Thrombin Stimulates Vascular Smooth Muscle Cell Polyamine Synthesis by Inducing Cationic Amino Acid Transporter and Ornithine Decarboxylase Gene Expression

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Abstract—Thrombin, a serine protease, is a potent mitogen for vascular smooth muscle cells (SMCs), but its mechanism of action is not known. Since L-ornithine is metabolized to growth-stimulatory polyamines, we examined whether thrombin regulates the transcellular transport and metabolism of L-ornithine by vascular SMCs. Treatment of SMCs with thrombin initially (0 to 2 hours) decreased L-ornithine uptake, whereas longer exposures (6 to 24 hours) progressively increased transport. Kinetic studies indicated that thrombin-induced inhibition was associated with a decrease in affinity for L-ornithine, whereas stimulation was mediated by an increase in transport capacity. Thrombin induced the expression of both cationic amino acid transporter (CAT)-1 and CAT-2 mRNA. Furthermore, thrombin stimulated L-ornithine metabolism by inducing ornithine decarboxylase (ODC) mRNA expression and activity. The stimulatory effect of thrombin on both L-ornithine transport and ODC activity was reversed by hirudin, a thrombin inhibitor, and was mimicked by a 14–amino acid thrombin receptor–activating peptide. Thrombin also markedly increased the capacity of SMCs to generate putrescine, a polyamine, from extracellular L-ornithine. The thrombin-mediated increase in putrescine production was reversed by N\textsubscript{G}-methyl-L-arginine, a competitive inhibitor of cationic amino acid transport, or by \(\alpha\)-difluoromethylornithine (DFMO), an ODC inhibitor. DFMO also inhibited thrombin-induced SMC proliferation. These results demonstrate that thrombin stimulates polyamine synthesis by inducing CAT and ODC gene expression and that thrombin-stimulated SMC proliferation is dependent on polyamine formation. The ability of thrombin to upregulate L-ornithine transport and direct its metabolism to growth-stimulatory polyamines may contribute to postangioplasty restenosis and atherosclerotic lesion formation. (Circ Res. 1998;83:217-223.)

Key Words: thrombin ■ L-ornithine ■ polyamine ■ proliferation

Thrombin is a multifunctional serine protease that is activated at sites of blood vessel injury. Apart from its central role in hemostasis and thrombosis, thrombin has been implicated in the pathogenesis of postangioplasty restenosis and atherosclerosis. Numerous studies have demonstrated that thrombin is a potent mitogen for vascular smooth muscle cells (SMCs). Thrombin stimulates the release of growth factors from platelets and vascular cells and directly induces SMC proliferation. However, the mechanism by which thrombin mediates SMC growth is not known. Recent studies indicate that the formation of polyamines plays an integral role in the mitogenic response of vascular SMCs. SMC proliferation is preceded by increases in cellular polyamine content, and inhibition of polyamine synthesis prevents cell growth. Furthermore, the exogenous addition of polyamines to SMCs stimulates DNA synthesis.

The polyamines putrescine, spermidine, and spermine are naturally occurring organic cations found in all eukaryotic cells. Putrescine is generated from the cationic amino acid L-ornithine via a decarboxylation reaction catalyzed by the enzyme ornithine decarboxylase (ODC), whereas the successive polyamines, spermidine and spermine, are formed by the sequential transfer of an aminopropyl group from S-adenosylmethionine. Induction of ODC activity and polyamine synthesis correlate with cell growth in various experimental conditions. Both arterial injury–induced and platelet-derived growth factor (PDGF)-mediated SMC proliferation is associated with a striking increase in ODC activity. Moreover, pharmacological blockade of ODC inhibits both arterial injury–mediated and PDGF-mediated SMC proliferation, indicating that ODC activity is essential for SMC growth.

