Thrombin induces endothelial arginase through AP-1 activation

Weiwei Zhu,1,2,3 Unni M. Chandrasekharan,1,3 Smarajit Bandyopadhyay,1,3 Sidney M. Morris, Jr.,3 Paul E. DiCorleto1,3 and Vikram S. Kashyap1,2

Departments of 1Cell Biology and 2Vascular Surgery, The Cleveland Clinic Foundation, Cleveland, Ohio; and 3Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

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Thrombin induces endothelial arginase through AP-1 activation. Am J Physiol Cell Physiol 298: C952–C960, 2010. First published December 23, 2009; doi:10.1152/ajpcell.00466.2009.—Arterial thrombosis is a common vascular disease caused by occlusion of a vessel via an obstructing thrombus and is associated with tremendous morbidity and mortality. Although thrombi can be removed by mechanical means and regional thrombolytic infusion, endothelial dysfunction can persist. Thrombus-associated vasospasm and rethrombosis after successful removal of the original thrombus remain vexing clinical problems (37). In addition to regulating vascular tone and blood pressure, the vascular endothelium has antithrombotic properties and modulates interactions between the blood vessel wall, circulating leukocytes, and platelets. Nitric oxide is a powerful antithrombogenic molecule and vasodilator produced by endothelial nitric oxide synthase (eNOS) through l-arginine (33, 31, 49). Endothelial dysfunction contributes significantly to vasospasm and rethrombosis (1, 4, 10). In addition to eNOS, l-arginine is also a substrate of arginase, the final enzyme of the hepatic urea cycle hydrolyzing l-arginine to urea and L-ornithine (21, 22). Arginase is constitutively expressed in endothelial cells of different vascular beds by two isoforms, arginase I and II. In rat endothelium, arginase I is the predominant isoform expressed, whereas arginase II is the major isoform present in mouse vascular endothelium, human aortic endothelial cells, and human umbilical vein endothelial cells (HUVEC) (6, 32, 41, 43, 44, 54). Increasing evidence has recognized arginase as a novel target for therapy in vascular disease. Upregulation of arginase activity and/or expression was reported in atherosclerosis-prone mice and endothelial cells of patients with pulmonary arterial hypertension; underlying oxidized low-density lipoproteins, ischemia-reperfusion, obesity, diabetes, and aging induced endothelial dysfunction (14, 15, 41, 43–45, 55). Thrombin-enhanced arginase enzyme activity in HUVEC was found to occur via a Rho pathway-dependent mechanism (32).

Using acetylcholine-stimulated endothelium-dependent relaxation (EDR), our initial studies in several animal models revealed that acute arterial thrombosis causes endothelial dysfunction. Lack of blood flow (ischemia) alone without intraluminal thrombus did not affect EDR. Nitric oxide levels were decreased accordingly, and l-arginine administration restored EDR, suggesting normal eNOS function and that impaired l-arginine availability underlies arterial thrombosis-induced endothelial dysfunction (12, 23, 39). We first investigated the mechanism of this pathology in HUVEC. eNOS activity and expression as well as l-arginine transport were not affected by thrombin, thrombin receptor agonist peptide (TRAP), or fibrin treatment. Interestingly, high-dose thrombin treatment or TRAP increased both arginase II activity and expression, while fibrin exposure had no appreciable effect (56). We continued to investigate the mechanism using cell culture-mediated studies and an animal model of thrombosis. Recently, we found that exposure of rat aortic endothelial cells (RAECs) to thrombin upregulated arginase I mRNA and protein expression. Furthermore, both specific and nonspecific arginase inhibitors ameliorated endothelial dysfunction after thrombosis in rats (27, 56). However, the molecular mechanisms of thrombin-induced upregulation of arginase I are unknown.

To understand how expression of the arginase I gene is induced in response to thrombin, we have examined the role of thrombin on arginase I promoter and enzyme activity using transfected RAECs in this study. Our objective was to identify the thrombin-responsive element in the arginase I promoter and to reveal the transcription factors and signaling pathways involved.

