

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

Xiu-Fen Ming, MD/PhD<sup>1</sup>; Christine Barandier, PhD<sup>1</sup>; Hema Viswambharan, MSc<sup>1</sup>; Brenda R. Kwak, PhD<sup>3</sup>; François Mach MD<sup>3</sup>; Lucia Mazzolai, MD<sup>4</sup>; Daniel Hayoz, MD<sup>4</sup>; Jean Ruffieux, BS<sup>1</sup>; Sandro Rusconi, PhD<sup>2</sup>; Jean-Pierre Montani, MD<sup>1</sup>; Zhihong Yang, MD<sup>1</sup>

Vascular Biology, Department of Medicine, Division of Physiology<sup>1</sup> and Biochemistry<sup>2</sup>,  
University of Fribourg, Switzerland, Division of Cardiology, University Hospital Geneva<sup>3</sup>  
and Division of Hypertension and Vascular Medicine, CHUV, Lausanne<sup>4</sup>, Switzerland

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*Address for correspondence:*

Prof. Dr. Zhihong Yang  
Vascular Biology  
Department of Medicine  
Division of Physiology  
University of Fribourg  
Rue du Musée 5  
CH-1700, Fribourg  
Switzerland  
Telephone: 0041/26/300 85 93  
Telefax: 0041/26/300 96 36  
E-mail: [zhihong.yang@unifr.ch](mailto:zhihong.yang@unifr.ch)

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### Abstract

**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 1.9-fold increase ( $p < 0.001$ ) at 1 U/ml for 24 hours. The effect of thrombin was prevented by C3 exoenzyme or HMG-CoA reductase inhibitor fluvastatin that inhibit RhoA or by ROCK inhibitors Y-27632 and HA-1077. Adenoviral expression of constitutively active RhoA or ROCK mutants enhanced arginase activity (ca. 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<sup>-/-</sup> compared to wild type mice. Interestingly, L-arginine (1 mmol/L), despite a significantly higher eNOS expression in aortas of ApoE<sup>-/-</sup> mice, evoked a more pronounced contraction which was reverted to a greater vasodilation by the arginase inhibitor L-norvaline (20 mmol/L) compared to the wild type animals ( $n=5$ ,  $p < 0.001$ ).

**Conclusions:** Thrombin enhances arginase activity via RhoA/ROCK in human endothelial cells. Higher arginase enzymatic activity is involved in atherosclerotic endothelial dysfunction in ApoE<sup>-/-</sup> mice. Targeting vascular arginase may represent a novel therapeutic possibility for atherosclerosis.

**Key words:** Atherosclerosis - cells – cardiovascular disease – endothelium-derived factors – signal transduction

### Condensed Abstract

In human endothelial cells, thrombin stimulates arginase enzymatic activity which was prevented by inhibition of RhoA/ROCK pathway. Adenoviral expression of active mutant of RhoA or ROCK mimics thrombin's effect. Arginase activity, eNOS expression and RhoA level are increased in atherosclerotic aortas of ApoE<sup>-/-</sup> mice. Inhibition of arginase by L-norvaline caused a greater vasorelaxation to L-arginine in atherosclerotic aortas of ApoE<sup>-/-</sup> compared to wild type mice. Thus, higher arginase enzymatic activity is involved in endothelial dysfunction in atherosclerosis. Targeting arginase in the vasculature may represent a novel therapeutic possibility for atherosclerosis.

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.<sup>1</sup> 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.<sup>2,3</sup> Among other mechanisms such as regulation of co-factors, eNOS gene expression, and enzymatic activation, the availability of the substrate L-arginine is an important limiting factor for NO production.<sup>4</sup> Emerging evidence demonstrates that arginase, an enzyme in the urea cycle, competes with NOS for the substrate L-arginine and thus reduces NO production as shown in macrophages and vascular endothelial cells.<sup>4-7</sup> This mechanism has been recently implicated in diabetic erectile dysfunction, aging-associated endothelial dysfunction, allergen-induced deficiency of NO and airway hyperresponsiveness in asthma.<sup>8-10</sup>

Two types of mammalian arginase namely arginase I and II encoded by different genes exist.<sup>11</sup> Arginase I, located in the cytoplasm, is expressed most abundantly in the liver, while arginase II is a mitochondrial enzyme and is mostly expressed in extrahepatic tissues<sup>12-14</sup>. 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.<sup>7,15,16</sup> Depending on cell types studied, activity or gene expression of the two isozymes are either constitutively expressed or induced, which regulates NO production. In porcine coronary and rat aortic endothelial cells arginase I is constitutively expressed,<sup>7,17</sup> whereas arginase II is induced in response to lipopolysaccharide.<sup>17</sup> 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.<sup>8</sup> These observations suggest a potential role of arginase II in negative regulation of NO production. However, the exact type of the isozymes 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 vasculature.<sup>18,19</sup> With respect 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.<sup>19-21</sup> These data suggest that RhoA/ROCK may affect endothelial function at multiple levels. Therefore, this study was designed to determine whether 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.

