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

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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^ω-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 NO 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^ω-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-

ABBREVIATIONS: L-Arg, L-arginine; NO, nitric oxide; CAT, cationic amino acid transporter; NOHA, N^ω-hydroxy-L-arginine; NOS, NO synthase; iNOS, inducible NOS; mtNOS, mitochondrial NOS; NOS3, endothelial NO synthase; HUVEC, human umbilical vein endothelial cell; Nor-NOHA, N^ω-hydroxy-nor-L-arginine; PBS, phosphate-buffered saline.
NO synthase (NOS3), we investigated, in HUVECs, the effects of the NOHA derivative thapsigargin on NO synthesis in the absence of extracellular L-Arg. Reduced availability of endothelial NO is related to overexpression of the constitutive NOS of cell membrane, the iNOS, and the mtNOS. L-Arg is also converted into ornithine and urea by the cytosolic arginase I and the mitochondrial arginase II. As indicated by nonarrows (T), the two arginase isoforms are inhibited by high concentration of NOHA (100 μM) and by Nor-NOHA in the range of 10 μM.

In preeclampsia, the reduced L-Arg level of chorionic villi is associated with overexpression of NO synthase 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 (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 activate NOS3 (Hardy and May, 2002) (Fig. 1). This so-called phenomenon “L-Arg paradox” was explained by the colocalization of NOHA 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-Duflilho et al., 2003). The NO-dependent relaxation of blood vessels rings, induced by various agonists, is also usually measured without L-Arg in the bathing medium. In addition, different arginase inhibitors induce endothelium-dependent relaxation (Berkowitz et al., 2003). These data show altogether the existence of functional L-Arg pools, which are independent of the stimulus. Indeed, two distinct pools are accessible to NOS3: one is freely exchangeable, and the other one is not (Simon et al., 2003). Our previous observation that histamine- and thrombin-activated NO release were similarly increased by extracellular L-Arg indicates constant distribution of exchangeable L-Arg pools in human umbilical vein endothelial cells (HUVECs) (David-Duflilho et al., 2001).

To determine whether the arginases contribute to regulate the L-Arg accumulation within a pool that is accessible to NOS3, we investigated, in HUVECs, the effects of the NOHA derivative Nω-hydroxy-nor-L-Arg (Nor-NOHA), which is 40 times more potent than NOHA to inhibit the arginase (Tenu et al., 1999). In addition, we characterized which arginase isoform is constitutively expressed in HUVECs because Nor-NOHA does not display selectivity toward the cytosolic and mitochondrial isoforms (Custot et al., 1997).

**Materials and Methods**

**Materials.** The L-Arg derivatives NOHA and Nor-NOHA were synthesized as described previously (Moali et al., 2000). Primers and TRizol were purchased by Invitrogen (Carlsbad, CA). 2',7'-Dichlorodihydrofluorescein diacetate was from Molecular Probes (Eugene, OR). [2,3,4-3H]L-Arg ([3H]L-Arg) (41 Ci/mmol) was purchased by PerkinElmer Life and Analytical Sciences (Boston, MA).

**Cell Culture.** Endothelial cells were isolated from segments of human umbilical cord vein as described previously (Lantoine et al., 1998). They were cultured in medium 199 (40%) and RPMI 1640 (40%) containing 2.4 mM glutamine and 620 μM L-arginine and supplemented with 20% fetal calf serum as described previously (Lantoine et al., 1998). The medium was renewed every 2 days until confluence (4–5 days). HUVECs were detached by incubation for 1 to 2 min at room temperature with 0.01% trypsin and 0.004% EDTA, washed, and cultured until confluence. The culture medium was renewed 24 h before the experiments. For some experiments, cells were left in glutaamine-free medium. For the experiments, cells were washed twice and incubated with phosphate buffer, pH 7.4, containing 5 mM glucose, 0.5 mM MgCl₂, and 1 mM CaCl₂ (PBS-MgCa).

**RT-PCR.** Total RNA was extracted from HUVECs grown onto 60-mm plastic dishes using the TRizol reagent according to the manufacturer’s instructions. The cDNA was synthesized from 1 μg of total RNA by incubation for 15 min at 42°C with 2.5 U/μl murine leukemia virus reverse transcriptase (Promega, Madison, WI). The cDNA was amplified by PCR using specific primers for arginase I sense primer, 5′-CTGTTTTCG GACCTGGTCG-3′; arginase I antisense primer, 5′-CAGTCTATGGATGGGGGCTTA-3′ (381 bp); arginase II sense primer, 5′-CTATGACCAATCTCTCAGT-3′; arginase II antisense primer, 5′-CTTTGACTACACTCCACCTT-3′ (643 bp) (Rouzaud et al., 1999). The PCR reaction mixture (25 μl) contained 2 mM MgCl₂, PCR buffer II, and AmpliTag DNA polymerase (PE Applied Biosystems) at 25 mU/μl and each primer at 0.2 μM. Amplification was performed in a programmable thermal controller (model PTC-100; MJ Research, Inc., Watertown, MA). Samples of cDNA for arginase I (6 μl) and arginase II (3 μl) were amplified with the following primers: arginase I sense primer, 5′-GAGTTGCTGACTGGCTC-3′; arginase I antisense primer, 5′-CAGTCTATGGATGGGGGCTTA-3′; arginase II sense primer, 5′-CTATGACCAATCTCTCAGT-3′; arginase II antisense primer, 5′-CTTTGACTACACTCCACCTT-3′.

