Inhibition of Thrombin-Induced Vascular Endothelial Growth Factor Production in Human Neuroblastoma (NB-1) Cells by Argatroban

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Key Words
Argatroban • Neuronal cells • Thrombin • Vascular endothelial growth factor

Abstract
Thrombin, a serine protease that plays a pivotal role in blood coagulation, wound healing, and angiogenesis, has also been implicated in the mitogenesis of various cell types. Previously, we showed that thrombin and the thrombin receptor agonist peptide (TRAP-14; SFLRNPNKDKEYPF) for protease-activated receptor 1 (PAR1) induce vascular endothelial growth factor (VEGF) secretion in PC-12 cells. In this study, we show that thrombin and TRAP-14 also stimulate VEGF secretion in the human NB-1 neuroblastoma cells. In these cells, we further show that thrombin-induced VEGF secretion was blocked by cycloheximide and actinomycin D, indicating that de novo protein synthesis is essential for this process. Reduced thrombin-induced VEGF secretion upon treatment with LY294002, calphostin C, or BAPTA, further suggests that the process is dependent on phospha-tidyl-inositol-3-kinase, protein kinase C, and calcium. However, the complete loss of thrombin-induced VEGF production upon treatment with argatroban, a derivative of arginine and a potent anticoagulant/antithrombin agent, supports the notion that argatroban serves as a useful therapeutic tool for thrombin-associated pathologic conditions. Here, it appears that argatroban may be effective in controlling disorders linked to thrombin-induced VEGF production in neuronal cells.

Introduction
Thrombin is a multifunctional serine protease, which is generated proteolytically from prothrombin by factor Xa in the final step of the blood coagulation cascade. Thrombin converts fibrinogen to fibrin, activates platelets and several coagulation factors, and plays a crucial role in thrombosis and hemostasis [1, 2]. These cellular effects of thrombin are mediated, at least in part, through the activation of the thrombin receptor that is coupled to G protein. The full name of ‘G protein’ is GTP-binding protein because in the active state it binds to GTP (guanosine triphosphate). There are two types of G proteins: heterotrimeric G proteins and monomeric G proteins (or small G proteins). G-protein-coupled receptors are coupled to heterotrimeric G proteins. The heteromeric G protein consists of three subunits: α, β and γ. In the inactive state, the α subunit binds to GDP and the three sub-
units are attached together. When the α subunit binds to GTP, its affinity to the βγ subunits is decreased, resulting in their dissociation. The separated α and/or βγ subunits can then interact with their effectors. There are at least three protease-activated receptors (PARs) that have been identified as thrombin receptors, PAR-1, PAR-3, and PAR-4 [3–5]. Like other G-protein-coupled receptors, the three PAR family members consist of a single polypeptide with seven membrane-spanning domains and an extended extracellular N-terminus. However, unlike the receptors for most cellular growth factors, PAR-1 does not require the traditional ligand-receptor complex formation for activation. Instead, the receptor serves as a substrate for proteolytic digestion, yielding an irreversibly activated form on the cell surface to convey additional cell signaling. Thrombin cleaves PAR-1 between Arg41 and Ser42. Cleavage of this site results in a new truncated N-terminus, starting with the amino acid serine-42. The first six to fourteen amino acids (SFLLRNPNDKYEPF) in the newly generated N-terminal domain play a role in intramolecular ligand activation, and have been found to be an agonist for PAR-1 activation [3, 6]. Even in the absence of thrombin, this peptide binds to the second loop of its transmembrane domain, and mimics thrombin activity [7].

Vascular endothelial growth factor (VEGF) is a cytokine encoded by a single gene that generates at least four protein products from alternative splicing of the mRNA: VEGF 121, VEGF 165, VEGF 189, and VEGF 206 [8], the numbers corresponding to the amount of amino acids. It is well documented that VEGF is a potent angiogenic factor and is specific for endothelial cell migration. In addition to tumor angiogenesis [9], enhanced expression of VEGF has also been implicated in rheumatoid arthritis [10], wound healing [11], diabetic retinopathy [12], and atherosclerosis [13], indicating that VEGF may play a critical role in pathophysiological states.

It has been suggested that thrombin generated following injury to the central nervous system disrupts the blood-brain barrier [14], which ensures homeostasis of the brain microenvironment. Similarly, VEGF has been shown to induce the permeability of the blood-brain barrier in vitro and in vivo [15, 16].

Argatroban [(2R,4R)-4-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl]-L-arginyl-2-piperidine-carboxylic acid monohydrate] is a derivative of arginine that competitively binds to the active site of thrombin (fig. 1). Argatroban has an anticoagulant effect [17, 18], and abrogates thrombus formation in heparin-induced thrombocytopenia with thrombosis syndrome [19]. Recently, argatroban has been approved for clinical use in certain disease conditions. In Japan, argatroban is used for the treatment of chronic peripheral arterial obstructive disease and acute ischemic stroke, and in the United States, it has recently been approved for use in the treatment of thrombosis in patients with heparin-induced thrombocytopenia. Data presented in this study support the concept that argatroban is a useful therapeutic tool for thrombin-associated pathologic conditions, including those in neuronal cells.