Although ODC activity is often believed to be rate-limiting in the polyamine biosynthetic pathway, recent studies indicate that L-ornithine availability also plays a crucial role in regulating polyamine synthesis. The steady-state intracellular level of L-ornithine markedly influences polyamine formation and DNA synthesis in neoplastic cells. In addition, we have demonstrated that PDGF and lysophosphatidylcholine-induced polyamine synthesis and vascular SMC mitogenesis are dependent on the transcellular transport of L-ornithine. The transport of cationic amino acids, such as L-ornithine, by...
vascular SMCs is mediated by the system y' carrier. This particular transport system is characterized by its high affinity for cationic amino acids, its Na⁺ independence, and the stimulation of transport by substrate on the opposite (trans) side of the membrane. Recently, the proteins responsible for the activity of the system y' carrier have been cloned and designated as cationic amino acid transporter (CAT)-1, CAT-2, and CAT-2A. Both CAT-1 and CAT-2 are high-affinity (Kₘ ≈ 100 μmol/L) low-capacity transporters for cationic amino acids, whereas CAT-2A is an alternate splice variant of CAT-2 that possesses low affinity but high transport capacity. In a previous study, we found that vascular SMCs express mRNA for both the high-affinity transporters, CAT-1 and CAT-2, but do not express message for CAT-2A.

Since polyamines play a critical role in mediating cell growth, the present study examined whether thrombin regulates polyamine synthesis. We now report that thrombin coordinately induces the gene expression of CAT proteins and ODC, resulting in markedly increased production of polyamines by vascular SMCs. In addition, we show that thrombin-stimulated SMC proliferation is dependent on polyamine synthesis.

Materials and Methods

Materials

Fetal calf serum, l-ornithine, trichloroacetic acid, penicillin, streptomycin, SDS, EDTA, elastase, collagenase, trypsin, hirudin, pyridoxal-5-phosphate, ammonium hydroxide, chloroform, formamide, methanol, Triton X-100, Tris, TES, HEPES, ninhydrin spray, and thin-layer chromatography plates (silica gel-25) were purchased from Sigma Chemical Co; actinomycin D, ribonuclease A, and ribonuclease T₁ were from Boehringer Mannheim; human α-thrombin was from US Biochemicals; thrombin receptor–activating peptide (TRAP [SFLRNPNDKYEPF]) was synthesized at the Baylor College of Medicine Protein Chemistry Core Facility (Houston, Tex); plasmin was from Kabi; MEM was from ICN Biomedicals; guanidine isothiocyanate and CsCl were from GIBCO; DTT, thiorphan (TRAP [SFLLRNPNDKYEPF]), and ribonuclease T₁ were from Ambion Inc; ODC cDNA was from American Type Culture Collection; bicinchoninic acid protein assay was from Pierce; Gene Screen Plus membranes were from New England Nuclear; α-difluoromethylornithine (DFMO) was generously provided by Dr Ekkhardt H.W. Bohme (Hoechst Marion Rousell, Cincinnati, Ohio); [¹⁴C]l-ornithine (55 Ci/mmol) was from American Radiolabeled Chemicals; and [¹³C₄]l-ornithine (0.25 μCi/mmol) and 0.2 mg soluble protein in Tris buffer (20 mmol/L, pH 7.4) were from Amshers Corp.

SMC Culture

Vascular SMCs were isolated by elastase and collagenase digestion of thoracic aortas obtained from adult 12-week-old male rats and characterized by morphological and immunological criteria. Cells were cultured serially in MEM containing Earle’s salts, 5.6 mmol/L glucose, 2 mmol/L l-glutamine, 20 mmol/L TES-NaOH, 20 mmol/L HEPES-NaOH, 100 U/mL penicillin, and 100 U/mL streptomycin. Subcultured strains were used between passages 6 to 28. When cells reached confluence, the culture media were replaced with serum-free media containing BSA (0.1%) for 24 hours and then exposed to the various treatment regimens.

l-Ornithine Transport

l-Ornithine transport was determined by measuring the influx of radiolabeled l-ornithine into SMCs, as previously described. Cells grown on 12-well plates were washed with HEPES buffer (140 mmol/L choline chloride, 5.0 mmol/L KCl, 1.0 mmol/L MgCl₂, 0.9 mmol/L CaCl₂, 5.6 mmol/L d-glucose, and 25 mmol/L HEPES, pH 7.4) and then incubated for 45 seconds in 0.5 mL HEPES buffer containing [³H]l-ornithine (50 μmol/L, 1 μCi). Transport activity was terminated by aspirating the media and rapidly washing the cells with ice-cold HEPES buffer. Cells were allowed to dry, and the cell-associated radioactivity was extracted with 500 μL of 0.2% SDS in 0.2N NaOH and then assayed by liquid scintillation spectrometry (Tri-Carb liquid scintillation analyzer, model 1900 TR, Packard). Protein in the NaOH extracts was measured using the bicinchoninic acid method with serum albumin as the standard. To correct for nonspecific uptake or binding to the cell surface, cells were incubated in parallel wells with HEPES buffer containing 10 mmol/L unlabeled l-ornithine, the fraction of the radioactivity of the cells was determined, and this fraction was then subtracted from each data point.