Address for reprint requests and other correspondence: V. S. Kashyap, The Cleveland Clinic Foundation, 9500 Euclid Ave., S40, Cleveland, OH 44195 (e-mail: kashyav@ccf.org).
MATERIALS AND METHODS

Cell culture. RAECs were primary cultured as described previously (27). Isolated RAECs were initially plated onto T75 flasks in a 37°C, humidified, 5% CO2 incubator. They were maintained in DMEM medium (Sigma, St. Louis, MO) containing 15% fetal bovine serum, 0.009% heparin, and 0.015% endothelial cell growth supplement. The cells were grown to confluence before initiation of experiments and were used between passages 3 and 5. Transfection experiments were performed using TargenF-2 and peptide enhancer (Targeting System) as described previously (5). Adult male Sprague-Dawley rats weighing 350 to 500 mg were used for isolated RAECs. Animals were handled and cared for under Cleveland Clinic guidelines and in compliance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996), and the Association for Assessment and Accreditation of Laboratory Animal Care International. The experimental protocols were approved by the Cleveland Clinic Institutional Animal Care and Use Committee and were performed in accordance with the recommendations of Good Laboratory Practices.

Arginase activity assay. Arginase activity in cell lysates was determined according to the established protocol (24, 34). In this assay, arginase activity was determined as the conversion of L-[guanidine-14C] arginine to [14C]urea, which was converted to 14CO2 by urease and trapped as Na214CO3 for scintillation counting. Arginase activity was determined according to the established protocol (24, 34). In this assay, arginase activity was determined as the conversion of L-[guanidine-14C] arginine to [14C]urea, which was converted to 14CO2 by urease and trapped as Na214CO3 for scintillation counting. Arginase activity was expressed as pmol urea formed per hour per milligram of protein at 37°C.

Arginase I-luciferase reporter constructs. 5’-Deletion arginase I promoter-luciferase (Luc) constructs—–4.78, -3.29, -2.78, and -1.41 kb arginase I promoter-Luc—were used and have been described previously (17). The -3.11 kb arginase I promoter-Luc was generated by BamHII/NcoI digestion of the -3.29-kb arginase I promoter-Luc, followed by religation.

Internal deletion mutants were made using standard PCR techniques. The primers used to create mutant sites were as follows (mutated base pairs are in lower case): CCAAT transcription factor-nuclear factor 1 mutant, forward 5’-ACATACCATGGCCCTGAGGTTCTCCCTTGCTGTATG-3’; AP-1 mutant, forward 5’-ACATACCATGGCCCTGAGGTTCTCCCTTGCTGTATG-3’; AP4-2 mutant, forward 5’-ACATACCATGGCCCTGAGGTTCTCCCTTGCTGTATG-3’; AP4-1 mutant, forward 5’-ACATACCATGGCCCTGAGGTTCTCCCTTGCTGTATG-3’. The genomic DNA fragments were isolated, purified, and amplified by PCR (5). The primer sequences were 5’-ACATACCATGGCCCTGAGGTTCTCCCTTGCTGTATG-3’ and 5’-CACTACCATGGGCTCTAGATGTTCC-3’.

Deletion arginase I promoter-Luc constructs were made by internal deletion using restriction enzymes. The genomic DNA fragments were isolated, purified, and amplified by PCR (5). The primer sequences were 5’-ACATACCATGGCCCTGAGGTTCTCCCTTGCTGTATG-3’ and 5’-ACATACCATGGGCTCTAGATGTTCC-3’.

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To directly determine the effect of thrombin on arginase I transcription, RAECs were cotransfected with a ~4.8 kb murine arginase I promoter-Luc construct and a plasmid containing β-galactosidase driven by the SV40 promoter to normalize for transfection efficiency (17). Forty-eight hours after transfection, cells were treated with different concentrations of thrombin for 4 h, and then luciferase and β-galactosidase activities were determined. Ten and 30 U/ml thrombin exposure induced a 3.2 ± 1.4- and 6.4 ± 0.5-fold increase in luciferase activity compared with control, whereas 30 U/ml of thrombin induced a 64.4 ± 0.5-fold increase. n = 3; *P < 0.05 vs. vehicle (concentration 0).

