Thrombin Stimulates Human Endothelial Arginase
Enzymatic Activity via RhoA/ROCK Pathway
Implications for Atherosclerotic Endothelial Dysfunction

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Background—Arginase competes with endothelial nitric oxide synthase (eNOS) for the substrate L-arginine and decreases NO production. This study investigated regulatory mechanisms of arginase activity in endothelial cells and its role in atherosclerosis.

Methods and Results—In human endothelial cells isolated from umbilical veins, thrombin concentration- and time-dependently stimulated arginase enzymatic activity, reaching a 1.9-fold increase (P<0.001) at 1 U/mL for 24 hours. The effect of thrombin was prevented by C3 exoenzyme or the HMG-CoA reductase inhibitor fluvastatin, which inhibit RhoA, or by the ROCK inhibitors Y-27632 and HA-1077. Adenoviral expression of constitutively active RhoA or ROCK mutants enhanced arginase activity (~3-fold, P<0.001), and the effect of active RhoA mutant was inhibited by the ROCK inhibitors. Neither thrombin nor the active RhoA/ROCK mutants affected arginase II protein level, the only isozyme detectable in the cells. Moreover, a significantly higher arginase II activity (1.5-fold, not the protein level) and RhoA protein level (4-fold) were observed in atherosclerotic aortas of apoE−/− compared with wild-type mice. Interestingly, L-arginine (1 mmol/L), despite a significantly higher eNOS expression in aortas of apoE−/− mice, evoked a more pronounced contraction, which was reverted to a greater vasodilation by the arginase inhibitor L-norvaline (20 mmol/L) compared with the wild-type animals (n=5, P<0.001).


Key Words: atherosclerosis ■ cells ■ cardiovascular diseases ■ endothelium-derived factors ■ signal transduction

Vascular endothelial cells produce nitric oxide (NO) from the precursor substrate L-arginine via endothelial NO synthase (eNOS). NO plays a critical role in regulation of vascular tone and maintenance of vascular integrity. Decreased production or bioavailability of NO is a common mechanism involved in the pathogenesis of various cardiovascular diseases, such as atherosclerosis, hypertension, and diabetic vasculopathy. Among other mechanisms, such as regulation of cofactors, eNOS gene expression, and enzymatic activation, the availability of the substrate L-arginine is an important limiting factor for NO production. Emerging evidence demonstrates that arginase, an enzyme in the urea cycle, competes with NO synthase for the substrate L-arginine and thus reduces NO production, as shown in macrophages and vascular endothelial cells. This mechanism was recently implicated in diabetic erectile dysfunction, aging-associated endothelial dysfunction, and allergen-induced deficiency of NO and airway hyperresponsiveness in asthma.

Two types of mammalian arginase exist, arginase I and II, encoded by different genes. Arginase I, located in the cytoplasm, is expressed most abundantly in the liver, whereas arginase II is a mitochondrial enzyme and is expressed primarily in extrahepatic tissues. Recent studies demonstrate that the activity of arginase can be regulated in many cell types, including vascular endothelial cells, smooth muscle cells, and macrophages by various cytokines. According to the cell types studied, activity or gene expression of the 2 isozymes are either constitutively expressed or induced, which regulates NO production. In porcine coronary and rat aortic endothelial cells, arginase I is constitutively expressed, whereas arginase II is induced in response to lipopolysaccharide. In human diabetic corpus cavernosum,
arginase II expression is significantly increased, and inhibition of this enzyme enhanced NO-dependent relaxation of penile corpus cavernosum smooth muscle.8 These observations suggest a potential role of arginase II in negative regulation of NO production. However, the exact type of the isoforms expressed in human vascular endothelial cells has not yet been elucidated. Moreover, the regulatory mechanisms of arginase activity in endothelial cells and in atherosclerotic blood vessels are unknown.

Recent research provides compelling evidence that the small G protein RhoA plays an important role in regulation of various cellular functions in the vasculature.18,19 With regard to its role in the regulation of NO production, studies from our own and other laboratories demonstrated that RhoA, via its downstream effector ROCK, plays an important role in regulation of eNOS gene expression and enzyme activity.19–21 These data suggest that RhoA/ROCK may affect endothelial function at multiple levels. Therefore, this study was designed to determine whether the RhoA/ROCK pathway is also involved in regulation of arginase activity or expression in cultured human endothelial cells and whether arginase plays a role in endothelial dysfunction in atherosclerosis.

Methods

Materials

All reagents were purchased from Calbiochem except the following: Fluvastatin was provided by Novartis; anti-RhoA monoclonal antibody was from Santa Cruz Biotechnology, Inc; anti-arginase I antibody was from BD Transduction Laboratory; anti–arginase II antibody was from Promega; and BCIP/NBT solution was purchased from Upstate Biotechnology Inc. Endothelial cell growth supplement pack was from PromoCell GmbH; all cell culture media and materials were purchased from Gibco BRL.

