Mitochondrial Arginase II Modulates Nitric-Oxide Synthesis through Nonfreely Exchangeable L-Arginine Pools in Human Endothelial Cells

Gökce Topal, Annie Brunet, Laurence Walch, Jean-Luc Boucher, and Monique David-Dufilho

Centre National de la Recherche Scientifique, Université Pierre et Marie Curie-Paris 6, Unité Mixte de Recherche 7131, Paris, France (G.T., A.B., L.W., M.D.-D.); and Centre National de la Recherche Scientifique, (J.-L.B.)

Received February 27, 2006; accepted June 23, 2006

ABSTRACT

Reduced synthesis of nitric oxide (NO) contributes to the endothelial dysfunction and may be related to limited availability of L-arginine, the common substrate of constitutive nitric-oxide synthase (NOS) and cytosolic arginase I and mitochondrial arginase II. To determine whether arginases modulate the endothelial NO synthesis, we investigated the effects of the competitive arginase inhibitor N-hydroxy-nor-L-arginine (Nor-NOHA) on the activity of NOS, arginases, and L-arginine transporter and on NO release at surface of human umbilical vein endothelial cells (HUVECs). In unstimulated cells, Nor-NOHA dose-dependently reduced the arginase activity with maximal inhibition at 20 μM. When HUVECs were stimulated by thrombin without extracellular L-arginine, Nor-NOHA dose-dependently increased the NOS activity and the NO release with maximal effects at 20 μM. Extracellular L-arginine also dose-dependently increased NO release and arginase activity. When HUVECs were stimulated by thrombin in the presence of 100 μM L-arginine, NOS activity and NO release were similar in untreated and Nor-NOHA-treated cells. However, despite activation of L-arginine uptake, the inhibition of arginase activity by Nor-NOHA was still significant. The depletion of freely exchangeable L-arginine pools with extracellular L-lysine did not prevent Nor-NOHA from increasing the NO release. This indicates the presence of pools, which are accessible to NOS and arginase, but not exchangeable. Interestingly, the mitochondrial arginase II was constitutively expressed, whereas the cytosolic arginase I was barely detectable in HUVECs. These data suggest that endothelial NO synthesis depends on the activity of arginase II in mitochondria and L-arginine carriers in cell membrane.

L-Arginine (L-Arg) is the precursor of the messenger nitric oxide (NO) involved in cell communication and signal transduction (Hanafy et al., 2001). As shown in Fig. 1, L-Arg enters the cell through the cationic amino acid transporter (CAT) (Mann et al., 2003). Inside the cell, L-Arg is oxidized into L-citrulline and NO with formation of the intermediate N-hydroxy-L-Arg (NOHA) by constitutive NO synthase (NOS) of cell membrane, cytosolic inducible NOS (iNOS) (Stuehr et al., 1991; Knowles and Moncada, 1994), and, in various tissues, constitutive mitochondrial NOS (mtNOS) (Elfving et al., 2002). L-Arg is also converted into urea and ornithine by the cytosolic arginase I and the mitochondrial arginase II (Wu and Morris, 1998) (Fig. 1). Although K_m values of arginases are 100-fold higher than those of NOSs, the two enzymes compete for L-Arg because the maximal rate of arginases are more than 1000 times those of NOSs (Wu and Morris, 1998).

In the vascular system, the competition between NOSs and arginases has been studied in response to inflammatory cytokines, when the iNOS produces high NO output (Buga et al., 1996; Suschek et al., 2003). Under such conditions, the substantial amounts of NOHA produced by iNOS inhibit the arginase activity (Buga et al., 1996) (Fig. 1). The intermediate NOHA substrate indeed inhibits purified arginases with a K_i in the range of 40 to 150 μM (Boucher et al., 1994; Daghigh et al., 1994).

