cell signaling. Thrombin cleaves the PAR1 between Arg41-Ser42. Once thrombin cleaves this site, a new truncated N terminus, starting with the amino acid serine, is unveiled. Interestingly, the first 6–14 amino acids (SFLLRNPNDKYEPF) in the newly generated N-terminal domain play a role in the intramolecular ligand activation mechanism, and have been found to be an agonist for PAR1 activation [3, 7]. Even in the absence of thrombin, this peptide binds to the second loop of its transmembrane domain, and mimics the action of thrombin [8]. It also mimics most of the cellular actions of thrombin such as cell proliferation [9], neurite retraction [10], and apoptosis [11]. PAR1 activation by thrombin triggers two main signaling events. The first event is thought to be involved in the inhibition of cAMP through interactions with the inhibitory G protein of the Gi class [12, 13]. The second event is stimulation of phospholipase C (PLC)-catalyzed hydrolysis of phosphoinositides, resulting in an increase in intracellular calcium, and activation of protein kinases [12, 14].

The family of protein kinases and mitogen-activated protein kinases (MAPKs) lie at the core of cell growth, differentiation, tumor angiogenesis, and survival-signaling pathways. Activation of protein kinase C (PKC) is generally modulated by intracellular calcium and diacylglycerol. Extracellular signal-regulating kinase (ERK1/2 or p44/42) is predominantly activated by mitogens through a Ras/Raf/MEK (MAPK kinase) signaling cascade [15]. However, phosphatidylinositol (PI3-K) is generally activated following membrane phospholipid turnover, and the activation of the serine-threonine kinase Akt is dependent on PI3-K in many cell types [16, 17]. NF-κB, a transcriptional factor which regulates most of the members of the cytokine family**,** has been found to be associated with the proliferation of smooth muscle cells after thrombin exposure [9]**.**

Vascular endothelial growth factor (VEGF) is a cytokine that regulates the proliferation, differentiation, and survival of microvascular endothelial cells. VEGF is encoded by a single gene that finally produces at least four protein products: VEGF 121, VEGF 165, VEGF 189, and VEGF 206 (according to the number of amino acids), generated by an alternative splicing of the mRNA [18]. VEGF is a potent angiogenic factor and is specific for endothelial cell migration. This increases the fenestration as well as the extravasation of plasma macromolecules. The enhanced expression of VEGF has been implicated in pathophysiological conditions associated with tumor angiogenesis [19], rheumatoid arthritis [20], wound healing [21], diabetic retinopathy [22], and atherosclerosis [23]. On the other hand, enhanced expression of the thrombin receptor has been shown in intimal/medial regions of the atherosclerotic vessels [24, 25]. Recently, thrombin has been shown to induce VEGF release in smooth muscle cells (SMCs). As the proliferation of SMCs may cause various diseases such as restenosis after percutaneous transluminal coronary angioplasty and atherosclerosis, attention has been drawn to thrombin-induced VEGF release in SMCs [26, 27], which suggests that VEGF may play a critical role in pathophysiological states. Thus, elucidation of the molecular mechanism of thrombin-induced VEGF release is needed to develop a therapeutic approach. The purpose of the present study was to obtain insights into the role of thrombin receptors and their downstream signaling pathways in thrombin-induced VEGF release in SMCs. Data presented in the present study suggest that PAR-1 and its downstream signaling pathway involving intracellular calcium and p44/42 mediate the thrombin-induced VEGF release in human vascular smooth muscle (HVSM) cells.

Materials and methods

Materials

Thrombin was purchased from Sigma (St Louis, Mo.). The MEK1 inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis [2-aminophenylthio] butadiene) was purchased from Promega (Madison, Wis.). A23187, BAPTA, LY294002, calphostin C, and pyrrolidinedithiocarbamate (PDTC) were purchased from Calbiochem (San Diego, Calif.). SN50 was purchased from Biomol (Plymouth, Mass.). Protease inhibitor (complete cocktail) was purchased from Roche Molecular Biochemicals (Indianapolis, Ind.). Antithrombin III, heparin sulfate and all other chemicals were purchased from Sigma. E5510 was kindly provided by Eisai Pharmaceutical Company (Tokyo, Japan).

Antibodies

MAPK assay kits (containing polyclonal antibodies against p44/42 MAPK/phos-p44/42 and antibodies against Akt/phos-Akt (serine 473 and threonine 308) were purchased from Cell Signaling Techonology (Beverly, Mass.).