## Materials and Methods

### Materials

All reagents were purchased from Calbiochem (Luzern, Switzerland) except the following: Fluvastatin was provided by Novartis (Basel, Switzerland); anti-RhoA monoclonal antibody from Santa Cruz Biotechnology, Inc. (Nunningen, Switzerland); anti-arginase I antibody from BD Transduction Laboratory; anti-arginase II rabbit polyclonal antibody was generated by Eurogentech, S.A, by immunization with a synthesized peptide containing the 16 C-terminal amino acid (AA 339 –354) of human arginase II; anti- $\alpha$ -tubulin antibody from Sigma; anti-mouse and –rabbit IgG alkaline phosphatase (AP) conjugate were purchased from Promega (Wallisellen, Switzerland); BCIP/NBT solution was purchased from Upstate Biotechnology Inc. (Lucerne, Switzerland). Endothelial cell growth supplement (ECGS) pack from PromoCell GmbH (Allschwill, Switzerland); all cell culture media and materials were purchased from Gibco BRL (Basel, Switzerland).

### Cell Culture

Endothelial cells were isolated from human umbilical veins (HUVEC) as previously described.<sup>21</sup> Cells of 1<sup>st</sup> to 4<sup>th</sup> passages were used.

### Generation of Recombinant Adenoviruses (rAd) and Adenoviral Infection

Generation of recombinant adenovirus expressing HA-tagged RhoA, ROCK mutants driven by the cytomegalovirus (CMV) promoter and the adenoviral infection of HUVECs were described previously.<sup>21</sup>

### Animals

13 male apolipoprotein E-deficient (ApoE<sup>-/-</sup>) mice and 13 male C57BL/6J wild type control mice (4-weeks-old) were obtained from Jackson Laboratory (USA). To accelerate lesion formation, animals were fed a Western type diet (Harlan TD88137, 21.2% total fat and 0.2% cholesterol) for 4 months.<sup>22</sup> At 5 months of age, animals were anesthetized with

pentobarbital (100 mg/kg i.p.), the entire aorta from the heart to the iliac bifurcation were removed, placed into cold (4°C) Krebs bicarbonate solution, dissected free from fat and adhering perivascular tissue. The isolated aorta was cut into two 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<sup>21</sup>. Protein concentration was determined by Bradford method (Bio-Rad). Animal handling and experimentation were approved by local animal ethical committee.

### **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 a modification of published procedure.<sup>16</sup>

### **Western blot for eNOS and arginase expression**

HUVEC and mouse aortic tissue extract preparation, SDS-PAGE, transfer of SDS gels to an Immobilon-P membrane (Millipore) were performed as previously described.<sup>21</sup> 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 decorated with a corresponding anti-mouse or anti-rabbit AP-conjugated secondary antibody and detected using BCIP/NBT substrate solution. Quantification of the signals was performed using NIH Image 1.62 software. In all the plotted graphics, each point represents the average value from three to five independent experiments.

### **eNOS activity assay**

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

### **Vascular reactivity studies**

The isolated descending thoracic aortas with intact endothelium from wild type and ApoE<sup>-/-</sup> mice were cut into rings (3 mm in length) which were suspended in a Multi-Myograph System (Model 610M, Danish Myo Technology A/S, Denmark) as described.<sup>23</sup> 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,<sup>5-9</sup> and then contracted with norepinephrine (0.3  $\mu$ mol/L). L-arginine (1 mmol/L) was added on top of the contraction.

### **Statistics**

Data are given as mean $\pm$ 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-test. Differences in mean values were considered significant at  $p < 0.05$ .

## Results

### Thrombin stimulates arginase activity in human endothelial cells

Stimulation of HUVECs with various concentrations of thrombin (0.1 U/ml 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, **Fig. 1**). Therefore, 1 U/ml of 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 **Fig. 2**, a significant increase in arginase activity was observed after 18 to 24 hours exposure of the cells to thrombin with a maximum effect at 24 hours (1.9-fold, n=3, p<0.001).

### The role of RhoA /ROCK in regulation of arginase activity

As shown in **Fig. 3A**, stimulation of arginase activity by thrombin (1 U/ml, 24 hours) in endothelial cells was inhibited by HMG CoA reductase inhibitor fluvastatin (1  $\mu\text{mol/L}$ ) or ROCK inhibitor Y-27632 (10  $\mu\text{mol/L}$ ) at the concentrations that inhibit RhoA or ROCK as shown by our previous study.<sup>24</sup> Neither thrombin nor the inhibitors significantly influenced arginase II protein level (**Fig. 3B**, arginase I was not detectable in the cells). Stimulation of arginase activity by thrombin was also inhibited by C3 exoenzyme (20  $\mu\text{g/mL}$ ) or by another ROCK inhibitor HA-1077 (10  $\mu\text{mol/L}$ , **Fig. 3C**; n=5, p<0.001 vs. thrombin alone), further demonstrating the role of Rho/ROCK pathway in thrombin-stimulated arginase activity in the cells. It is to notice that exposure of the cells to thrombin at 1 U/ml time-dependently suppressed eNOS protein level over 24 hours, an effect that was already significant at 6 hours (**Fig. 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 (**Fig. 4A**, n=7). Again, the arginase II protein level in the cells were not affected by the mutants (**Fig. 4B**, n=7). The increase in arginase activity stimulated by the active Rho63 mutant was abolished by the two different ROCK inhibitors Y-27632 (10  $\mu\text{mol/L}$ ) or HA-1077 (10  $\mu\text{mol/L}$ , **Fig. 4C**). The