Although continuous NO synthesis by the constitutive endothelial NOS isoform (NOS3) is essential for the vascular function, little is known about the role of the arginase iso-
forms in L-Arg supply to the NOS3. In preeclampsia, the reduced L-Arg level of chorionic villi is associated with overexpression of the mitochondrial arginase II and no change in CAT-1 expression (Noris et al., 2004). In hypertension, the reduced availability of endothelial NO is related to overexpression of arginase II likewise (Xu et al., 2004). In fact, the dependence of NOS3 on L-Arg is highly complex. Despite intracellular L-Arg concentrations that are high enough to saturate the NOS3, extracellular L-Arg supply is required to maximally activated NOS3 (Hardy and May, 2002) (Fig. 1). This so-called phenomenon “L-Arg paradox” was explained by the colocalization of NOS3 and CAT-1 within the membrane microdomain caveolae (McDonald et al., 1997). Although confirming the colocalization of the two proteins, a recent study shows no direct relationship between endothelial NO release and CAT-1-mediated L-Arg transport (Li et al., 2005). We previously demonstrated, in human and guinea pig endothelial cells, that the agonists histamine, thrombin, ATP, and endothelin-1 and the inhibitor of intracellular Ca2⁺ pumps thapsigargin activate NO synthesis in the absence of extracellular L-Arg (Schussler et al., 1996; David-Dufilho et al., 2001). The NO-dependent relaxation of blood vessel rings, induced by various agonists, is also usually measured without a further incubation of 7 min at 72°C after the last cycle. Each sample (5 μl) was electrophoresed on polyacrylamide gels (4–20% w/v acrylamide). Samples of cDNA for arginase I (6 μl) and arginase II (3 μl) were amplified with the following primers: arginase I sense primer, 5'-CTTTGTTTCG GACTTGCTCGG-3'; arginase I antisense primer, 5'-CACCCTATGGTATGGGGGCTTA-3' (381 bp); arginase II sense primer, 5'-TCTATGACCACTTCTAAGC-3' and arginase II antisense primer, 5'-ACTTCTGACTTCCCCACTT-3' (643 bp) (Rouzau et al., 1999). The PCR reaction mixture (25 μl) contained 2 mM MgCl₂, 1 μM deoxy-NTP, 1 μM ribonucleic acid, and 2.5 μM random hexamers. Samples were electrophoresed on polyacrylamide gels (4–20% Tris/boric acid/EDTA; Novex, San Diego, CA) and stained for 15 min with ethidium bromide (2.5 μg/ml) for densitometric analysis with NIH Image software (Scion Corporation, Frederick, MD).
phyrinic NO-selective microsensor with a Biopulse potentiostat (Tacussel Radiometer, Villeurbanne, France) as described previously (Lantoine et al., 1995). NO sensor was calibrated at the beginning and at the end of measurements in the presence of cells, by the addition of NO standard solutions as detailed previously (Lantoine et al., 1995). Before stimulation, cells were incubated for 5 min at 37°C in PBS-MgCa with or without L-Arg to record the basal current. Treatment of HUVECs for 30 min with NOHA or Nor-NOHA or lysine did not change the profiles of NO calibration curves. The NO release was expressed as the maximum of thrombin-oxidation current.

**Arginase Activity.** Arginase activity in whole cells was measured as the formation of $[^3H]$Urea from $[^3H]$.L-Arg according to a previously described protocol with some modifications (Buga et al., 1996). To study the dose-dependent effect of NOHA and Nor-NOHA, HUVECs seeded onto six-well plates were incubated for 30 min at 37°C with various concentrations of NOHA or Nor-NOHA in PBS-MgCa containing 600 μM unlabeled L-Arg and 0.5 μCi/ml $[^3H]$.L-Arg. To investigate the dose-dependent effect of L-Arg, HUVECs were first treated for 30 min with vehicle or Nor-NOHA directly in culture medium. They were then washed and incubated for 20 min at 37°C in PBS-MgCa containing various concentrations of unlabeled L-Arg and 0.5 μCi/ml $[^3H]$.L-Arg and for a further 10 min without or with thrombin. For both protocols, the reaction was stopped by adding cold stop buffer consisting of 500 μM urea, 2 mM EDTA, and 500 mM acetic acid, pH 4.5, and cells were frozen at −20°C from 2 h to some days. After thawing, cells were scraped and sonicated, and homogenates were applied to Dowex 50WX8-400 cation exchange resin (H form) (Acros Organics France, Noisy Le Grand Cedex, France) pre-equilibrated with stop buffer. Cell homogenates were gently mixed with resin and centrifuged at 4°C for 5 min at 100g before counting of $[^3H]$Urea amounts in Ultima Gold liquid scintillation using a β counter (PerkinElmer Life and Analytical Sciences).