ODC Assay

ODC activity was determined by measuring the release of ¹⁴CO₂ from [¹⁴C]l-ornithine, as previously described. SMCs were harvested in ice-cold Tris buffer (20 mmol/L Tris, 0.1 mmol/L EDTA, 2 mmol/L DDT, and 0.1 mmol/L pyridoxal-5-phosphate, pH 7.4), sonicated, and centrifuged at 14 000g for 20 minutes at 4°C. The supernatant (soluble fraction) was collected and stored at −70°C until use. The reaction mixture (400 μL) contained 250 μmol/L l-ornithine (0.25 μCi/mmol) and 0.2 mg soluble protein in Tris buffer (20 mmol/L, pH 7.4). Incubations were carried out in stopped glass tubes for 2 hours at 37°C. The ¹⁴CO₂ liberated by the decarboxylation of l-ornithine was trapped on a piece of filter paper impregnated with 2N NaOH, which was suspended above the reaction mixture. The reaction was stopped by the addition of trichloracetic acid (10% final concentration), and the filter was collected for liquid scintillation counting. Aliquots of the supernatant were measured for protein concentration by the Bradford assay using gamma globulin as the standard. Enzymatic activity was expressed in pmol CO₂ mg protein⁻¹ h⁻¹.

Polyamine Production

Polyamine formation was determined by incubating SMCs with [³H]l-ornithine and monitoring the intracellular formation of radioabeled putrescine. SMCs were plated onto 6-well plates and incubated with [³H]l-ornithine (20 μCi/mmol) for 8 hours. The reaction was stopped by removing the radiolabeled-containing media and washing the cells with ice-cold PBS (pH 7.4). Ice-cold Tris (20 mmol/L, pH 7.4) buffer containing Triton X-100 (0.01%) was then added to the monolayer, and cells were scraped, vortexed, and centrifuged at 10 000g for 1 minute. Aliquots of the supernatant were spotted onto thin-layer chromatography plates and developed in the solvent system chloroform:methanol:ammonium hydroxide:water (1:4:2:1, by volume). After drying, putrescine was detected by ninhydrin spray, and [³H]putrescine was identified by cochromatography with unlabeled putrescine, scraped, and quantified by liquid scintillation counting.

mRNA Analysis

Total cellular RNA was obtained by the guanidine isothiocyanate/CsCl procedure, and RNA concentration was determined by absorbance spectrophotometry at 260 nm. CAT mRNA levels were determined by solution hybridization/ribonuclease protection analysis. In brief, total RNA (15 μg) was hybridized with 5 × 10¹⁰ cpm of [³²P]UTP-labeled antisense CAT and GAPDH riboprobes (316 bp). The CAT-1 (195-bp) and CAT-2 (210-bp) antisense RNA probes were prepared as described earlier. Samples were incubated in hybridization buffer (65 mmol/L sodium citrate, 200 mmol/L sodium acetate, 0.5 mmol/L EDTA, and 55% formamide) for 16 hours at 45°C, followed by digestion with ribonuclease A (40 μg/mL) and ribonuclease T₁ (2 μg/mL) at room temperature for 30 minutes. Protected RNA was analyzed by electrophoresis using 6% polyacrylamide/8 mol/L urea gel. The gel was exposed overnight to x-ray film at −70°C in the presence of intensifying screens. The size of the
predicted nucleotide-protected fragment was confirmed using a 32P-labeled RNA molecular weight ladder.