Cell culture

HVSM cells were obtained from a human umbilical cord, as described previously by Nakajima et al. [9]**.** In brief, explants were grown in Early's 199 medium containing 10% fetal bovine serum (FBS) with appropriate antibiotics at 37 °C in a 5% CO_2 atmosphere and 95% air. Isolated SMCs were identified by their typical 'hill and valley' morphology in cultures and about 95–97% cells were found to express α-actin, the marker of vascular smooth muscles. Cells from passages 5–9 were used in all of the experiments.

Enzyme-linked immunosorbent assay for VEGF

VEGF levels in the culture media were measured by a newly developed colorometric enzyme-linked immunosorbent assay (ELISA) with slight modifications of the chemiluminescence enzyme immunoassay method [28]. The anti-VEGF/VPF IgG was used as a plate coating, and a peroxidase (POD)-conjugated Fab′ fragment of the antibody was used as a secondary antibody. The samples diluted with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) were added to the wells of 96-well dishes, and incubated for 1 h at 22 °C. After washing, the POD-conjugate was added, and the samples were incubated for 1 h at 22 °C, with o-phenylenediamine being used as a substrate for the reaction in a plate reader (Microplate reader M-Vmax; Molecular Devices).

RT-PCR analysis

HVSM cells were treated with thrombin and the thrombin receptor agonist peptide (TRAP) for the indicated periods. Total RNAs were extracted using an RNA extraction kit, TRIZOL (Gibco BRL, Rockville, Md.), and reverse transcription was done at 42 °C using reverse transcriptase (Takara, Osaka, Japan). One-twentieth of the volume of the RT product was amplified using Taq DNA polymerase (Promega), according to the manufacturer's instructions. The sense primer was 5′-GAGAATTCGGCCTCCGAA-3′ and the anti-sense primer was 5′-GAGCATGCCCTC-CTGCCC-3′ for VEGF. The PCR profile consisted of a 1-min initial denaturation at 94 °C, followed by 30 cycles, with each cycle comprising 1 min denaturation at 94 °C, 1 min extension at 72 °C, 1 min annealing at 63 °C, and finally 5 min extension at 72 °C, and the PCR products were separated in a 2% (w/v) agarose gel.

Northern blotting of VEGF

The HVSM cells were grown up to 60–80% confluence, and then treated with the indicated concentrations of thrombin or TRAPs, and incubated for the time indicated. Total RNAs were extracted with TRIZOL, and were analyzed by hybridization with a ^{32}P-labeled cDNA of VEGF probe.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined by dual-wavelength fura-2 microfluorometry combined with a digital camera. The SMCs were incubated with 2 µM fura-2 acetoxymethylester for 30 min at 37 °C in 5.6 mM glucose containing Krebs Ringer bicarbonate buffer (KRB) composed of 121.7 mM NaCl, 4.4 mM KCl, 1.2 mM KH_2PO_4, 2.0 mM CaCl2, 1.2 mM $MgSO_4$, 5.0 mM $NaHCO_3$, and 10 mM HEPES (pH 7.4). The cells were then mounted in a chamber, and superfused with KRB containing reagents at 37 °C. The cells were excited at 340 and 380 nm alternately every 5 s. Emission signals at 510 nm were detected with an silicon intensified (SIT) camera, and the ratio images were produced by an Argus-50 system (Hamamatsu Photonics, Hamamatsu, Japan).

Preparation of whole-cell lysates and Western blotting

Preparation of cell lysates and analysis of the activation of MAPKs were done as described by Sarker et al. [29].

Assay for immunoprecipitated PI3-K activity

The stimulated HVSM cells were collected and lysed with a cold lysis buffer (137 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 1% NP-40, 1 mM PMSF) and were immunoprecipitated by incubating with the anti-PI3-K antibody-bound agarose beads for 1 h at 4 °C. The washed beads were reacted with a solution [0.88 mM ATP containing 30 µl of γ-^{32}P-ATP (3000 Ci/mmol) and 20 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA] for 10 min at 37 °C, and then 20 µl of 6N HCl was added to each sample to stop the reaction. Samples were run onto a thin-layer chromatography (TLC) plate and the radiolabeled lipids were visualized by autoradiography.