Materials and Methods

Materials

BAPTA, LY294002, and calphostin C were purchased from Calbiochem (San Diego, Calif., USA). Human thrombin and other chemicals were purchased from Sigma (St. Louis, Mo., USA). Argatroban was kindly provided by Daiichi Pharmaceutical (Tokyo, Japan).

Cell Culture

Human neuroblastoma (NB-1) cells were maintained in DMEM and RPMI-1640 (1:1) containing 10% fetal bovine serum with appropriate antibiotics at 37°C in a 5% CO₂ atmosphere and 95% air. Cells used for experiments were exposed to DMEM/RPMI containing 0.2% FBS for the indicated periods.

ELISA for VEGF

The VEGF levels in the culture media were measured by a colorimetric ELISA with slight modifications of the chemiluminescence enzyme immunoassay method [20]. Briefly, an anti-VEGF/VPF IgG was used to coat the ELISA plates. Samples diluted in phosphate-buffered saline containing 1% BSA were added to the wells and incubated for 1 h at 22°C. After washing, a peroxidase...
(POD)-conjugated Fab’-fragment of the coating antibody was added, and the samples incubated for another hour at 22°C. O-Phenylene-diamine was used as a reaction substrate. The M-Vmax Microplate reader (Molecular Devices, Mass., USA) was used to detect reactivity.

**RT-PCR Analysis**

NB-1 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, Gaithersburg, Md., USA), and reverse transcription was performed at 42°C using reverse transcriptase (Takara, Japan). One twentieth of the volume of the RT product was amplified using Taq DNA polymerase (Promega, Madison, Wisc., USA), following the manufacturer’s instructions. The sense primer was 5’-GAGAATTCGGCCTCCGAA-3’, and the antisense primer was 5’-GAGCATGCCCTCCTGCCC-3’ for VEGF. The PCR profile consisted of a 1-min initial denaturation at 94°C, followed by 30 cycles, with each cycle comprising a 1-min denaturing step at 94°C, 1 min extension at 72°C, 1 min annealing at 63°C, and 5 min extension at 72°C. The PCR products were analyzed in a 2% (w/v) agarose gel.

**Results**

To further examine mediation of VEGF secretion by thrombin, we used the human neuroblastoma (NB-1) cells as a model system for our study. Figure 2a shows that an increase in VEGF release from NB-1 cells occurs upon addition of 0.3 U/ml thrombin and a further significant increase in VEGF production was observed following the addition of 1 U/ml thrombin. In figure 2b, we show that thrombin induced some VEGF release within 6 h of addition. A more remarkable and increasing VEGF production was noted 12, 18, and 24 h after thrombin addition. This was followed by a gradual decrease in VEGF release, which reached a plateau 36 h after thrombin addition (data not shown). Thus, in NB-1 cells, thrombin induced VEGF secretion in a dose- and time-dependent manner.

Since the protease-activated receptors, PAR-1 and PAR-3, which were shown to be present in NB-1 cells by RT-PCR (fig. 3a), have been identified as thrombin receptors [3–5], we examined the possibility that thrombin induces VEGF secretion through PAR activation. TRAP-14 (SFLLRNPNPKYEPF) induced VEGF production in a dose- and time-dependent fashion, respectively (fig. 3b, c), indicating that TRAP-14 mimicked the effects of thrombin on VEGF secretion from NB-1 cells.

Next, we sought to determine whether thrombin-induced VEGF production was dependent on protein and RNA syntheses. Figure 4 shows that NB-1 cells treated with the protein synthesis inhibitor, cycloheximide, or the transcription inhibitor, actinomycin D, prior to thrombin addition exhibited considerably reduced VEGF secretion. This result suggests that both protein and RNA syntheses are essential for thrombin-induced VEGF secretion from NB-1 cells.

Activation of the thrombin receptor is known to cause phosphatidylinositol-3-kinase (PI3-K) activation, which subsequently leads to an increase in intracellular calcium.
and protein kinase C (PKC) activation. In this study, we found that the calcium chelator, BAPTA, the PI3-K inhibitor, LY294002, and the PKC inhibitor, calphostin C, significantly reduced thrombin-induced VEGF secretion from NB-1 cells (fig. 5). The basal level of VEGF secretion was detected up to 24 h following addition of TRAP-14 (100 μM). Values are means ± SD from three separate experiments.