**NOS Activity.** NOS activity in whole cells was monitored by the conversion of $[^3H]$.L-Arg to $[^3H]$.L-citrulline according to a method previously described for cell homogenates (Lamas et al., 1991). To study the dose-dependent effect of NOHA and Nor-NOHA, HUVECs were treated for 30 min at 37°C with various concentrations of NOHA or Nor-NOHA in PBS-MgCa containing 600 μM unlabeled L-Arg and 1 μCi/ml $[^3H]$.L-Arg. The NOS activity was stimulated by incubating the cells for 10 min at 37°C in PBS-MgCa with thrombin. To examine the effect of extracellular L-Arg, cells were first treated for 30 min with vehicle, NOHA, or Nor-NOHA directly in culture medium. They were then washed and incubated for 20 min at 37°C in PBS-MgCa containing 100 μM unlabeled L-Arg and 0.5 μCi/ml $[^3H]$.L-Arg and for a further 10 min without or with thrombin. For both protocols, the reaction was stopped by adding cold stop buffer consisting of 2 mM L-citrulline, 5 mM EDTA, and 50 mM Na acetate, pH 7.4, and freezing. After thawing, scraping, and sonication of cells, homogenates were applied to Dowex 50WX8-400 (Na⁺ form) pre-equilibrated with NO stop buffer to separate L-citrulline from L-Arg. Cell homogenates were gently mixed with resin and centrifuged before counting $[^3H]$.L-citrulline as described above.

**L-Arg Uptake.** HUVECs seeded onto 12-well plates were first treated for 30 min with various concentrations of NOHA or Nor-NOHA. After washing, they were incubated for 20 min at 37°C with various concentrations of unlabeled L-Arg and 0.5 μCi/ml $[^3H]$.L-Arg in PBS-MgCa and for a further 10 min without or with thrombin. The stimulation was stopped by washing three times with ice-cold buffer (2 mM citrulline, 5 mM EDTA, and 50 mM Na acetate, pH 7.4) and freezing of cell monolayers. After thawing, homogenates were obtained by scraping and sonication of cells. Amount of $[^3H]$.L-Arg was counted as described above.

**Statistical Analysis.** Results are expressed as means ± S.E.M of n independent experiments performed with endothelial cells obtained from different donors. For arginase and NOS activity, each experiment was performed in duplicate. Multiple comparisons and dose-dependent effects were examined by one-way analysis of variance and post hoc Fisher’s test. Comparison of dose-response curves performed under two different experimental conditions was assessed by two-way analysis of variance. The kinetics parameters of NO synthesis and L-Arg uptake, $K_m$ and $V_{max}$ values, were calculated by fitting data to the Michaelis-Menten equation and assuming a single binding site in the range of concentrations studied.

**Results**

**Modulation of Arginase and NOS3 Activity by Nor-NOHA and NOHA.** Under our experimental conditions, NOHA had no significant effect on urea formation (Fig. 2, top), but its derivative Nor-NOHA dose-dependently inhibited the arginase activity ($p < 0.02$) with maximal effect from 20 μM (Fig. 2, bottom). In contrast, NOHA dose-dependently decreased the basal $[^3H]$.L-citrulline formation ($p < 0.02$), whereas Nor-NOHA had no effect (Fig. 3A). In thrombin-stimulated cells, both Nor-NOHA and NOHA dose-dependently increased the L-citrulline formation (Fig. 3B, $p = 0.038$ and 0.001, respectively). The dose-dependent effect of Nor-NOHA, however, was observed in a narrow range of concentrations, with an increase of 10% at 5 μM, of 35% at 10 to 20 μM, and no difference compared with untreated cells at 50 to 100 μM (Fig. 3B, left). The NOHA effects were maximal from 20 to 100 μM with an apparent EC₅₀ of 7 μM (Fig. 3B, right). When NO release at cell surface was taken as an indicator of NOS3 activity, the effects of NOHA were similar, whereas those of Nor-NOHA differed from the data obtained by detection of L-citrulline (Fig. 4). Both compounds dose-dependently increased the thrombin-activated NO release with maximal effects from 20 to 100 μM ($p = 0.001$ and 0.02 for NOHA and Nor-NOHA, respectively). From these experiments, we calculated an apparent $K_m$ of NOS3 for NOHA of 4 μM. As shown in Fig. 4 (bottom), extracellular L-Arg dose-dependently increased the thrombin-activated NO synthesis.
Interactions between Endothelial NO Synthase and Arginase