Results

The PAR1 but not PAR3 agonist mimics thrombin-induced VEGF release in the culture media of HVSM cells

To determine whether thrombin triggers VEGF release in vascular smooth muscle cells, HVSM cells were treated with the various concentrations of thrombin for 16 h. Figure 1A shows that VEGF release significantly increased to 377 ± 30.5 pg/ml when the cells were exposed to 10 U/ml of thrombin. The HVSM cells were then incubated with 10 U/ml of thrombin for the indicated periods. As shown in figure 1B, VEGF release was induced by thrombin as early as 4 h; however, the peak (413 ± 33.64 pg/ml) was 16 h after thrombin treatment, and then reached its plateau.

To determine the role of thrombin receptors in VEGF release, HVSM cells were treated with the PAR1 agonist peptide, SFLLRNPNDKYEPF, and the PAR3 agonist peptide, TFRGAP. The PAR1 but not the PAR3 agonist peptide induced VEGF release in HVSM cells. Like thrombin, PAR1 dose- dependently increased VEGF release. Figure 2A shows that the VEGF release was initiated by 10 µM SFLLRNPNDKYEPF, and significantly increased to 310 ± 30.6 pg/ml by 100 µM at 16 h after treatment. However, TFRGAP did not increase the VEGF release. Treatment of the cells with the scrambled peptide of PAR1 (SLLFRNPNDKYEPF) did not induce VEGF release (data not shown). The SFLLRNPNDKYEPF-induced VEGF release peaked 16 h after thrombin treatment (fig. 2B).

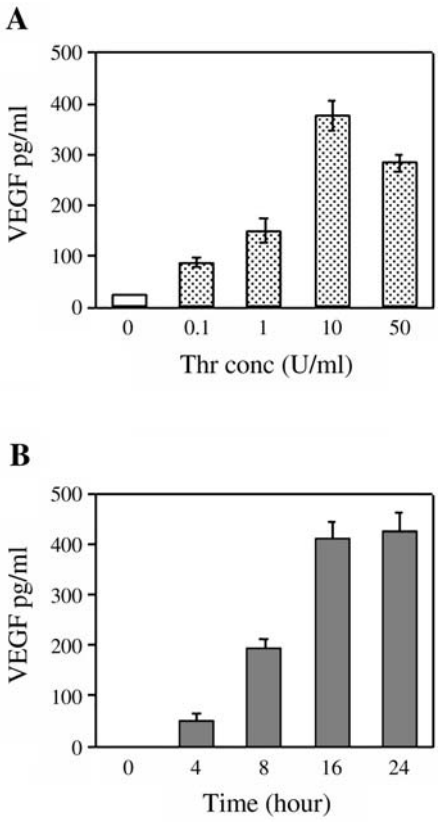

Figure 1. Thrombin dose- and time-dependently triggers VEGF release in HVSM cells. Results are given as the mean ± SD of three individual experiments. (*A*) Dose-dependency of thrombin-induced VEGF release. HVSM cells (1×10^5/well) were plated onto 12-well collagen-coated dishes. Supernatant was collected after 16 h of treatment and VEGF was measured by ELISA. (*B*) Time-dependency of VEGF release after exposure to thrombin (10 U/ml).

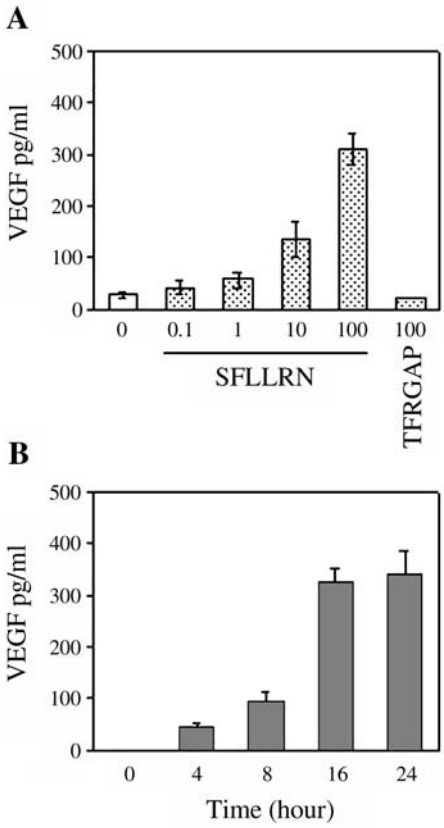

Figure 2. Effects of PARs on VEGF release in HVMS cells. Results are given as the mean ± SD of three individual experiments. (*A*) Dose-dependency of PAR agonists for VEGF release. HVSM cells (1×10^5/well) plated onto 12-well dishes were treated with micromolar concentrations of PAR1 agonist (SFLLRNPNDKYEPF) and PAR3 agonist (TFRGAP) and VEGF was measured by ELISA after 16 h. (*B*) Time-dependency of VEGF release after exposure to 100 μM SFLLRNPNDKYEPF (SFLLRN).