Cell Culture

Endothelial cells were isolated from human umbilical veins (HUVECs) as previously described.21 Cells of the first to fourth passages were used.

Generation of Recombinant Adenoviruses and Adenoviral Infection

Generation of recombinant adenovirus expressing HA-tagged RhoA, ROCK mutants driven by the cytomegalovirus promoter, and the adenoviral infection of HUVECs were described previously.21

Animals

Thirteen male apolipoprotein E–deficient (apoE−/−) mice and 13 male C57BL/6J wild-type control mice (4 weeks old) were obtained from Jackson Laboratory (Bar Harbor, Me). To accelerate lesion formation, animals were fed a Western-type diet (Harlan TD88137, 21.2% total fat and 0.2% cholesterol) for 4 months.22 At 5 months of age, animals were anesthetized with pentobarbital (100 mg/kg IP), and the entire aorta from the heart to the iliac bifurcation was removed, placed into cold (4°C) Krebs bicarbonate solution, and dissected free from fat and adhering perivascular tissue. The isolated aorta was cut into 2 parts. One part was used for analysis of arginase activity and the other for protein expression by Western blot. The isolated vessel was snap-frozen in liquid nitrogen. The tissues were homogenized and sonicated in arginase lysis buffer (see below) for arginase activity assay or in extraction buffer for analysis of protein expression by Western blot.21 Protein concentration was determined by Bradford method (Bio-Rad). The local animal ethics committee approved animal handling and experimentation.

Measurement of Arginase Activity

Arginase activity in the cells and aortic tissue lysates was measured by colorimetric determination of urea formed from L-arginine by modification of a published procedure.16

Western Blot for eNOS and Arginase Expression

HUVECs and mouse aortic tissue extract preparation, SDS-PAGE, and transfer of SDS gels to an Immobilon-P membrane (Millipore) were performed as previously described.21 The resultant membrane was incubated overnight with the corresponding primary antibody at 4°C with gentle agitation after blocking with 5% skimmed milk. The protein was labeled with a corresponding anti-mouse or anti-rabbit alkaline phosphatase–conjugated secondary antibody and detected by use of BCIP/NBT substrate solution. Quantification of the signals was performed with NIH Image 1.62 software. In all the plotted graphics, each point represents the average value from 3 to 5 independent experiments.

eNOS Activity Assay

eNOS activity was measured by L-citrulline production in HUVECs as previously described.21

Vascular Reactivity Studies

The isolated descending thoracic aortas with intact endothelium from wild-type and apoE−/− mice were cut into rings (3 mm in length), which were suspended in a Multi-Myograph System (model 610 M, Danish Myo Technology A/S) as described previously.23 To study the role of arginase in modulation of eNOS function, aortic rings with endothelium in parallel were incubated with or without the arginase inhibitor L-norvaline (20 mmol/L) for 1 hour,5–9 and then contracted with norepinephrine (0.3 μmol/L). L-Arginine (1 mmol/L) was added on top of the contraction.

Statistics

Data are given as mean±SEM. In all experiments, n equals the number of experiments or animals. Statistical analysis was performed with unpaired t test or ANOVA with Dunnett or Bonferroni post hoc test. Differences in mean values were considered significant at a value of P<0.05.

Results

Thrombin Stimulates Arginase Activity in Human Endothelial Cells

Stimulation of HUVECs with various concentrations of thrombin (0.1 to 2 U/mL) for 24 hours enhanced arginase activity, with the maximum effect at the concentration of 1 U/mL (1.9-fold increase above control, n=9, P<0.001; Figure 1). Therefore, 1 U/mL thrombin was used in the following experiments. The cells were then stimulated with thrombin (1 U/mL) at different time points from 5 minutes to 24 hours. As demonstrated in Figure 2, a significant increase in arginase activity was observed after 18 to 24 hours of exposure of the cells to thrombin, with a maximum effect at 24 hours (1.9-fold, n=3, P<0.001).