ODC mRNA levels were determined by Northern blotting. Total RNA (30 μg) was loaded on 1.2% gels containing 2.2 mol/L formaldehyde and fractionated by electrophoresis. RNA was blot-transferred to Gene Screen Plus membranes and prehybridized for 4 hours at 42°C in a solution containing deionized formamide (40%), 2× SSC, 5× Denhardt’s reagent, SDS (2%), and denatured salmon sperm DNA (100 μg/mL). Membranes were hybridized overnight at 42°C in prehybridization solution containing [32P]DNA probes (1 to 4×10^5 cpm/mL) for ODC and GAPDH. DNA probes were labeled with [α-32P]dCTP by random priming. After hybridization, membranes were washed twice with 0.1× SSC/0.1% SDS at 42°C for 20 minutes and then exposed to Kodak X-Omat film at −70°C in the presence of intensifying screens.

For both ribonuclease protection assays and Northern blotting, relative mRNA levels were quantified by scanning densitometry (LKB 2222-020 Ultrascan XL laser densitometer) and normalized with respect to GAPDH mRNA.

**SMC Proliferation**

SMCs were seeded at a density of 2.0×10^4 cells per well in 12-well plates in serum (10%)–containing media. After 24 hours, culture media were exchanged for serum-free media, and cells were incubated for an additional 48 hours. SMCs were then treated with thrombin in the presence or absence of DFMO. Media, with appropriate additions, were replenished every second day. Cell number determinations were performed after 4 days of treatment by dissociating cells with trypsin (0.025%) and EDTA (1 mmol/L) and counting cells in a calibrated Coulter Counter (model ZF, Coulter Electronics).

**Statistics**

Results are expressed as the mean±SEM. Statistical analysis was performed with the use of the Student 2-tailed t test and ANOVA when 2 treatments were compared. Values of P<0.05 were considered to be statistically significant.

**Results**

Vascular SMCs expressed constitutive L-ornithine transport activity (422±28 pmol·mg protein⁻¹·45 s⁻¹) that was increased by thrombin in a concentration-dependent manner (Figure 1). Time-course studies demonstrated that thrombin (10 nmol/L) had a biphasic effect on L-ornithine transport (Figure 2). Initially, thrombin exposure inhibited the transport of L-ornithine, but by 6 hours of thrombin treatment, a significant rise in transport was observed, and this was further increased after 24 hours of treatment. The stimulatory effect by thrombin on L-ornithine transport was mimicked by TRAP (Figure 3) and was blocked by the thrombin inhibitor hirudin (3 U/mL).
Thrombin Stimulates L-Ornithine Transport and Metabolism

Figure 5. Representative Eadie-Hofstee plot of saturable L-ornithine transport in vascular SMCs. Specific transport of [3H]L-ornithine (5 to 500 μmol/L) was measured for 45 seconds in control SMCs (C) and in SMCs pretreated with thrombin (10 nmol/L for 24 hours). Transport velocity was plotted as a function of velocity/L-ornithine concentration (μmol/L). Similar findings were made in 4 separate experiments.

Figure 6. Effect of thrombin on the expression of CAT mRNA in vascular SMCs. Ribonuclease protection analysis of CAT-1, CAT-2, and GAPDH mRNA from untreated SMCs (C) and SMCs stimulated with thrombin (10 nmol/L for 8 hours) is shown. Data are representative of 3 separate independent experiments.

Discussion

The present study demonstrates that thrombin stimulates polyamine synthesis in vascular SMCs and that polyamines contribute to the proliferative action of thrombin. Thrombin induces polyamine production by modulating the expression of the genes that regulate the transport and metabolism of L-ornithine. In particular, thrombin increases the intracellular metabolism to polyamines by stimulating the expression of the genes for L-ornithine. In particular, thrombin increases the transcellular transport of L-ornithine and its intracellular metabolism to polyamines by stimulating the expression of the genes for both CAT and ODC. These thrombin effects are specific receptor-mediated responses, since they are reversed by hirudin and are mimicked by a thrombin receptor–activating peptide. In addition, other serine proteases such as trypsin and plasmin have no effect on L-ornithine transport.

Treatment of vascular SMCs with thrombin increased the capacity of SMCs to generate the polyamine putrescine from extracellular L-ornithine in a concentration-dependent manner (Figure 10). The stimulatory effect of thrombin on putrescine synthesis was inhibited by the cationic amino acid transport inhibitor Nω-methyl-L-arginine (L-NMA, 10 mmol/L) and by the selective ODC inhibitor DFMO (2 mmol/L) (Figure 11B). In addition, DFMO (2 mmol/L) blocked the proliferative effect of thrombin (10 nmol/L) on vascular SMCs (Figure 12).