A: Basal

![Graph](Fig. 3. Dose-dependent effect of Nor-NOHA and NOHA on eNOS activity. A, basal [3H]L-citrulline formation in unstimulated HUVECs. B, thrombin-activated [3H]L-citrulline formation. Cells were incubated for 30 min at 37°C without (0) or with various concentrations of NOHA or Nor-NOHA in PBS-MgCa containing 600 μM unlabeled L-Arg and 1 μCi/ml [3H]-L-Arg (20 nM), washed, and stimulated with 2 U/ml thrombin for 10 min in the absence of L-Arg. Thrombin-induced [3H]L-citrulline formation was calculated by subtracting the basal values determined in the absence of thrombin. Results are from four independent experiments. *, p < 0.05; **, p < 0.01; and *** p < 0.001 compared with values of untreated cells.)

B: Thrombin

![Graph](Fig. 4. Dose-dependent effect of L-Arg, NOHA and Nor-NOHA on thrombin-activated NO release. Cells were untreated (0) or treated either for 30 min with NOHA or Nor-NOHA in culture medium or for 5 min with L-Arg in PBS-MgCa. They were then stimulated by 2 U/ml thrombin in PBS-MgCa without (NOHA, Nor-NOHA) or with L-Arg. Results are means of five to nine independent experiments. *, p < 0.05; **, p < 0.01; *** p < 0.001 compared with values from untreated cells.)

likewise, with an apparent $K_m$ of 6 μM and maximal effect from 50 μM (p = 0.008). Addition of 500 μM N-monomethyl-L-arginine to 20 μM Nor-NOHA or saturating concentrations of NO substrates inhibited the NO synthesis by 60 or 70%, respectively. The NO concentrations decreased from 28 ± 3 to 11 ± 3 nM in Nor-NOHA-treated cells (n = 5, p < 0.01), from 29 ± 3 to 8 ± 2 nM in NOHA-treated ones (n = 5, p < 0.001), and from 26 ± 3 to 8 ± 3 nM in the presence of L-Arg (n = 6, p < 0.01).

1-Arg Uptake and Arginase Activity. In both control and Nor-NOHA-treated cells, L-Arg uptake increased in a dose-dependent and saturable manner with increasing extracellular L-Arg concentrations (p < 0.001) but showed small nonsaturable components (Fig. 5A). In the range from 10 to 100 μM, the amounts of incorporated [3H]-L-Arg were higher in thrombin-stimulated cells (black circles) than in unstimulated ones (white circles; p = 0.03 for control and p = 0.008 for Nor-NOHA). As shown in Fig. 5B, the kinetic parameters calculated from the Lineweaver-Burk graphical representations did not significantly differ in unstimulated control ($K_m$ = 134 ± 35 μM and $V_{max}$ = 1.84 ± 0.23 nmol/mg protein/min) and Nor-NOHA-treated cells (172 ± 20 μM and 1.75 ± 0.08 nmol/mg protein/min). In addition, thrombin reduced the $K_m$ values in both control (91 ± 22 μM) and Nor-NOHA-treated cells (102 ± 14 μM) with no significant changes in $V_{max}$. In control cells, the thrombin-activated L-Arg uptake was associated with saturation of arginase activity from 100 μM L-Arg (Fig. 6, black circles). In the absence of thrombin, the basal arginase activity was directly proportional to the extracellular L-Arg concentrations (Fig. 6, white circles). In Nor-NOHA-treated cells, the basal arginase activity was more markedly inhibited in the absence of L-Arg (80%) than in its presence (40%), with constant inhibition from 50 to 200 μM (Fig. 7A). Extracellular L-Arg indeed dose-dependently reduced the Nor-NOHA-induced inhibition of arginase (Fig. 7B; p = 0.004), when thrombin increased its uptake. The decrease in urea formation was still significant at 100 μM (p = 0.005), but there was no longer any inhibition at 200 μM. Furthermore, the effects of 20 μM Nor-NOHA and 100 μM L-Arg on thrombin-stimulated NOS3 activity were not cumulative: 14 ± 6 versus 12 ± 3 pmol/mg/min [3H]-L-citrulline (n = 4) and 30 ± 3 versus 34 ± 3 nM NO (n = 8) in the absence and presence of Nor-NOHA, respectively.