Role of RhoA/ROCK in Regulation of Arginase Activity

As shown in Figure 3A, stimulation of arginase activity by thrombin (1 U/mL, 24 hours) in endothelial cells was inhibited by the HMG-CoA reductase inhibitor fluvastatin (1
arginase activity by thrombin was also inhibited by C3 exoenzyme (20 μmol/L) or by another ROCK inhibitor, HA-1077 (10 μmol/L, Figure 3C; n=5, P<0.001 versus thrombin alone), further demonstrating the role of the Rho/ROCK pathway in thrombin-stimulated arginase activity in the cells. It should be noted that exposure of the cells to thrombin at 1 U/mL, time-dependently suppressed eNOS protein level over a period of 24 hours, an effect that was already significant at 6 hours (Figure 3D). Furthermore, adenovirus-mediated expression of the active mutant of RhoA (Rho63) or ROCK (CAT) enhanced arginase activity in the cells, whereas the negative mutant of RhoA (Rho19) or ROCK (RB) alone had no effect (Figure 4A, n=7). Again, the arginase II protein level in the cells was not affected by the mutants (Figure 4B, n=7). The increase in arginase activity stimulated by the active Rho63 mutant was abolished by the 2 different ROCK inhibitors, Y-27632 (10 μmol/L) and HA-1077 (10 μmol/L, Figure 4C). The inhibitors alone had no significant effects on the basal arginase activity (Figure 4C). We have shown previously \(^2\) that previously the eNOS protein level is markedly downregulated by the active RhoA or ROCK mutants 24 hours after transduction.

Increased Arginase Activity and RhoA Expression in Atherosclerosis

Given the above observations, we tested whether arginase activity is increased in atherosclerosis. ApoE\(^{-/-}\) atherosclerotic mice were used for this purpose. Indeed, apoE\(^{-/-}\) mice fed a cholesterol-rich diet for 4 months developed atherosclerotic lesions throughout the aorta\(^2\) and showed a significantly higher arginase activity than aortas isolated from age-matched wild-type mice (1.5-fold increase, n=5, P<0.01; Figure 5A). In addition, the arginase II protein level was comparable in the aortas of the 2 groups, and arginase I protein was not detectable in the mouse aortas (data not shown). Interestingly, the RhoA protein level was remarkably increased (4-fold) in the aortas of apoE\(^{-/-}\) mice compared with wild-type animals (Figure 5B).

Arginase and eNOS Activity

The role of arginase in regulating eNOS activity was further investigated. In HUVECs, eNOS activity as measured by L-citrulline production was blunted by the eNOS inhibitor N\(^{\text{G}}\)-nitro-L-arginine methyl ester (0.1 mmol/L) and also by thrombin (1 U/mL, 24 hours, n=5, 50±9% decrease, P<0.001; Figure 6A). Cotreatment of the cells with the arginase inhibitor L-norvaline\(^5\) (20 mmol/L, 24 hours), however, did not significantly reverse eNOS activity (Figure 6A). In mouse aortas, L-arginine (1 mmol/L) caused vasoconstriction (in contrast to rats in which L-arginine caused vasorelaxation; data not shown) that was more pronounced in apoE\(^{-/-}\) mice (38±6%) than in wild-type animals (17±4%, n=5, P<0.01; Figure 6B), although eNOS expression was significantly higher in apoE\(^{-/-}\) than in wild-type mice (Figure 6C; P<0.05). Remarkably, the contraction was converted to a more pronounced relaxation by the arginase inhibitor L-norvaline (20 mmol/L, 1 hour) in atherosclerotic apoE\(^{-/-}\) mice (−21±5%) than in wild-type animals (−6±2%, Figure 6B, n=5; P<0.001).

Discussion

Emerging evidence demonstrates that arginase is present in various cell types and is involved in negative regulation of NO production, as reported in macrophages\(^{24,27}\) and endothelial cells.\(^7,17,28\) Our present study demonstrates that in human endothelial cells, arginase activity is significantly induced by thrombin. The induction of arginase activity can be inhibited by the HMG-CoA reductase inhibitor fluvastatin, which inhibits RhoA activation by geranylgeranylation of the enzyme,\(^19\) and also by C3 exoenzyme, which inactivates RhoA.

Figure 1. Thrombin dose-dependently stimulates arginase activity in HUVECs. HUVECs were first serum-starved in 0.2% FCS culture medium for 24 hours, followed by treatment with various concentrations of thrombin as indicated for 24 hours. Cells were then extracted in arginase lysis buffer and subjected to arginase activity assay as described in Methods. Columns represent mean±SEM of 3 experiments (*)P<0.05, **P<0.01, ***P<0.001 vs untreated cells, ie, time zero).

Figure 2. Time course of thrombin-induced arginase activity. HUVECs were treated with thrombin (1 U/mL) for different times as indicated, extracted, and assayed for arginase activity. Columns represent mean±SEM of 3 experiments (*)P<0.05, **P<0.01, ***P<0.001 vs untreated cells, ie, time zero).
Moreover, inhibition of ROCK, a downstream effector of RhoA, either by Y-27632 or HA-1077 abolished the effect of thrombin, suggesting that thrombin stimulates arginase activity in human endothelial cells through the RhoA/ROCK pathway. This conclusion is supported by the experiments showing that adenovirus-mediated ectopic expression of the constitutively active mutant of RhoA (Rho63) or ROCK (CAT) but not the negative mutants significantly enhanced arginase activity in the cells. Furthermore, the effect of Rho63 was abolished by the ROCK inhibitor Y-27632 or HA-1077.