The PAR1 agonist peptide mimics the thrombin-induced message level of VEGF in HVSM cells

To examine whether PAR1 receptor activation induces VEGF mRNA, HVMS cells were initially treated with thrombin or SFLLRNPNDKYEPF over time and VEGF expression was determined by RT-PCR and Northern blotting. As shown in figure 3A, thrombin induced expression of VEGF mRNA within 30 min and this expression was sustained until 15 h after thrombin addition as determined by Northern blotting (fig. 3B). Like thrombin, the PAR1 agonist peptide SFLLRNPNDKYEPF markedly elicited the expression of VEGF mRNA (fig. 3C). However, PAR3 was found to be ineffective in influencing the expression of VEGF mRNA (data not shown), further demonstrating further that thrombin triggers the expression of the message level of VEGF through PAR1 activation.

PAR1 triggers VEGF release through increasing the intracellular calcium level in HVSM cells

To determine the intracellular signaling pathways in thrombin-induced VEGF release, we first investigated whether thrombin receptor activation could enhance intracellular calcium concentration. As shown in figure 4A, the agonist of PAR1, SFLLRNPNDKYEPF, noticeably up-regulated intracellular $[Ca^{2+}]_i$. In contrast, the agonist of PAR3, TFRGAP, failed to elicit intracellular calcium accumulation, indicating that intracellular calcium may play a key role in thrombin-induced VEGF release. To test whether the increase in intracellular calcium led to an increase in VEGF release, the cells were then exposed to the calcium ionophore A23187 for 16 h. Figure 4C shows that 1 μM A23187 strikingly triggered VEGF release, suggesting that intracellular calcium plays a critical role in the thrombin-induced VEGF release.

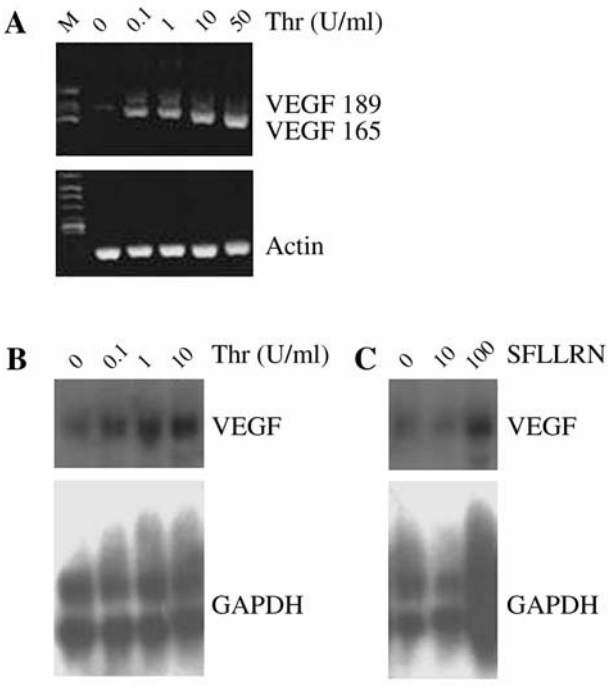

Figure 3. Up-regulation of VEGF mRNA in thrombin- and SFLLRNPNDKYEPF-treated HVSM cells. (*A*) Dose-dependency of thrombin effect on expression of VEGF mRNA. HVSM cells plated onto 10-cm collagen-coated dishes were treated with thrombin. (*B, C*) Increase in VEGF mRNA determined by Northern blotting 15 h after treatment with thrombin (U/ml) and SFLLRNPNDKYEPF (micromolar concentrations). Blots are representative of three independent experiments.