In subsequent kinetic studies, saturable uptake of radiolabeled L-ornithine (5 to 500 μmol/L) was measured. As evident from a representative Eadie-Hofstee plot (Figure 5), high-affinity uptake of L-ornithine by vascular SMCs was mediated by a single carrier. Data from several experiments (n = 5) indicated that this transporter had a Michaelis constant (K_m) of 116.2 ± 14.2 μmol/L and a maximum transport velocity (V_max) of 612 ± 65 pmol · mg protein^{-1} · 45 s^{-1}. Pretreatment of vascular SMCs with thrombin (10 nmol/L) for 24 hours significantly increased both the K_m (226.3 ± 17.2 μmol/L, P < 0.05) and V_max (1952 ± 212 pmol · mg protein^{-1} · 45 s^{-1}, P < 0.05) of the L-ornithine transporter.

Treatment of vascular SMCs with thrombin for 8 hours stimulated the expression of mRNA for both cationic amino acid transporters, CAT-1 and CAT-2, in a concentration-dependent manner (Figure 6). Thrombin (10 nmol/L) induced an -4- and 5-fold increase in the steady-state levels of CAT-1 and CAT-2 mRNA, respectively. Incubation of SMCs with actinomycin D (2 μg/mL) resulted in a rapid decay of CAT-1 (t_{1/2} ≈ 150 minutes) and CAT-2 (t_{1/2} ≈ 60 minutes) mRNA. Thrombin had no effect on the stability of CAT-1 or CAT-2 message (data not shown).

Incubation of vascular SMCs with thrombin also induced ODC activity. Significant increases in enzyme activity were evident 4 hours after thrombin (10 nmol/L) addition, reached maximum activity at 8 hours (≈-20-fold), and then declined toward basal levels by 24 hours (Figure 7A). Increases in ODC activity were dependent on the concentration of thrombin (Figure 7B) and were mimicked by TRAP (Figure 8). In addition, hirudin selectively reversed the thrombin-mediated increase in ODC activity but had no effect on the TRAP-induced rise in activity (Figure 8). Treatment of SMC with cycloheximide or actinomycin D abolished the thrombin-stimulated increase in ODC activity (Figure 9).

Untreated control SMCs did not express ODC message; however, treatment of SMCs with thrombin induced, in a concentration-dependent fashion, the expression of 2 distinct ODC transcripts of ≈ 2.6 and 2.2 kb, respectively (Figure 10). Thrombin (10 nmol/L)-stimulated an ≈ 12-fold increase in the larger (2.6-kb) transcript, whereas the smaller (2.2-kb) transcript increased ≈ 10.5-fold.

The present study demonstrates that thrombin stimulates polyamine synthesis in vascular SMCs and that polyamines contribute to the proliferative action of thrombin. Thrombin induces polyamine production by modulating the expression of the genes that regulate the transport and metabolism of L-ornithine. In particular, thrombin increases the transcellular transport of L-ornithine and its intracellular metabolism to polyamines by stimulating the expression of the genes for both CAT and ODC. These thrombin effects are specific receptor-mediated responses, since they are reversed by hirudin and are mimicked by a thrombin receptor–activating peptide. In addition, other serine proteases such as trypsin and plasmin have no effect on L-ornithine transport.

Treatment of vascular SMCs with thrombin stimulates the transport of L-ornithine in both a time- and concentration-dependent manner. Kinetic experiments indicate that high-affinity (K_m = 120 μmol/L) L-ornithine transport is mediated by a single carrier system and that thrombin increases both
the \( V_{\text{max}} \) and \( K_m \) of this transport system. These kinetic data suggest that the thrombin-induced decrease in \( \text{L-ornithine} \) uptake observed at early time points likely arises from a thrombin-mediated decrease in affinity of the transporter. In contrast, the increase in \( \text{L-ornithine} \) transport observed at later time points may result from the de novo expression of additional transport proteins. In accordance with this, we found that cycloheximide blocks thrombin-induced transport. Interestingly, incubation of SMCs with cycloheximide for 24 hours had no effect on basal transport activity, indicating that CAT proteins have a low rate of turnover.