Two isoforms of arginases, arginase I and II, were reported to be expressed in vascular endothelial cells. In endothelial cells of porcine coronary arterioles and rat aortas, arginase I is constitutively expressed, whereas arginase II is inducible in response to lipopolysaccharide or cytokines. Our present study, however, showed abundant basal levels of arginase II, whereas arginase I is not detectable in HUVECs, suggesting that arginase II is the major isozyme in HUVECs. It should be noted that neither thrombin nor the active mutants of RhoA/ROCK did modulate arginase II expression (arginase I is not inducible in the cells), suggesting that activation of arginase by thrombin or active RhoA/ROCK mutants occurs at the level of enzyme activity rather than on gene expression. Similar findings were also reported in rat endothelial cells, in which expression of arginases was not modified by cytokines, although the enzymatic activity was stimulated. It is conceivable that the enzyme might be modified biochemically by the RhoA/ROCK pathway, which alters either the enzymatic activity or the affinity of the enzyme to its substrate. A third isoform of arginase might exist in endothelial cells and cannot be ruled out under our experimental conditions. This aspect certainly warrants further investigation.

Furthermore, we demonstrated that arginase enzymatic activity was significantly increased in atherosclerotic aortas.
The higher arginase activity in the atherosclerotic aortas was associated with higher RhoA protein levels, suggesting a role of RhoA in upregulation of arginase activity. A definite characterization of the role of RhoA in stimulation of arginase activity in atherosclerosis in vivo could not be performed at this stage because of lack of specific RhoA inhibitors applicable in living mice. Nevertheless, the results obtained from cultured HUVECs support the role of RhoA in stimulation of arginase activity.

Previous studies have indicated an important role of increased arginase activity, even a moderate increase of 1.5- to 2-fold, in endothelial dysfunction in aged rats and in human diabetic erectile dysfunction. We further investigated the role of increased arginase activity in regulating NO production in cultured endothelial cells and in atherosclerotic aortas of apoE−/− mice. In HUVECs treated with thrombin, eNOS activity was significantly reduced. However, this stimulation of arginase activity in atherosclerosis in vivo could not be performed at this stage because of lack of specific RhoA inhibitors applicable in living mice.
reduction of eNOS activity was not significantly reversed in the presence of the arginase inhibitor L-norvaline. It is most likely because the eNOS protein level was simultaneously suppressed by thrombin, as demonstrated by Figure 3D, and also by our previous studies.20,21 Alternatively, inhibition of cofactors of eNOS by thrombin might also be involved.

To our surprise, L-arginine induced vasoconstriction in mouse aorta, which contrasts with the observation in rats (data not shown; see Reference 9) and humans,31 in which it evoked vascular relaxation. The contraction induced by L-arginine is much more pronounced in atherosclerotic apoE–/– mice compared with control animals. The results may imply that in the mouse aortas, particularly in the atherosclerotic apoE–/– aortas, L-arginine may be metabolized by arginase to certain vasoconstrictive intermediate products. Most interestingly, the contractions were converted to relaxations in the presence of the arginase inhibitor L-norvaline, an effect that was significantly greater in atherosclerotic apoE–/– mice than wild-type animals, demonstrating a dominant role of increased arginase activity in regulation of endothelial NO production in atherosclerosis. It should be mentioned that eNOS gene expression is significantly higher in atherosclerotic aortas (see Figure 6C), further supporting the concept that endothelial dysfunction in atherosclerosis is caused by a decreased NO bioavailability.13 The higher eNOS expression in atherosclerosis would make an efficient NO production possible when arginase is inhibited. This may implicate arginase as a potential therapeutic target for treatment of atherosclerotic vascular disease. It is also important to point out that eNOS protein levels were not suppressed in atherosclerotic aortas of apoE–/– mice despite higher RhoA level in these blood vessels. It is conceivable that the suppressing effect of RhoA on eNOS protein expression might be compensated by other mechanisms in vivo.

In conclusion, our present study provides evidence for the role of the RhoA/ROCK pathway in stimulation of arginase activity in human endothelial cells. The increased arginase activity is associated with higher RhoA expression and is involved in endothelial dysfunction in atherosclerosis. Targeting arginase in the vasculature may represent a novel therapeutic strategy for treatment of atherosclerosis.

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