inhibitors alone had no significant effects on the basal arginase activity (**Fig. 4C**). We have previously shown<sup>21</sup> that eNOS protein level is markedly down-regulated 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<sup>-/-</sup> atherosclerotic mice were used for this purpose. Indeed, ApoE<sup>-/-</sup> mice fed a cholesterol rich diet for 4 months developed atherosclerotic lesions throughout the aorta,<sup>22</sup> and showed a significant higher arginase activity than aortas isolated from age-matched wild type mice (1.5-fold increase, n=5, p<0.01, **Fig. 5A**). In addition, the arginase II protein level was comparable in the aortas of the two groups and arginase I protein was not detectable in the mice aortas (data not shown). Interestingly, RhoA protein level was remarkably increased (4-fold) in the aortas of ApoE<sup>-/-</sup> mice as compared to wild type animals (**Fig. 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 eNOS inhibitor L-NAME (0.1 mmol/L) and also by thrombin (1 U/ml, 24 hours, n=5, 50±9% decrease, P<0.001, **Fig. 6A**). Co-treatment of the cells with arginase inhibitor L-norvaline<sup>5</sup> (20 mmol/L, 24 hours), however, did not significantly reverse eNOS activity (**Fig. 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<sup>-/-</sup> (38±6%) than in wild type animals (17±4%, n=5, p<0.01, **Fig. 6B**), although eNOS expression was significantly higher in ApoE<sup>-/-</sup> than in wild type mice (**Fig. 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<sup>-/-</sup> mice (-21±5%) than in wild type animals (-6±2%, **Fig. 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<sup>25-27</sup> and endothelial cells.<sup>7,17,28</sup> 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<sup>19</sup>, and also by C3 exoenzyme which inactivates RhoA by ADP ribosylation<sup>29</sup>. Moreover, inhibition of ROCK, a downstream effector of RhoA, either by Y-27632 or HA-1077 abolished the thrombin's effect, suggesting that thrombin stimulates arginase activity in human endothelial cells through 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 namely 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,<sup>7,17</sup> whereas arginase II is inducible in response to lipopolysaccharide or cytokines.<sup>17</sup> Our present study, however, showed abundant basal level of arginase II, whereas arginase I is not detectable in HUVEC, suggesting that arginase II is the major isozyme in HUVECs. It is to note 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, where expression of arginases was not modified by cytokines, although the enzymatic activity was stimulated.<sup>30</sup> It is conceivable that the enzyme might be modified biochemically by RhoA/ROCK pathway, that either alters 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 condition. This aspect certainly warrants further investigation.

Furthermore, we demonstrated that arginase enzymatic activity was significantly increased in atherosclerotic aortas of ApoE<sup>-/-</sup> mice. The higher arginase activity in the atherosclerotic aortas was associated with higher RhoA protein level 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 due to 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. Similar to cultured HUVECs, no difference in arginase II expression was observed between control mice and atherosclerotic ApoE<sup>-/-</sup> mice, and arginase I was not detectable in the mice aortas (data not shown), suggesting an upregulation of arginase II enzymatic activity in atherosclerosis.

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,<sup>8,9</sup>. We further investigated the role of increased arginase activity in regulating NO production in cultured endothelial cells and in atherosclerotic aortas of ApoE<sup>-/-</sup> mice. In HUVECs treated with thrombin, eNOS activity was significantly reduced. However, this reduction of eNOS activity was not significantly reversed in the presence of arginase inhibitor L-norvaline. It is most likely due to the fact that eNOS protein level was simultaneously suppressed by thrombin, as demonstrated by figure 3D and also by our previous studies<sup>20,21</sup>. Alternatively, inhibition of co-factors of eNOS by thrombin might also be involved.

To our surprise, L-arginine induced vasoconstriction in mice aorta, which contrasts with the observation in rats (data not shown and see ref. 9) and humans<sup>31</sup>, where it evoked vascular relaxation. The contraction induced by L-arginine is much more pronounced in atherosclerotic ApoE<sup>-/-</sup> mice compared to control animals. The results may implicate that in the mice aortas, particularly in the atherosclerotic ApoE<sup>-/-</sup> 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 which was significantly greater in atherosclerotic ApoE<sup>-/-</sup> mice compared to wild type animals, demonstrating a dominant role of increased arginase activity in regulation of endothelial NO production in atherosclerosis. It is worth to mention that eNOS gene expression is significantly higher in atherosclerotic aortas (see figure 6C), further supporting the concept that endothelial dysfunction in atherosclerosis is due to a decreased NO bioavailability<sup>32</sup>. 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 level was not suppressed in atherosclerotic aortas of ApoE<sup>-/-</sup> 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 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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### Legends to the Figures

**Figure 1. Thrombin dose-dependently stimulates arginase activity in HUVECs.**

HUVECs were first serum starved in 0.2% FCS culture medium for 24 h, followed by treatment with various concentrations of thrombin as indicated for 24 h. Cells were then extracted in arginase lysis buffer and subjected to arginase activity assay as described in materials and methods. Columns represent mean  $\pm$  SEM of 9 experiments (\* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs. untreated cells, i.e. dose 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  $\pm$  SEM of 3 experiments (\* $P$ <0.05, \*\*\* $P$ <0.001 vs. untreated cells, i.e. time zero).