Studies in our laboratory and others have shown that high-affinity transport of cationic amino acids by vascular SMCs is mediated by CAT-1 and CAT-2.\(^{12,33} \) The present study demonstrates that thrombin stimulates a comparable induction of CAT-1 and CAT-2 mRNA expression. Although the molecular mechanism by which thrombin induces CAT gene expression is not known, it likely involves the transcriptional activation of the CAT genes, since the transcriptional inhibitor actinomycin D blocks this effect. Moreover, thrombin has no effect on the stability of CAT mRNA.

The coexpression of both CAT-1 and CAT-2 mRNA in vascular SMCs after thrombin treatment is also observed after the administration of PDGF or angiotensin and suggests a general mechanism by which growth factors increase intracellular \( \text{L-ornithine} \) levels.\(^{12,33} \) Interestingly, thrombin induces PDGF synthesis from SMCs,\(^6,7 \) raising the possibility that the thrombin effect is secondary to PDGF release. Although neutralizing antibodies to PDGF fail to modulate thrombin-mediated increases in \( \text{L-ornithine} \) uptake, SMC-derived PDGF may exert its effect in an intracrine manner, which would be unaffected by neutralizing antibodies.

In addition to stimulating transcellular \( \text{L-ornithine} \) transport, thrombin stimulates the intracellular metabolism of \( \text{L-ornithine} \) to polyamines in vascular SMCs. Thrombin induces ODC activity in a concentration- and time-dependent manner. Increases in ODC activity are paralleled by increases in ODC mRNA, suggesting that thrombin stimulates ODC gene transcription. The capacity of the transcriptional inhibitor, actinomycin D, to inhibit thrombin-stimulated ODC activity is consistent with this notion. The induction of 2 ODC mRNA species in vascular SMCs is also in agreement with previous studies using different cells and/or stimuli.\(^{18,34,35} \) The 2 ODC mRNA species appear to arise from the alternative use of 2 different polyadenylation signals.\(^8,37 \)

The coinduction of CAT and ODC proteins by thrombin may provide a mechanism by which increased levels of substrate (\( \text{L-ornithine} \)) are provided to SMCs during activation of the ODC enzyme. In this respect, treatment of vascular SMCs with thrombin results in a prominent increase in the capacity of SMCs to generate putrescine. This thrombin-mediated effect is blocked by the cationic amino acid transport inhibitor L-NMA and by the selective ODC inhibitor DFMO, indicating that both the transcellular transport of \( \text{L-ornithine} \) and intracellular ODC activity are limiting factors.
that regulate the capacity of thrombin to generate polyamines in vascular SMCs. Interestingly, L-ornithine uptake is dissociated from polyamine synthesis at later time points. Whereas thrombin stimulates a transient elevation in ODC activity that gradually returns toward basal levels after 24 hours of exposure, L-ornithine transport activity progressively increases during this time. The increase in transport activity at this time may serve to provide the necessary amino acids required for the synthesis of new proteins during cell growth. In support of this proposal, enhanced CAT gene expression has also been demonstrated in numerous proliferating cells, including hepatocytes, lymphocytes, and various tumor cells.

The capacity of thrombin to regulate cationic amino acid transport and metabolism in vascular SMCs may be of pathophysiological importance. After local injury of the blood vessel wall, thrombin is generated from its circulating zymogen prothrombin. Thrombin would augment L-ornithine uptake and metabolism, resulting in cellular polyamine production and SMC proliferation. In addition, our earlier studies demonstrating that thrombin inhibits inducible NO synthase expression in vascular SMCs would further promote intimal proliferation by inhibiting the synthesis of the anti-proliferative molecule NO from the cationic amino acid L-arginine. Thus, the combined ability of thrombin to stimulate polyamine synthesis and inhibit NO generation may contribute to its SMC proliferative action at sites of vascular injury.

In conclusion, these studies demonstrate that thrombin stimulates SMC polyamine biosynthesis by coordinately inducing the expression of the genes for both CAT and ODC. In addition, they show that thrombin-stimulated SMC proliferation is dependent on polyamine synthesis. The capacity of thrombin to upregulate L-ornithine transport and to direct its metabolism to polyamines may contribute to postangioplasty restenosis and atherosclerotic lesion formation.

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