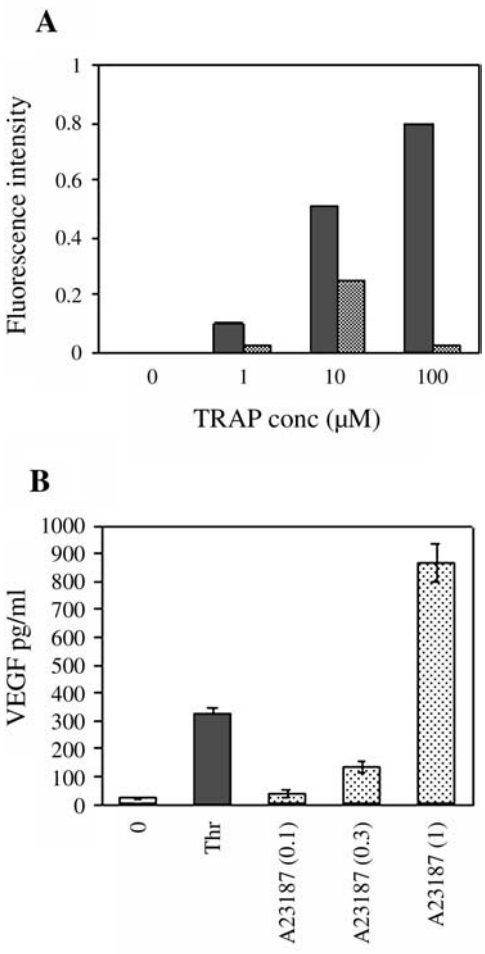

Figure 4. The Calcium ionophore A23187 triggers VEGF release in HVSM cells. (*A*) PAR1 but not PAR3 up-regulates intracellular calcium. HVSM cells were seeded onto cover ships and treated with TRAPs. Solid bars represent the PAR1 agonist (SFLLRN) and hatched bars represent the PAR3 agonist (TFRGAP). (*B*) A23187 triggers VEGF release in HVSM cells. HVSM cells (1×10^5/well) plated onto 12-well collagen-coated dishes were treated with A23187 (micromolar concentrations) for 16 h. Results are given as the mean ± SD of three individual experiments.

Thrombin triggers the PI3-K-Akt signaling cascade in HVSM cells

To determine whether thrombin-induced VEGF release was dependent on the PI3-K signaling cascade, HVSM cells were exposed to 10 U/ml thrombin. PI3-K was activated as early as 3 min, followed by a noticeable increase 10 min after thrombin exposure (fig. 5A). Activation of PI3-K has been documented to increase Akt activity. To investigate whether Akt was activated in thrombin-treated cells, the activation of Akt was determined by immunoblotting using a phospho-specific antibody against Akt serine 473 and threonine 308. As shown in figure 5B (upper and middle panels), thrombin caused the phosphorylation of Akt both at serine 473 and threonine 308, 5 min after thrombin exposure.

p44/42 but not the PI3-K-Akt or NF-κB signaling module mediates thrombin-induced VEGF release

We next investigated whether thrombin activated p44/42 MAPK in HVSM cells. Cells were exposed to 10 U/ml of thrombin, and activation of p44/42 MAPK was monitored at various time points. A slight activation of p44/42 MAPK was observed 2.5 min after the addition of thrombin, followed by a noticeable increase 5 min after thrombin exposure (fig. 6A). The thrombin-induced p44/42 activation was drastically abolished by the MEK1/2 inhibitor U0126 (fig. 6B). Like thrombin, SFLLRNPNDKYPEF and A23187 activated p44/42 within 5 min of exposure (fig. 6C). This is evidence that thrombin receptor activation leads to an increase in cytosolic $[Ca^{2+}]_i$ which, in turn, activates PKC. Thrombin receptor activation also results in the activation of NF-κB [9]. We then undertook a pharmacological blockade study to observe whether the inhibition of thrombin/ thrombin receptor, chelation of Ca^{2+}, inhibition of PKC, PI3-K, p44/42, or NF-κB activation blocks thrombin-induced VEGF release. HVSM cells were pretreated with anti-thrombin, heparin, a synthetic thrombin receptor inhibitor E5510 [9], a cell-permeable calcium chelator BAPTA, a PKC inhibitor calphostin C, the PI3-K inhibitor LY294002, a p44/42 MAPK inhibitor U0126, and the NF-κB inhibitor