**Western Blot Analysis.** Proteins of cell homogenates were resolved by SDS-polyacrylamide gel electrophoresis. HUVECs grown onto 60-mm plastic culture dishes were lysed in an ice-cold buffer containing: 150 mM NaCl, 50 mM Tris, 1 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM aprotinin, and 1% Nonidet P-40. The lysates were cleared by centrifugation for 15 min at 12,000g at 4°C, and proteins were resolved by electrophoresis on 8% SDS-polyacrylamide gels. The proteins were transferred electrophoretically onto nitrocellulose membranes. Nonspecific binding sites were blocked by 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 before overnight incubation of membranes at 4°C with primary antibodies against arginase I or II (1:1000 dilution), rinsed, and further incubated for 90 min at room temperature with horseradish peroxidase-linked anti-rabbit IgG secondary antibody. The immunoreactive bands were detected by an enhanced chemiluminescence system (Super Signal West Pico; Pierce Chemical, Rockford, IL) and quantified by densitometry using Image J 1.33 software.

**Measurements of Nitric Oxide.** The NO released at the cell surface was measured by differential pulse amperometry at a por-
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-activated oxidation current.

Arginase Activity. Arginase activity in whole cells was measured as the conversion of [3H]L-Arg to [3H]L-citrulline 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]-Arg to [3H]-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 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 2 mM L-citrulline, 5 mM EDTA, and 50 mM Na acetate, pH 7.4, and freezing. After thawing, cells were scraped and sonicated, and homogenates were applied to Dowex 50WX8–400 cation exchange resin (H form) 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]-Arg to [3H]-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, cells were scraped and sonicated, and homogenates were applied to Dowex 50WX8–400 (Na⁺ form) pre-equilibrated with NOS 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.

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, Kₘ and Vₘₐₓ, 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 Kₘ of NOS3 for NOHA of 4 μM. As shown in Fig. 4 (bottom), extracellular L-Arg dose-dependently increased the thrombin-activated NO synthesis...
likewise, with an apparent $K_m$ of 6 $\mu$M and maximal effect from 50 $\mu$M ($p = 0.008$). Addition of 500 $\mu$M $N$-monomethyl-$L$-arginine to 20 $\mu$M 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 $\mu$M, the amounts of incorporated $[\text{H}]$-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 ($K_m = 134 ± 35 \mu$M and $V_{max} = 1.84 ± 0.23 \text{nmol/mg protein/min}$) and Nor-NOHA-treated cells ($172 ± 20 \mu$M and $1.75 ± 0.08 \text{nmol/mg protein/min}$). In addition, thrombin reduced the $K_m$ values in both control (91 ± 22 $\mu$M) and Nor-NOHA-treated cells (102 ± 14 $\mu$M) 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 $\mu$M 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 $\mu$M (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 $\mu$M ($p = 0.005$), but there was no longer any inhibition at 200 $\mu$M. Furthermore, the effects of 20 $\mu$M Nor-NOHA and 100 $\mu$M L-Arg on thrombin-stimulated NOS3 activity were not cumulative: 14 ± 6 versus 12 ± 3 pmol/mg/min $[\text{H}]$-citrul- line ($n = 4$) 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 by 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 NOS activation 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 NOS activity may rationally result from increased L-Arg availability in the nonexchangeable pool because this NOHA derivative is not a 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 reported for CAT-1 (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.

**Fig. 9.** RT-PCR analysis for human arginase I and II mRNAs. Reverse transcriptase samples of arginase I (6 $\mu$L) were amplified with 40 cycles and those of arginase II (3 $\mu$L) with 35 cycles of amplification. Amplified PCR products were detected at 381- and 643-bp bands for arginase I and II, respectively. Data are from four independent cultures.

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 reported for CAT-1 (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.
Acknowledgments
We thank L. Jouret, G. Cohen, and colleagues from maternities of Notre Dame de Bon Secours Hospital and Institut Mutualiste Montsouris for collecting umbilical cords.

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