Although argatroban is known for blocking thrombin activity, this effect is not well characterized in neuronal cells. Thus, we sought to determine whether argatroban could inhibit thrombin-induced VEGF secretion from NB-1 cells. Figure 6 shows that increasing concentrations of argatroban resulted in a corresponding decrease in thrombin-induced VEGF secretion from NB-1 cells. At
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1 μM argatroban, thrombin-induced VEGF secretion approached basal levels, suggesting that argatroban can completely block the effect of thrombin in neuronal cells.

To determine whether time is crucial to achieve the inhibitory effects of argatroban on thrombin-induced VEGF secretion, NB-1 cells were incubated with argatroban either prior to or following thrombin exposure. As shown in figure 7, pre-incubation or treatment with argatroban within 10 min following thrombin addition blocked the stimulation of VEGF secretion by thrombin. A considerable inhibition of VEGF secretion was also observed when argatroban was added 30 min after thrombin exposure. However, no significant inhibitory effect was noted when argatroban treatment was delayed for 60 min or more following thrombin exposure.

Discussion

Thrombin is a well-established mitogen that is generated in the coagulation cascade following tissue injury. Apart from its role in the mitogenic division of various cell types, thrombin has been implicated in wound repair,
inflammation, brain edema, and reactive gliosis following CNS injury [14]. Thrombin has also been found to induce the synthesis and secretion of nerve growth factor [22]. In addition, we and others have reported that thrombin induces VEGF production in vascular smooth muscle cells and in PC12 cells [21, 23, 24]. Incidentally, both thrombin and VEGF have been shown to alter the permeability of the blood-brain barrier [14–16]. Furthermore, enhanced VEGF expression has also been observed in tumor angiogenesis [9], rheumatoid arthritis [10], wound healing [11], diabetic retinopathy [12], and atherosclerosis [13]. Thus, it appears that VEGF plays an important role in a number of disease conditions.

In this study, we found that thrombin induces VEGF secretion from NB-1 cells. It is well established that thrombin generally exerts its cellular activities through the cleavage of the PAR-1, PAR-3, or PAR-4 thrombin receptor [3–5]. Since the sequence of TRAP-14, which enhances thrombin-induced VEGF secretion from NB-1 cells, corresponds to the cleavage product from PAR-1 digestion by thrombin, our results imply that thrombin triggered VEGF secretion via PAR-1 activation. This result is consistent with the finding that a high level of TRAP is essential to observe the cellular effects of thrombin [25, 26]. In contrast, PAR-3, which has been identified as a second thrombin receptor, acts as a cofactor for PAR-4, which is normally expressed in platelets [27]. Moreover, a PAR-3 agonist does not induce VEGF production in NB-1 cells (data not shown).

Thrombin receptor activation can cause membrane phosphatidyl turnover, which results in the generation of phosphatidylinositol and diacylglycerol. The latter two activate PKC in most of the mitogenic signaling pathways. There are several lines of evidence that PI3-K binds to the endoplasmic reticulum, and increases intracellular calcium, resulting in the activation of calcium-dependent kinases, including PKC. Characterization of thrombin-induced VEGF secretion from NB-1 cells revealed that activation of PI3-K and PKC, and intracellular calcium, are critical for this process, which requires de novo protein synthesis. Together, our results suggest that PI3-K, PKC and Ca2+ mediate thrombin-induced VEGF release in NB-1 cells.

Reduction in secondary microthrombi and improved regional collateral cerebral blood flow is the proposed mechanism of inhibition of thrombin activity. Argatroban is a direct thrombin inhibitor which binds to the catalytic site of the thrombin molecule. It binds rapidly and reversibly to both clot-bound and soluble thrombin. The relatively short elimination half-life of argatroban (39–51 min) and its reversible binding allow rapid achievement of therapeutic effect on initiation of therapy and rapid restoration of normal hemostasis upon cessation of therapy [28]. Argatroban given after thrombus formation by intraperitoneal implantation of an osmotic pressure pump was shown to reduce infarct size and neurologic deficits on day 3 and microthrombogenesis on day 1, and to raise the regional cerebral blood flow on day 1, at a plasma level of 0.2–0.6 μM. Argatroban was considered to exert its effects by salvaging neuronal cells in the ischemic penumbra and suppressing extension of the infarction into the penumbra by keeping blood vessels patent, mainly through the inhibition of microthrombogenesis [29]. Therefore, the concentration of argatroban used in the present study has a potential therapeutic benefit, supporting its clinical usefulness in the treatment of acute-phase cerebral thrombosis. However, the role of argatroban in inhibiting the effect of thrombin, particularly in neuronal cells, requires further investigation in order to fully understand its therapeutic potential. Our finding that argatroban blocks thrombin-induced VEGF secretion from NB-1 cells suggests that argatroban may be an effective tool in controlling diseases linked to thrombin-induced VEGF production in neuronal cells.

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