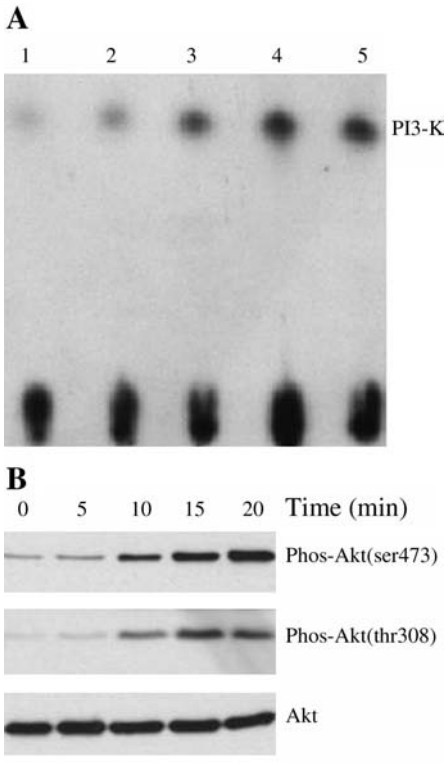

Figure 5. Thrombin activates PI3-K and Akt in HVSM cells. Cells plated onto 60- mm dishes (3×10^5/dish) were exposed to thrombin (10 U/ml). (*A*) Time-dependency of PI3-K activation. Lane1, control; lane 2, thrombin 1 min; lane 3, thrombin 3 min; lane 4, thrombin 10 min; lane 5, platelet-derived growth factor 3 min (positive control). (*B*) Thrombin time-dependently triggers phosphorylation of Akt at serine 473 (upper panel) and threonine 308 (middle panel). Blots are representative of three individual experiments.

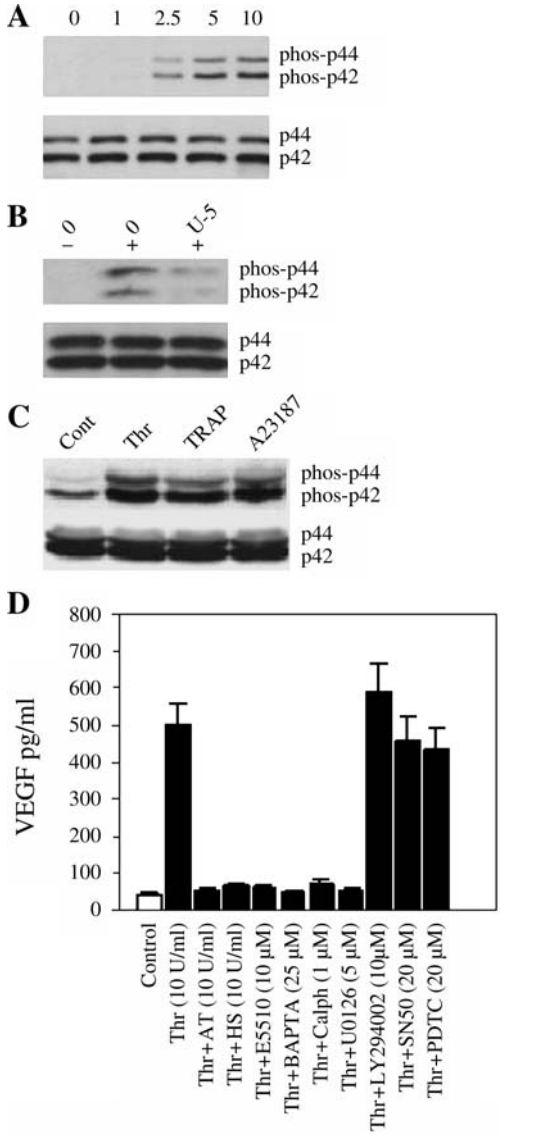

Figure 6. Effects of various agents on ERK activation and VEGF release in HVSM cells. (*A*) Time-dependency of p44/42 activation in thrombin-treated cells. (*B*) U0126 blocks thrombin-induced p44/42 activation. Cells were pretreated with 5 μM U0126 for 30 min followed by thrombin (10 U/ml) addition for 5 min. (*C*) SFLLRNPNDKYEPF (TRAP) and A23187 activate p44/42 in HVSM cells. Cells were treated with thrombin (10 U/ml), SFLLRNPNDKYEPF (TRAP, 100 μM) or A23187 (1 μM) for 5 min. (*D*) Antithrombin (AT), heparin (HS), and inhibitors of the thrombin receptor (E5510), PKC (Calph), p44/42 (V0126) and a calcium chelator (BAPTA) block thrombin-induced VEGF release in HVSM cells. Cells were exposed to the agents for 30 min prior to the addition of thrombin (10 U/ml). VEGF release was determined after 16 h of thrombin exposure. Values given are the mean ± SD of three independent experiments. LY294002, a PI3-K inhibitor; SN50, NF-κB inhibitor; PDTC, NF-κB inhibitor.