**Figure 3. Thrombin induces arginase activity via RhoA/ROCK.**

(A) stimulation of arginase activity by thrombin (1 U/ml, 24 hours) was inhibited by pretreatment of the cells with fluvastatin (FS, 1  $\mu$ mol/L) or Y-27632 (Y, 10  $\mu$ mol/L) for 1 h 30 minutes or (C) by C3 exoenzyme (20  $\mu$ g/mL, 20 hours) or HA-1077 (10  $\mu$ mol/L, 1 hour). (B) A representative blot revealing arginase II expression was not modified by various substances used in panel A (the expression of arginase I was not detectable in HUVECs). Columns represent mean  $\pm$  SEM of 5 independent experiments. (D) eNOS protein level was significantly decreased after 6 hours of exposure to thrombin (1 U/ml). \*\*\* $P$ <0.001 for comparison between groups as indicated or vs. control (0 h) in panel D.

**Figure 4. Active RhoA or ROCK stimulates arginase activity.**

(A) HUVECs were infected with recombinant adenovirus (rAd) expressing HA-tagged LacZ, active RhoA (Rho63), dominant negative RhoA (Rho19), active ROCK (CAT) or dominant negative ROCK (RB) as indicated, at titers ranging from 100 to 150 multiplicity of infection (MOI), and incubated

in 0.2% FCS RPMI-1640 supplemented with ECGS for 48 h. Cells were then extracted without any treatment and assayed for arginase activity or (B) subjected to Western blot for arginase expression. A representative blot revealing arginase II expression is shown in B. Columns represent mean  $\pm$  SEM of 7 independent experiments. (C) Rho63 induced activation of arginase activity was inhibited by the ROCK inhibitors Y-27632 (Y) or HA-1077 (HA). \*\*\*P<0.001 for comparison between groups as indicated or vs. LacZ.

**Figure 5. Increased arginase activity and RhoA level in atherosclerotic aortas of ApoE<sup>-/-</sup> mice:** The isolated aorta was cut in two sections. One section was homogenized and sonicated in arginase lysis buffer for analysis of arginase activity (A), the other section in extraction buffer for analysis of RhoA expression by Western blot (B). The equal amount of protein loading was ensured by ponceau S staining of the membrane after protein transfer. Each lane in panel B represents aortic sample from one individual animal. WT: wild type. \*P<0.05 and \*\*P<0.01 vs. WT.

**Figure 6: Regulation of eNOS activity by arginase:** (A) In cultured HUVECs, the eNOS inhibitor L-NAME (LN, 0.1 mmol/L) or thrombin (Thr, 1 U/ml, 24 hours) decreased eNOS activity as measured by L-citrulline production, which was not significantly reversed in the presence of arginase inhibitor L-norvaline (20 mmol/L, 24 hours, n=5). (B) In aortic rings pre-contracted with norepinephrine (0.3  $\mu$ mol/L), L-arginine (1 mmol/L) caused a more pronounced vasoconstriction in ApoE<sup>-/-</sup> mice than in wild-type (WT) animals. In the presence of arginase inhibitor L-norvaline the contractions were converted to a greater vascular relaxation in ApoE<sup>-/-</sup> mice than in WT animals (n=5). (C) eNOS expression was significantly higher in ApoE<sup>-/-</sup> mice than in the WT animals. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. control or WT.