To further investigate the thrombin responsive element(s), a series of 5′-deletion arginase I promoter-Luc constructs were used. RAECs were cotransfected with five different 5′-deletion arginase I promoter-Luc constructs: −4.78, −3.29, −3.11, −2.78 and −1.41 kb arginase I promoter-Luc and SV40 promoter β-galactosidase plasmids (17). Forty-eight hours after transfection, cells were treated with thrombin (30 U/ml) for 4 h; luciferase and β-galactosidase activities were then determined. Luciferase activity fold induction showed that the thrombin-responsive element was located between −3.29 kb and −3.11 kb in the arginase I promoter (Fig. 2A).

According to the prediction by a bioinformatics search (MacVector, Cary, NC), there are four different consensus sequences within this region: two AP-4, one AP-1, and one CTF-NF1. Mutants (Fig. 2B as shown in lowercase above the underlined sequences) for the consensus sequence sites predicted by the bioinformatics search were designed and created. RAECs were cotransfected with these four mutant arginase I promoter-Luc constructs, as well as β-galactosidase plasmids to normalize for the transfection efficiency. Relative luciferase activities were determined. The AP-1 sequence located at −3.157 bp was identified as the thrombin-responsive regulatory element (Fig. 2B).

Binding of c-Jun and ATF-2 to the AP-1 site. In addition to the luciferase assay using mutant arginase I promoter-Luc, we further performed EMSA to confirm that this AP-1 site was the thrombin-responsive regulatory element. Nuclear extracts were prepared from vehicle and thrombin-treated RAECs. Wild-type and mutant double-stranded oligonucleotide probes were designed to encompass the AP-1 binding element (−3.157 bp) and labeled with biotin. As shown in Fig. 3A, nuclear extracts from RAECs treated with thrombin (30 U/ml; 30 min) formed a specific complex with oligonucleotide containing the wild-type AP-1 site, whereas no specific complex formation was observed for nuclear extracts from untreated RAECs or with the oligonucleotide containing the mutant AP-1 sequence. To distinguish specific from nonspecific protein-DNA complex formation, incubations were performed in the presence of extra unlabeled probe (Fig. 3A).

To dissect the protein composition of this complex, supershift assays were performed with labeled wild-type AP-1 probe. Different AP-1 protein antibodies from Jun, Fos, ATF, and Jun dimerization partner (JDP) families and a control antibody were added to the nuclear extracts before electrophoresis to detect further decreases in electrophoretic mobility. The addition of c-Jun and ATF-2 antibodies to the EMSA reaction mixture resulted in the inhibition of the specific complex formation (Fig. 3B), indicating that c-Jun and ATF-2 are the proteins binding to this site in RAECs. However, there were no changes in DNA-protein binding with other AP-1 antibody-incubated nuclear extracts (data not shown). TRAP was used as a positive control. Because TRAP is a thrombin receptor-specific agonist peptide with the NH2 terminus of the tethered ligand, this implicated the role of the thrombin receptor on AP-1-mediated arginase I transcription.

The AP-1 site is responsible for endogenous c-Jun, ATF-2 binding in vivo. To determine whether c-Jun and ATF-2 are associated with the arginase I promoter upon stimulation by thrombin in vivo, ChiP assays were performed with specific c-Jun and ATF-2 antibodies by using chromatin from untreated and thrombin-treated RAECs. With primers amplifying sequences spanning from position −3.296 bp to −3.111 bp of the arginase I promoter, which includes the AP-1 site, an 186-bp DNA fragment was amplified from anti-c-Jun and anti-ATF-2 chromatin precipitates in thrombin-treated cells (Fig. 4, top). However, anti-c-Jun and anti-ATF-2 precipitates from untreated control RAECs resulted in little or no DNA amplifica-
tion. Appropriate primers for the region spanning from positions 2,340 bp to −2,157 bp (contains one AP-4 and one CTF-NFI site) were also used to amplify chromatin immunoprecipitated with anti-c-Jun and anti-ATF-2. In contrast to positions 3,296 bp to 3,111 bp of the arginase I promoter, no DNA fragment was detected from either control or thrombin-treated samples (Fig. 4, bottom). These results demonstrate that thrombin induced specific binding of c-Jun and ATF-2 to the 3,157 bp AP-1 site in RAECs.