SN50 or PDTC for 30 min, followed by thrombin addition for 16 h. Although LY294002, U0126, SN50 and PDTC failed to inhibit thrombin-induced VEGF release, U1026, BAPTA, and calphostin C markedly blocked thrombin-induced VEGF release, implicating intracellular calcium, PKC, and p44/42 in thrombin-induced VEGF release in HVSM cells. None of these inhibitors alone showed any cytotoxic effects on SMCs (data not shown).

Discussion

Blood coagulation is initiated following vessel injury, leading to the generation of thrombin and thrombus formation. Clot-bound thrombin exerts a pro-coagulant activity, and may further enhance the local thrombin concentration. Moreover, inflammation usually stimulates the coagulation cascade and thrombin generation. Thrombin may increase the release of VEGF in pathological states, leading to worsening of vascular complications. VEGF acts as a chemotactic factor for macrophages, major players in atherosclerosis. Recently, VEGF has been shown to enhance progression of atherosclerotic plaques [30, 31]. On the other hand, expression of tissue factor, an initiator of the intrinsic blood coagulation cascade, has been reported to be up-regulated by VEGF in

endothelial cell surfaces [32, 33]. In addition, thrombin receptor activation has been suggested to be correlated with the progression of vascular diseases such as atherosclerosis and restenosis [24, 25]. Thus, these results suggest that VEGF release in HVSM cells by thrombin may play a crucial role in vascular remodeling.

VEGF 189 and VEGF 206 are stored in the membrane whereas VEGF 121 and VEGF 165 are secreted. Although thrombin treatment induced an increase in both the message and protein level of VEGF (in the conditioned media) as determined by RT-PCR/Northern blotting and ELISA, respectively, the intracellular level of VEGF was not increased after thrombin exposure as determined by immunoblotting (data not shown). We have no current explanation for this phenomenon; however, one possibility is that once VEGF is synthesized, it is released into the media and functions in an autocrine or paracrine manner. Thrombin mediates its regulatory effects by activating cell surface receptors belonging to the PAR family. In addition to PAR1, thrombin transduces its cellular signaling through PAR3 [3, 5, 6]. We observed that cells treated with 100 μM of the PAR1 agonist peptide, SFLLRNPNDKYEPF, noticeably triggered VEGF release. The PAR1 agonist peptide was also found to increase VEGF mRNA in HVSM cells, consistent with the result obtained by using ELISA. A study by Nakajima et al. [9] reported that thrombin at a concentration of 10 U/ml induced SMC proliferation, which is blocked by E5510, an inhibitor of PAR1. In agreement with this finding, we observed that expression of VEGF and release of VEGF into the culture medium peaked when cells were treated with 10 U/ml of thrombin. In the present study, we also observed that thrombin-induced VEGF release was blocked by the PAR1 inhibitor E5510 (data not shown), suggesting that thrombin exerts its cellular functions through the activation of PAR1 in HVSM cells. By contrast, the PAR3 agonist peptide TFRGAP did not elicit VEGF release at a concentration similar to or higher than PAR1. Thus, this study demonstrates for the first time that PAR1 but not PAR3 mediates thrombin-induced VEGF release. The reason for the inability of PAR3 to mediate VEGF release in HVSM cells is not clear, but may be related to the modulation of adenyl cyclase. Elevated cAMP levels inhibit SMC proliferation [13, 34]. Coupling to Gi and the subsequent inhibition of adenyl cyclase may contribute to the efficacy of PAR1 in mediating the mitogenic response to thrombin. Conversely, the inability of PAR3 to inhibit adenyl cyclase may be one possible explanation for its lower potency compared to PAR1.