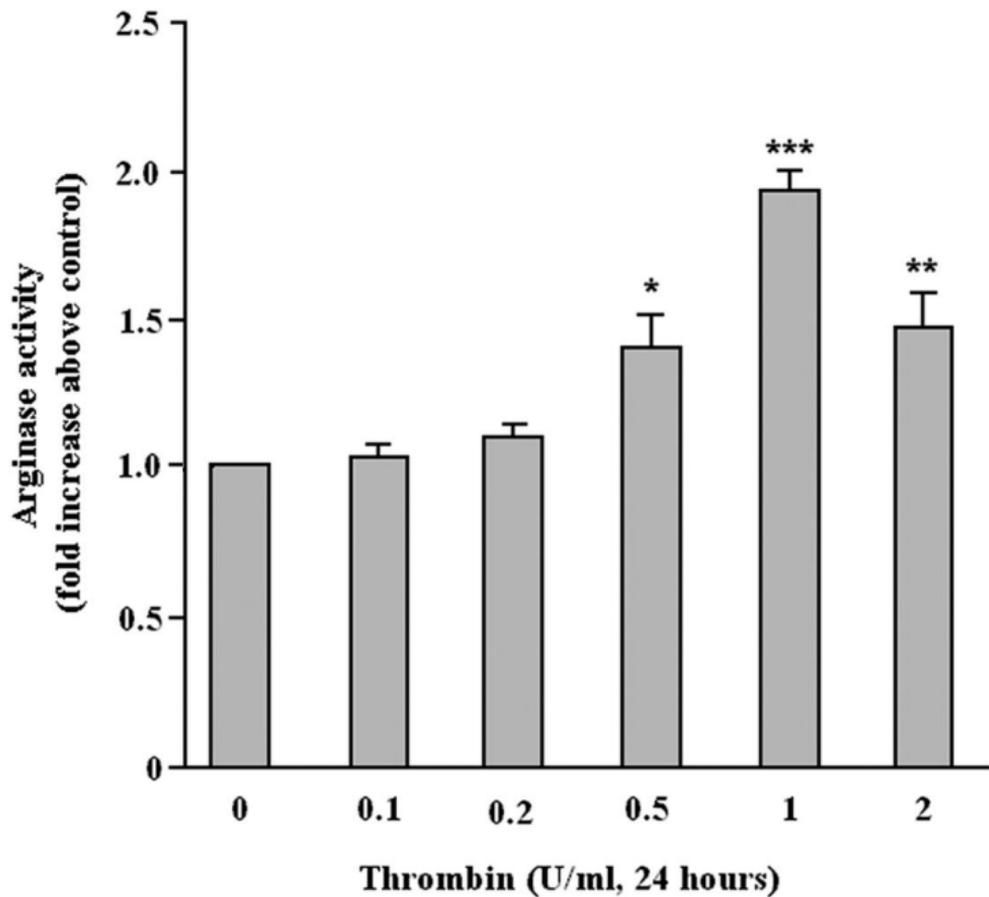


Fig. 1 Ming et al

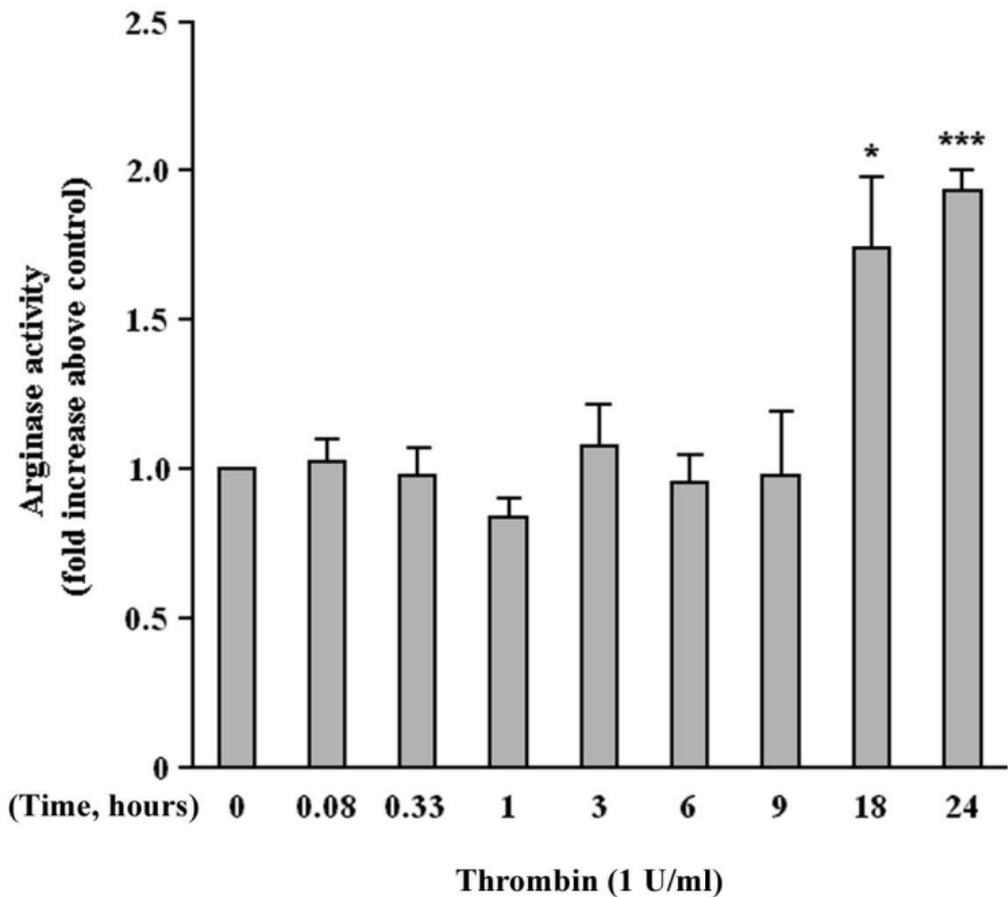


Fig. 2 Ming et al

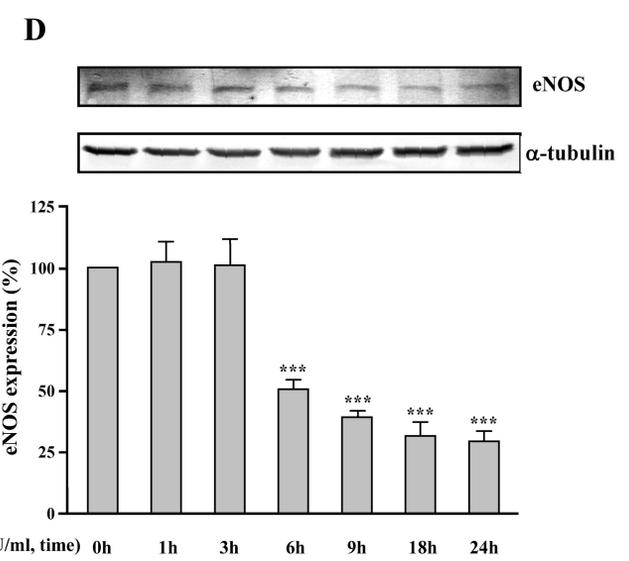