NO Synthesis, L-Arg Pools, and Arginases. L-Arg competes with L-lysine to enter the endothelial cell and it is exchanged with L-glutamine to go out (Broer et al., 2000; Hardy and May, 2002). When HUVECs were incubated with high L-lysine concentrations to deplete the freely exchangeable L-Arg pools, the thrombin-activated NO release was decreased by 45% in control and by 53% in the presence of extracellular L-Arg, thereby suppressing its activator effect (Fig. 8A). When cells were incubated with L-lysine together with Nor-NOHA, the activator effect of the arginase inhibitor was maintained and the NO release was reduced by 28% only. Depleting the cells of glutamine did not affect the thrombin-activated NO release from untreated and Nor-
NOHA-treated cells (Fig. 8B). However, it abolished the effects of extracellular L-Arg and L-lysine.

**Arginase Expression.** The arginase II mRNA was constitutively expressed in unstimulated HUVECs (Fig. 9, top blot). For arginase I, a twice higher amount of reverse transcriptase samples and more amplification cycles were required to detect some traces of amplified PCR products (Fig. 9, bottom blot). Such a result was confirmed by immunoblotting with specific antibodies of each isoform. HUVECs expressed trace detectable level of arginase I and significant amount of arginase II protein (640 ± 97 arbitrary units, n = 3).

**Discussion**

The new finding of our study is that NOS3 competes with the mitochondrial arginase II for getting supply of L-Arg into a specific internal pool. Our results show that inhibition of the mitochondrial arginase isofrom allows L-Arg to accumulate inside a pool that is accessible to the NOS3 but not exchangeable with the extracellular amino acids, suggesting a role for mitochondria in endothelial NO synthesis.
The present study confirms the existence of two distinct L-Arg pools in HUVECs. The delivery of extracellular substrate to the NOS3 through a specific compartment is indicated by the fact that extracellular L-Arg increases the NO synthesis despite cellular saturating concentrations for the enzyme (Hardy and May, 2002). In our study, extracellular L-Arg and NOHA dose-dependently increased the thrombin-activated NO release at cell surface with $K_{m}$ values in the range of those previously reported (Stuehr et al., 1991; Hardy and May, 2002). The demonstration of a nonfreely exchangeable pool has been shown by significant residual L-Arg when endothelial cells were depleted of this amino acid in exchange with extracellular L-lysine (Closs et al., 2000). Under such conditions, we observed significant residual NO release and Nor-NOHA-activated NO synthesis. Nor-NOHA induced NO synthesis from 5 to 20 $\mu$M only, but these values are in the range of the IC_{50} found in macrophages (Tenu et al., 1999). At upper concentrations, Nor-NOHA mediated NO formation by a NOS-independent mechanism as has been previously observed for both NOHA and Nor-NOHA in rat aorta (Vetrovsky et al., 2002; Beranova et al., 2005). At 20 $\mu$M, the Nor-NOHA-induced increase in NO activity may rationally result from increased L-Arg availability in the nonexchangeable pool because this NOHA derivative is not a NOS3 substrate (Mansuy and Boucher, 2004). Our observation of noncumulative effects of Nor-NOHA and L-Arg on thrombin-activated NO suggests balance between the two internal L-Arg pools to ensure maximal NO synthesis.

In cells from the human endothelial cell line EA.hy926, the L-Arg pool that is not subject to exchange with extracellular L-lysine appears to get a stock of L-Arg from citrulline recycling (Simon et al., 2003). We demonstrate here the presence in HUVECs of a L-Arg pool that is accessible to both NOS3 and arginase and not exchangeable with extracellular L-lysine. In fact, a highly active system of ornithine-citrulline exchange is present in the mitochondrial membrane. Two isoforms of the mitochondrial ornithine carrier are expressed in a wide range of human tissues and transport L-ornithine, L-arginine, and L-citrulline by exchange (Fiermonte et al., 2003). When the arginase activity is negligible, this carrier exports the ornithine from the mitochondria for polyamine biosynthesis in the cytosol. In isolated mitochondria, 90% of the mitochondria-bound arginase activity is located on the outer membrane and hydrolyzes the cytosolic L-Arg into urea and ornithine, which is converted into citrulline inside the matrix (Nissim et al., 2005). In the current study, the arginase activity and the Nor-NOHA inhibitory power depended on the L-Arg uptake. In addition, the arginase activity was associated with significant constitutive expression of both mRNA and protein of only arginase II, demonstrating the participation of mitochondria in intracellular L-Arg compartmentalization. In agreement with two previous studies performed in HUVECs and human pulmonary artery endothelial cells (Bachetti et al., 2004; Xu et al., 2004), we observed barely detectable expression of the cytosolic arginase I. This suggests a role for the mitochondria in regulation of endothelial NO synthesis. It is noteworthy that the localization of NOS3 on the cytoplasmic face of the outer mitochondrial membrane has been demonstrated in HUVECS (Gao et al., 2004). Inhibition of the arginase II may lead to L-Arg accumulation into mitochondria by exchange with ornithine through specific carrier and therefore to increased activity of the endothelial mtNOS during cell stimulation.