Thrombin receptor activation causes at least three common signaling modules downstream from the thrombin receptor: (i) PLC isoforms that increase cytosolic $[Ca^{2+}]_i$ and activate PKCs, (ii) the PI3-K/Akt pathway, and (iii) the ERK class of MAPKs. The balance of signal intensities, kinetics, and potential cross-regulations between these pathways may define the direction of VEGF release in HVSM cells. In addition to triggering VEGF release, PAR1 significantly increased the intracellular calcium concentration in HVSM cells, while PAR3 failed to do so. These results suggest that intracellular calcium may mediate the thrombin-induced VEGF release in HVSM cells. This notion was supported by the observation that the thrombin-induced VEGF release was markedly blocked by the intracellular calcium chelator BAPTA. Further support was provided by the finding that cells stimulated with A23187 showed a striking increase in VEGF release. Thrombin exerts a number of cellular functions in a manner dependent on calcium [35, 36]. To the best of our knowledge, we report for the first time that thrombin triggers VEGF release in a fashion dependent on intracellular calcium, where PAR1 but not PAR3 plays a regulatory role in the HVSM cells.

The diverse actions of thrombin are possible because thrombin triggers a number of parallel-signaling pathways. ERK and Akt signaling pathways have been reported to cross-talk to each other, and have been implicated in thrombin-induced cell proliferation and survival signaling pathways. Evidence has been gathered which shows that PI3-K-Akt inhibits the Raf-MEK-ERK signaling pathways [37]. The disruption of PI3-K/Akt signaling augmented platelet-derived growth-factor-induced Raf-kinase activity, resulting in sustained p44/42 phosphorylation [34]. The thrombin-induced VEGF release in HVSM cells was associated with the activation of p44/42. Our result is in line with the report that thrombin induced p44/42 activation in rat SMCs [34]. Inhibition of the p44/42 activation by U0126 resulted in an almost complete block of VEGF release. Conversely, thrombin caused the activation of PI3-K and the subsequent phosphorylation of Akt (at serine 473 and threonine 308). The thrombin-induced VEGF release was not changed in HVSM cells that were preincubated with LY294002 or wortmannin, potent and specific inhibitors of PI3-K. Moreover, the activation of p44/42 was not affected by LY294002 or wortmannin (data not shown). However, Huang et al. [38] have shown a positive correlation of PI3-K and thrombin-induced VEGF secretion in human FS4 fibroblasts, DU145 prostate cells, and CHRF megakaryocytes. We have no explanation for the discrepancy of these results but p44/42 or PI3-K may mediate thrombin-induced VEGF release in a fashion dependent on cell type. We have reported that the proliferation of HVSM cells triggered by the PAR1 agonist peptide was associated with the activation of NF-κB [9]. Consistent with this finding, we found that thrombin receptor activation led to the activation of NF-κB; however, inhibition of the NF-κB activation or the translocation of NF-κB to the nucleus did not affect thrombin-induced VEGF release (data not shown). Although thrombin-induced NF-κB activation is documented in HVSM

cells, our data rule out the possibility of the involvement of NF-κB in thrombin-induced VEGF release.

In patients with acute coronary syndromes, an arterial injury, which occurs either spontaneously or is induced by coronary interventions such as percutaneous transluminal coronary angioplasty, is associated with massive platelet activation and thrombin generation, often followed by the formation of an occlusive thrombus. A large amount of VEGF is released, predominantly at the site of thrombus formation, suggesting a role for VEGF in acute coronary syndromes [39]. VEGF expressed by the SMCs and foamy macrophages in the atherosclerotic intimas can act as a local and endogenous regulator of endothelial cell functions, including intimal neovascularization, in atherosclerotic lesions of human coronary arteries [40]. As the neovascularization is the critical step in atherosclerosis, we would like to suggest that thrombin may worsen atherosclerosis by inducing VEGF release. Under these conditions, PAR1 might be activated, augmenting thrombin-induced VEGF production and intimal hyperplasia, which is an essential component of venous graft disease [41]. A potent antagonist of PAR1 has been reported to attenuate vascular restenosis following balloon angioplasty in rats [42]. The present study reveals that PAR1 mediates thrombin-induced VEGF release at the receptor level in HVSM cells. In addition, intracellular calcium and p44/42 appear to be key players downstream from the receptor signaling in thrombin-induced VEGF release in HVSM cells. Thus, the control of PAR1 activation and intracellular calcium may be a therapeutic target in thrombin-induced VEGF release in pathological states.

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