The necessary role of c-Jun and ATF-2 on thrombin-induced arginase upregulation. To understand the specific requirement for c-Jun and ATF-2 on thrombin-induced arginase upregulation, we performed loss-of-function studies with specific siRNA. RAECs were transfected with control, c-Jun, and ATF-2 siRNA (designed and produced by Santa Cruz) 48 h before incubation with thrombin. Protein was then harvested for immunoblotting. We first examined the specificity of our siRNAs for selective knockdown of the target c-Jun and ATF-2 proteins. We found that each siRNA was specific for its target. c-Jun siRNA knocked down the c-Jun protein without affecting ATF-2. A similar result was obtained with ATF-2 siRNA (Fig. 5, A and B). To examine the role of c-Jun and ATF-2 on thrombin-induced arginase I induction, we did the immunoblot for arginase I in the same cell lysates. In control siRNA-transfected cells, thrombin induced arginase I upregulation. In contrast, in cells transfected with c-Jun or ATF-2 siRNA, thrombin did not lead to arginase I upregulation (Fig. 5, A and C). These data demonstrate the central roles of c-Jun and ATF-2 in the upregulation of arginase I in RAECs in response to thrombin exposure.

MAP kinase mediates the effect of thrombin on AP-1 activation. Phosphorylation by MAP kinase is thought to be a common event in the activation of AP-1 proteins. To determine whether thrombin induces phosphorylation of c-Jun and ATF-2, we treated RAECs with thrombin for 0, 5, 10, 15, 20, and 30 min and then immunoblotted for phospho-c-Jun and phospho-ATF-2. Thrombin induced a transient phosphorylation of these two transcription factors. Maximal phosphorylation of c-Jun was observed at 20 min and returned to basal level at 30 min. Phosphorylation of ATF-2 occurred after 5 min, and declined after 10 min. No change in total expression of JNK, c-Jun, and ATF-2 was observed at any time point (Fig. 6A).

In mammals, it was reported that two major MAP kinases can phosphorylate and activate c-Jun and ATF-2. They are JNK and the p38 MAP kinase (26). We assessed JNK and p38 MAP kinase phosphorylation in response to thrombin in RAECs treated with...
thrombin (30 U/ml) at different time points. Immunoblot analyses were used to determine protein abundance. Thrombin induced a transient phosphorylation of SAPK/JNK and p38 MAP kinase. Maximal activation of SAPK/JNK was observed after 10 min and declined to basal level within 30 min. p38 MAP kinase phosphorylation started at 5 min, maximized at 15 min, and declined after 15 min (Fig. 6B).

We then hypothesized that JNK phosphorylation induced c-Jun phosphorylation while p38 MAP kinase was responsible for ATF-2 phosphorylation. To test this hypothesis, we examined the effect of specific JNK inhibitor SP600125 and p38 MAP kinase inhibitor SB202190 on c-Jun and ATF-2 phosphorylation. Preincubation with SP600125 (10^{-6} mol/l) inhibited thrombin-induced c-Jun phosphorylation, whereas SB202190 (10^{-6} mol/l) inhibited ATF-2 phosphorylation (Fig. 6C). These data indicated JNK and p38 MAP kinase phosphorylated c-Jun and ATF-2, respectively, which induced the activation of AP-1. To further determine the effect of p38 MAP kinase inhibition on thrombin-induced arginase I transcription, RAECs were incubated with both thrombin and SB202190. Immunoblot analysis was then performed. In control cells, thrombin induced arginase I upregulation, which was prevented by SB202190 exposure (Fig. 6D).