In EA.hy926 cells, the other L-Arg pool is freely exchangeable with extracellular amino acids through the CATs (Closs et al., 2000). We measured in HUVECs a $K_{m}$ of L-Arg uptake, which was 20-fold higher than that of NOS3 for extracellular L-Arg but in the range of that reported for CAT-1 (Mann et al., 2003). This indicates that L-Arg transport through CAT-1 cannot be limiting for maximal NOS3 activity. Despite direct interactions of NOS3 with CAT-1, NO release appeared to be independent of CAT-1-mediated L-Arg transport into bovine aortic endothelial cells (Li et al., 2005). Nonetheless, in vivo experiments demonstrate the requirement of extracellular L-Arg transport through CAT-1 for normal vasodilatory responses of rat small intestinal arterioles to stimuli (Zani and Bohlen, 2005). HUVECs express the plasma membrane transporters for CAT-1 and y^+LAT (Sala et al., 2002; Mann et al., 2003). The latter system is responsible for L-Arg efflux in exchange with glutamine in polarized cells and is also transporter for L-lysine that competes with L-Arg (Broer et al., 2000). Interestingly, the $K_{m}$ of y^+LAT for L-Arg is in the range of that of NOS3 (Mann et al., 2003). In our study, the participation of y^+LAT in substrate supply to the NOS3 is suggested by the suppression in glutamine-depleted cells of L-Arg and L-lysine effects on NO release. In unstimulated endothelial cells, NOS3 is colocalized with CAT-1 in the plasma membrane microdomain caveolae (McDonald et al., 1997; Shaul, 2002; Li et al., 2005). In response to stimuli, the activated CAT-1 may transport L-Arg inside the cell and the y^+LAT transporter would specifically deliver the extracellular substrate to the NOS3 located in the caveolae membrane.

In conclusion, our results show that endothelial NO synthesis depends on the activity of cell membrane L-Arg carriers and mitochondrial arginase II through two types of L-Arg pools. We suggest that the freely exchangeable pools are caveolae, whereas the nonexchangeable ones are mitochondria. The question arises whether different NOS isoforms are responsible for NO synthesis from the two types of pools. It is now well established that NOS3 is located within membrane caveolae (Shaul, 2002). The existence of a mitochondrial NOS has been demonstrated in various tissues, including liver and heart, where it is catalytically active (Elfering et al., 2002). In vascular endothelial cells, NO facilitates the production of mitochondrial reactive oxygen species for cell signaling and to prevent accumulation of the hypoxia-inducible factor-1 (Quintero et al., 2006). Thus, endothelial mitochondria may act as signaling organelles under the control of NO. In response to various agonists, endothelium may synthesize NO through the NOS3 to regulate cell communication at the vessel wall and through the mitochondrial NOS to regulate the $O_{2}$ consumption.
Acknowledgments

We thank L. Joutel, G. Cohen, and colleagues from maternities of Notre Dame de Bon Secours Hospital and Institut Mutualiste Montsouris for collecting umbilical cords.

References


Address correspondence to: Monique David-Dufilho, Unité Mixte de Recherche 7131, Centre National de la Recherche Scientifique-UPMC, Groupe Hospitalier HEGP-Broussais, 102 rue Didot, 75014 Paris, France. E-mail: Monique.dufilho@brs.aphp.fr

Downloaded from jpet.aspetjournals.org on April 13, 2017