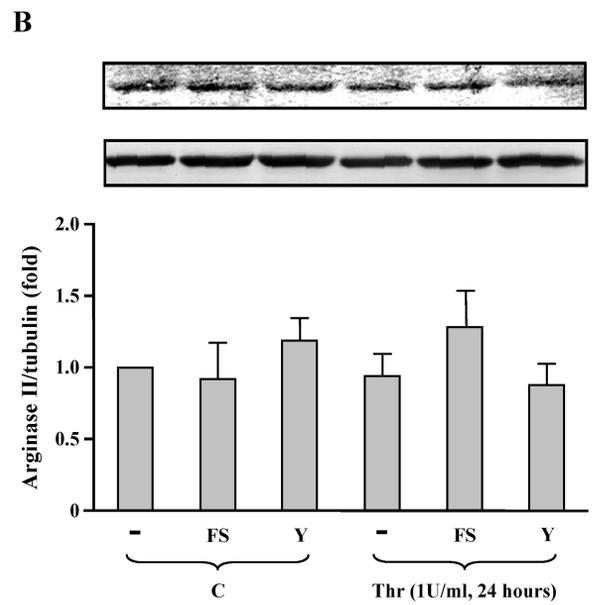
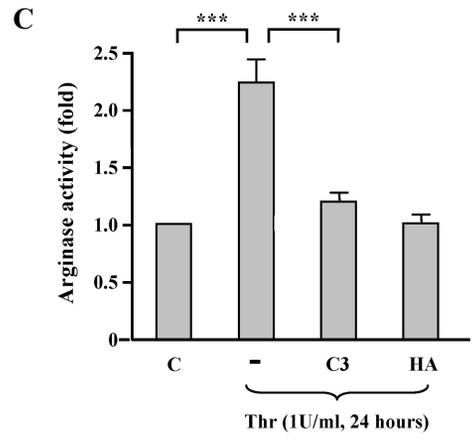
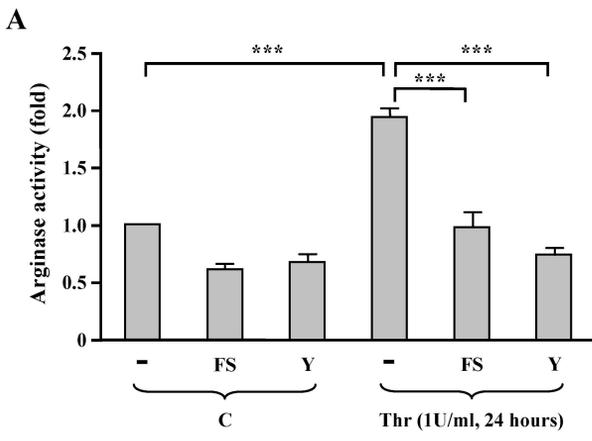