**DISCUSSION**

We have previously shown that arterial thrombosis leads to endothelial dysfunction secondary to decreased nitric oxide bioactivity, and that this can be ameliorated via L-arginine supplementation or arginase blockade (12, 23, 27, 39, 56). Of note, rat endothelium exposed to thrombin for 6 h led to increased arginase I mRNA (6.8-fold) and arginase I protein levels (2.1-fold) (27). We believe that thrombin plays a central role in thrombus-induced endothelial dysfunction and investigated the molecular mechanisms of arginase changes after thrombin exposure. Thrombin is a coagulation system protease present at the sites of vascular injury. In addition to catalyzing the conversion of soluble fibrinogen into an insoluble fibrin clot, thrombin is a potent endothelial cell agonist inducing several genes in endothelial cells (47, 52). Previously, a 9-bp thrombin-responsive element was identified in the platelet-derived growth factor B-chain gene promoter and its binding protein, thrombin-inducible nuclear factor, which belongs to the Y box protein family (46, 48). Our recent studies demons-
strated that the MAP kinase phosphatase-1 gene is thrombin responsive in HUVEC (9, 25). Here we report on the molecular mechanisms of thrombin-induced arginase upregulation. In particular, 1) the effect of thrombin on arginase I promoter activity and enzyme activity was assessed, 2) a thrombin-responsive element in RAECs was identified, 3) the transcription factors involved in thrombin-induced arginase I upregulation were determined, and 4) the signaling pathway mediating thrombin-induced arginase I upregulation in endothelial cells was defined. Our study reveals that after endothelial exposure to thrombin, c-Jun and ATF-2 are recruited to the AP-1 consensus sequence in the arginase I promoter and that arginase I transcription is upregulated.

Generation of a hemostatic clot requires conversation of fibrinogen to fibrin, which involves local pH, ionic strength, calcium concentration, fibrinogen, and other variables (8, 16, 36, 42). However, in situ thrombin concentration has the most profound physiological influence on fibrin clot formation (53). During a coagulation process, the concentration of free thrombin present can range from less than 0.1 U/ml to greater than 50 U/ml (2, 3, 11). In this study, we found that thrombin, at concentrations that mimic the circumstances of acute arterial thrombosis, induced arginase transcription. Thrombin signaling in endothelium is mediated by a family of 7-transmembrane G protein-coupled receptors called protease-activated receptors (PARs). This can result in multiple phenotype changes, including cell shape, permeability, vasomotor tone, migration, and angiogenesis (30). Thrombin-induced transcription changes have been well studied in low concentration, typically less than 1 to 2 U/ml. However, our finding suggests that there may be another group of genes that are specifically induced by higher, yet still pathologically relevant levels of thrombin, and arginase may represent one of those genes in endothelial cells. Alternatively, thrombin receptor 4 (PAR 4) requires high concentrations of thrombin if its coreceptor PAR 3 is absent or dysfunctional.

By using different mutant constructs, we identified an AP-1 site located 3,157 bp upstream of the transcription start site of arginase I promoter. The sequence of this AP-1 site matches the AP-1 consensus sequence GAGTCA. AP-1 is a group of structurally and functionally related members of the Jun, Fos protein family and some members of ATF and JDP subfamilies, which form dimeric complexes (51). A broad range of physiological and pathological stimuli, including cytokines, growth factors, stress, and oncogenic signals, could activate AP-1. In human vascular endothelial cells, peroxisome proliferator-activated receptor activators were reported to inhibit thrombin-induced endothelin-1 production through AP-1 signaling pathway (13). Suppression of transcription factors Egr-1, AP-1, and NF-κB was reported to inhibit thrombin-induced tissue factor gene activation (38). Thrombin and tumor necrosis factor-α synergistically stimulate tissue factor expression in endothelial cells through c-Fos and c-Jun (28). In this study, EMSA and supershift assay revealed that this AP-1-DNA complex is composed of c-Jun and ATF-2. We also demonstrated that suppression of c-Jun and ATF-2 by specific siRNA results in a significant inhibition of arginase protein expression in thrombin treated RAECs. Thus, the novel finding that thrombin modulates endothelial arginase gene expression through AP-1 binding via transcription factors c-Jun and ATF-2 indicates that AP-1 may play a central role in endothelial dysfunction after thrombosis.
Endothelial cells are the first cells in an artery to encounter circulating thrombin, one of the key stimuli leading to the activation of multiple signaling pathways. In the current study, we characterized the role of the stress-activated family of MAP kinases in thrombin-induced arginase expression via AP-1 activation. Previously, JNK-induced phosphorylation of Thr69 and Thr71 was found essential for the transcription activation of ATF-2 in response to proinflammatory cytokines or ultraviolet radiation (18, 29). In wild-type embryonic fibroblasts, it was reported that p38 MAP kinase was not rate limiting for phosphorylation of ATF-2. However, in JNK-deficient cells, p38 MAP kinase substituted for JNK phosphorylation of ATF-2 (35). In our study, we found thrombin-induced phosphorylation of ATF-2 is earlier than JNK phosphorylation. Incubation with SP600125 did not prevent the phosphorylation of ATF-2 Thr69/71, but incubation of SB202190 was sufficient to prevent ATF-2 phosphorylation by thrombin. These observations indicate that in RAECs, p38 MAP kinase, but not JNK, phosphorylates ATF-2 in response to thrombin. It remains to be determined whether this is a thrombin-specific mechanism in endothelial cell signaling.

ATF-2 and c-Jun are key components of AP-1 and function as homodimers or heterodimers. c-Jun-ATF-2 heterodimers activate the expression of many target genes in response to a variety of cellular and environmental signals (7, 18, 22, 50). By ultraviolet cross-linking and immunoprecipitation, we demonstrated that a heterodimer of transcription factors c-Jun and ATF-2 is bound to the AP-1 site in the arginase I promoter following stimulation by thrombin. Thrombin stimulation of endothelial cells induced marked activation of SAPK/JNK and p38 MAP kinase, which was followed by transient phosphorylation of both c-Jun and ATF-2. Regulation of AP-1 activity can be achieved by changes in the transcription level of AP-1 subunits, control of the stability of their mRNAs, posttranslational processing, turnover of preexisting or newly synthesized AP-1 subunits, and specific interactions between AP-1 proteins and other transcription factors and cofactors (19). A previous report has shown that a low concentration of thrombin (1 U/ml) stimulates HUVEC arginase enzyme activity via the RhoA/ROCK pathway, without affecting the arginase protein level (32). Our current study also showed an increased activity of arginase in endothelial cells exposed to a higher dose of thrombin (30 U/ml). However, this was via MAP kinase-mediated phosphorylation, AP-1 activation, and arginase upregulation.

There are limitations to the current study. Additional studies in animal/preclinical models may be needed to confirm our observations and extend our understanding of this process in the in vivo environment. But, we believe the results presented here combined with previous animal studies provide compelling evidence for arginase’s role in thrombosis-induced endothelial dysfunction (27). The predominant isoform of arginase in rats is arginase 1 and thus, this work may not translate to mechanistic relevance in humans where arginase 2 predominates. Further work in human endothelial cells and harvested arterial tissue is warranted. Lastly, arginase activation via nontranscriptional pathways should be investigated. Thus, alternative molecular pathways may play a role in thrombin’s effects and are worthy of future study.

Thrombolytic treatments are able to restore blood flow in occluded peripheral arteries. However, vasospasm and rethrombosis complicate a large fraction of treated individuals which may reflect endothelial dysfunction after thrombosis (37, 40). The present findings have important clinical relevance. Our understanding of endothelial dysfunction may lead to adjuncts that prevent suboptimal clinical outcomes. Currently, we are evaluating l-arginine supplementation in human arteries (unpublished observations). The results of the present study provide the first direct evidence supporting a key role for the transcription factor AP-1 in mediating endothelial arginase, an enzyme that can modulate l-arginine levels. Our findings highlight the arginase pathway as a new therapeutic target in limiting endothelial dysfunction.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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