Fig. 3 Ming et al

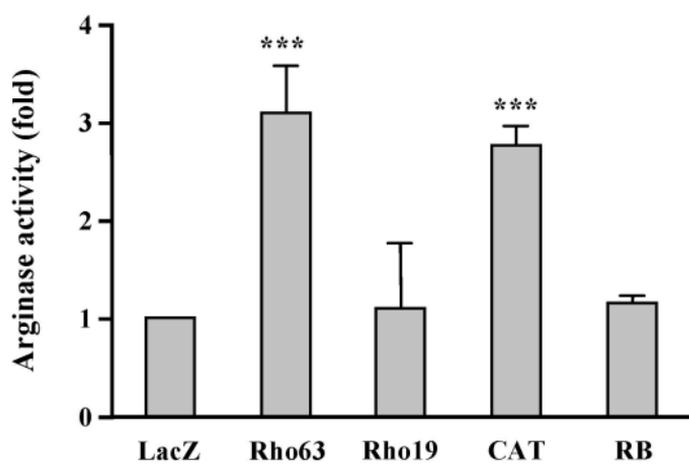
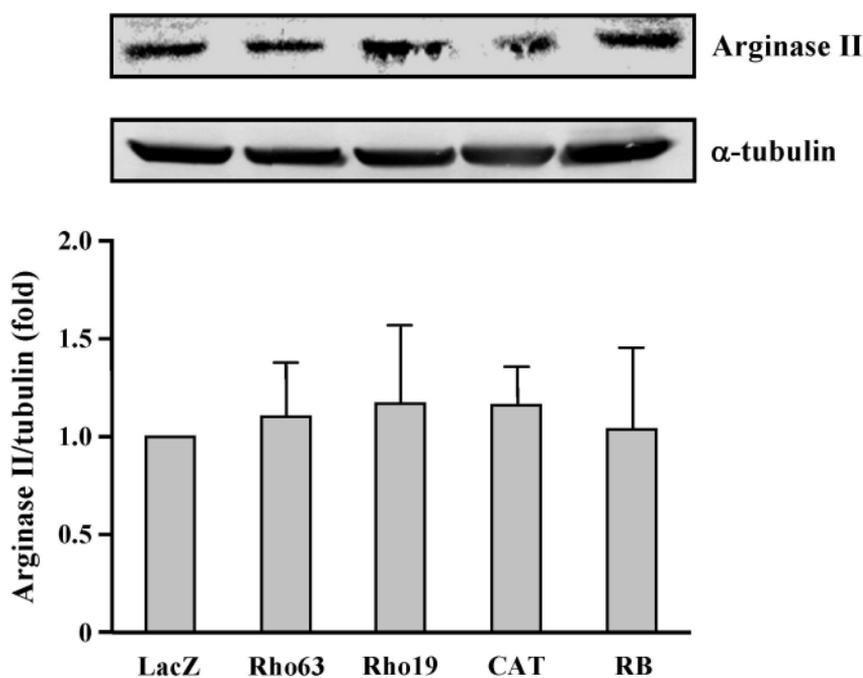
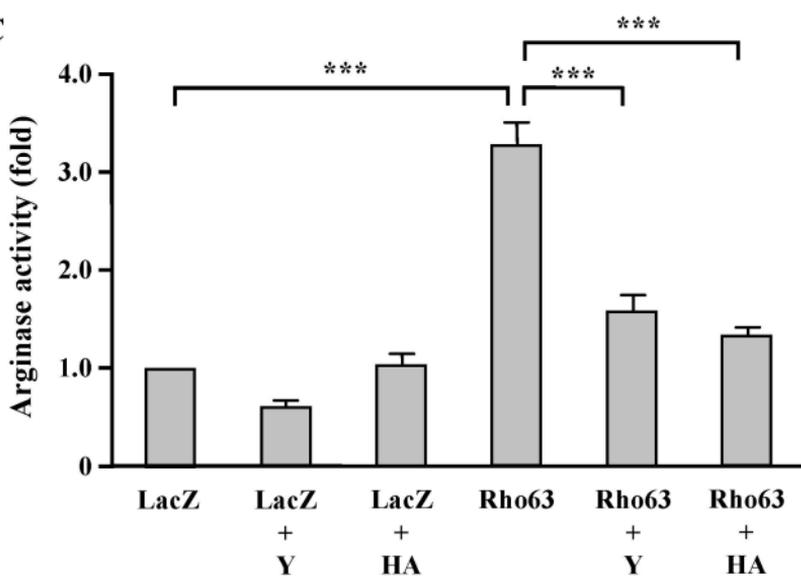
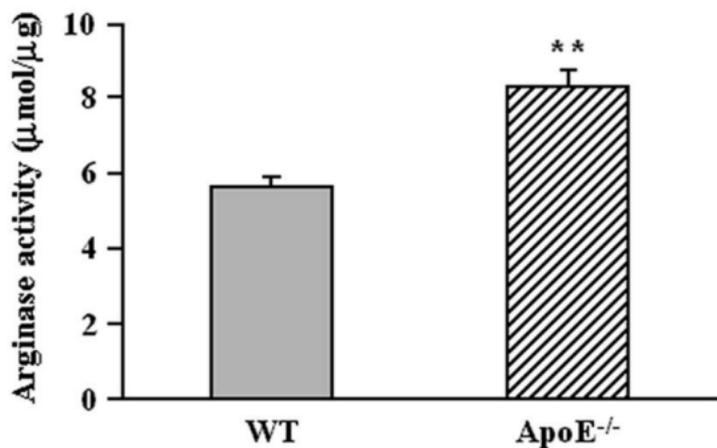
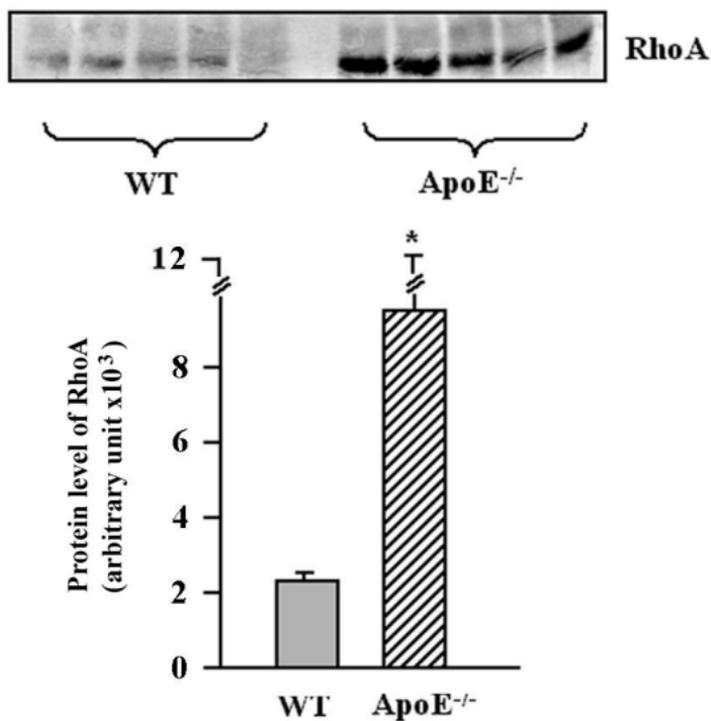
**A****B****C**

Fig. 4 Ming et al

**A****B****Fig. 5** Ming et al

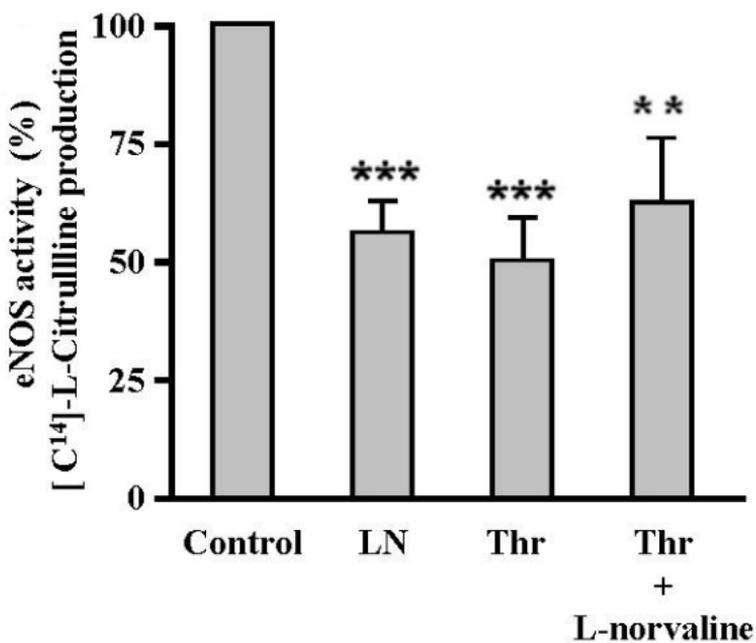
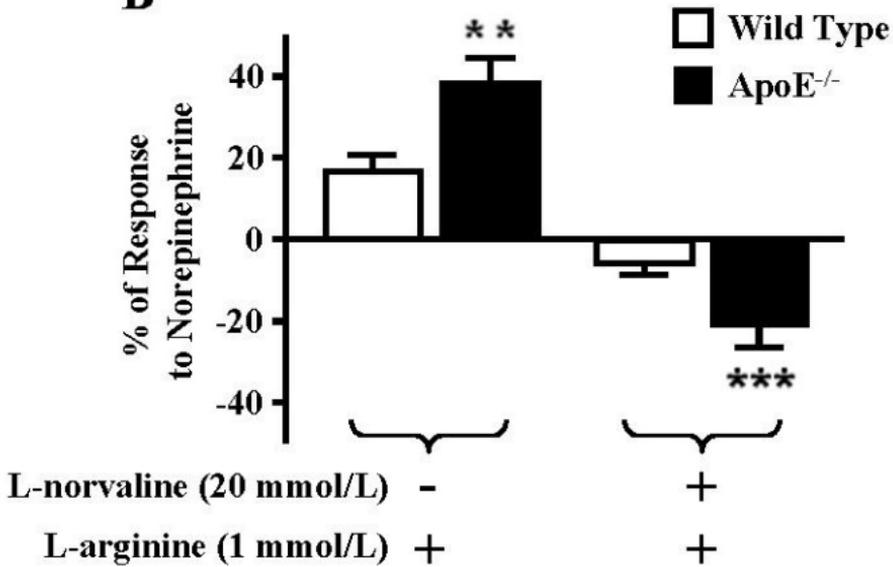
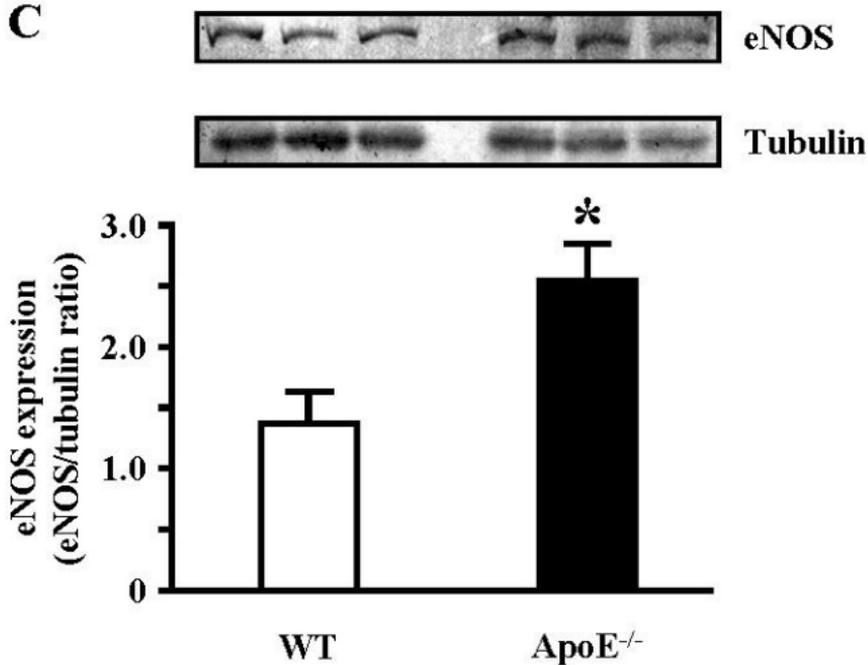
**A****B****C**

Figure 6, Ming et al