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Mitochondrial arginase II modulates nitric oxide synthesis through non-freely exchangeable L-arginine pools in human endothelial cells

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Running title: Interactions between endothelial NO synthase and arginase

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Abbreviations: L-Arginine, L-Arg; DCF, dichlorofluorescein; H_2DCF, 2',7'-dichlorodihydrofluorescein; HUVEC, human umbilical vein endothelial cell; L-NMMA, *N*-monomethyl-L-Arginine; NO, Nitric oxide; NOS3, endothelial NO synthase; NOHA, N^{ω}-hydroxy-L-Arginine**;** Nor-NOHA, N^{ω}-hydroxy-nor-L-Arginine.

Cardiovascular section

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 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.

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Introduction

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^{w}-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) (Elfering 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). Though Km values of arginases are 100-fold higher than those of NOSs, the two enzymes competes 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 NOS substrate indeed inhibits purified arginases with a Ki in the range of 40-150 µM (Boucher et al., 1994; Daghigh et al., 1994).

Though 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 isoforms 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

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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 co-localization of NOS3 and CAT-1 within the membrane microdomains caveolae (McDonald et al., 1997). While confirming the co-localization 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 Ca^{2+} pumps thapsigargin activate NO synthesis in the absence of extracellular L-Arg (Schussler et al., 1996; David-Dufilho et al., 2003). The NO-dependent relaxation of blood vessel 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 internal 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-Dufilho 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 N^{ω}-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).

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METHODS

Materials

The L-Arg derivatives NOHA and Nor-NOHA were synthesized as previously described (Moali et al., 2000). Primers and TRIzol® were purchased by Invitrogen (Carlsbad, CA). 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) was from Molecular probes (Eugene, OR). [2,3,4-^{3}H]-L-Arg ([^{3}H]L-arg) (41Ci/mmol) was purchased by Perkin Elmer Life Science (Boston, MA).

Cell culture

Endothelial cells were isolated from segments of human umbilical cord vein as previously described (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 previously described (Lantoine et al., 1998). The medium was renewed every 2 days until confluence (4-5 days). HUVECs were detached by incubation for 1-2 min at room-temperature with 0.01% trypsin and 0.004% EDTA, washed and cultured until confluence. The culture medium was renewed twenty four hours before the experiments. For some experiments, cells were depleted in glutamine by incubation for 2 h at 37°C in glutamine-free medium. For the experiments, cells were washed twice and incubated with phosphate buffer, pH 7.4, containing 5mM glucose, 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$ (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

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virus reverse transcriptase (PE Applied Biosystems, Foster City, CA) in 20 µl of PCR buffer II containing 5 mM $MgCl_2$, 1 mM of deoxy-NTP, 1 U/µl ribonuclease inhibitor, and 2.5 mM random hexamers. Samples of cDNA for arginase I (6 µl) and arginase II (3 µl) were amplified with the following primers: arginase I sense primer, 5'-CTTGTTTCG GACTTGCTCGG-3'; arginase I antisense primer, 5'-CACTCTATGTATGGGGGCTTA-3' (381-bp) and arginase II sense primer, 5'-TCTATGACCAACTTCCTACTC-3'; arginase II antisense primer, 5'-CTTCTGACTACTCCCCACTT-3' (643-bp) (Rouzaut et al., 1999). The PCR reaction mixture (25 µl) contained 2 mM $MgCl_2$, PCR buffer II, AmpliTaq 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). Sample denaturation at 95°C for 2 min was followed by 40 PCR cycles for arginase I and 35 cycles arginase II of 30s at 95°C, 30s at 60°C and 90s at 72°C and a further incubation of 7 min at 72°C after the last cycle. Each sample (5 µl) was electrophoresed on polyacrylamide gels (4–20% Tris/boric acid/EDTA, Novex, San Diego, CA) and stained for 15 min with ethidium bromide (2.5µg/mL) for densitometric analysis with NIH Image software (Scion Image).

Western blot analysis

Proteins of cell homogenates were resolved by SDS-PAGE. HUVECs grown onto 60 mm plastic culture dishes were lysed in an ice-cold buffer containing: in mM, NaCl 150, Tris 50, EDTA 1, NaF 50, sodium orthovanadate 1, phenylmethylsulfonyl fluoride 1, aprotinin 10µg/ml, leupeptin 10 µg/ml, pepsatin 10 µg/ml and 1% Nonidet P-40. The lysates were cleared by centrifugation for 15 min at 12000g at 4°C and proteins resolved by electrophoresis on 8% SDS-polyacrylamide gels. The proteins were transferred electrophoretically onto nitrocellulose membranes. Non specific binding sites were blocked

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by 5% non-fat 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) 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 porphyrinic NO-selective microsensor with a Biopulse potentiostat (Tacussel-Radiometer) as previously described (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 either 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-induced oxidation current.

Arginase activity

Arginase activity in whole cells was measured as the formation of [^{3}H]Urea from [^{3}H]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 6 well plates were incubated for 30 min at 37°C with various concentrations of NOHA or Nor-NOHA in PBS-MgCa containing 600 µM unlabelled L-Arg and 0.5 µCi/ml [^{3}H]L-Arg. To investigate the dose-dependent effect of L-Arg, HUVECs were first treated

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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 unlabelled L-Arg and 0.5 µCi/ml [^{3}H]L-Arg and for 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, 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) pre-equilibrated with stop buffer. Cell homogenates were gently mixed with resin and centrifuged at 4°C for 5 min at 100 g before counting of [^{3}H]Urea amounts in Ultima Gold liquid scintillation using a ß counter (Packard Bioscience).

NOS activity

NOS activity in whole cells was monitored by the conversion of [^{3}H]L-Arg to [^{3}H]L-citrulline according to a method previously described for cell homogenates (Lamas et al., 1991). To study the dose-dependent effect of nor-NOHA and NOHA, HUVECs were treated for 30 min at 37°C with various concentrations of NOHA or Nor-NOHA in PBS-MgCa containing 600 µM unlabelled L-Arg and 1 µCi/ml [^{3}H]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 unlabelled L-Arg and 0.5 µCi/ml [^{3}H]L-Arg and for 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, 5mM 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 NOS stop buffer to separate L-citrulline from L-Arg. Cell homogenates were gently mixed with resin and centrifuged before counting [^{3}H]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 unlabelled L-Arg and 0.5 µCi/ml [^{3}H]L-Arg in PBS-MgCa and for further 10 minutes without or with thrombin. The stimulation was stopped by washing 3-times with ice-cold buffer (2mM citrulline, 5mM EDTA and 50mM Na acetate, pH 7.4) and freezing of cell monolayers. After thawing, homogenates were obtained by scraping and sonication of cells. Amount of [^{3}H]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.

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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, upper panel), but its derivative Nor-NOHA dose-dependently inhibited the arginase activity (p=0.02) with maximal effect from 20 µM (Fig. 2, lower panel). In contrast, NOHA dose-dependently decreased the basal [^{3}H]L-citrulline formation (p=0.02) while 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 p=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 10-20 µM, and no difference compared to untreated cells at 50-100 µM (Fig. 3B, left panel). The NOHA effects were maximal from 20 to 100 µM with an apparent EC_{50} of 7 µM (Fig. 3B, right panel). When NO release at cell surface was taken as an indicator of NOS3 activity, the effects of NOHA were similar while 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 p=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 (lower panel), extracellular L-Arg dose-dependently increased the thrombin-activated NO synthesis likewise, with an apparent Km of 6 µM and maximal effect from 50 µM (p=0.008). Addition of 500µM L-NMMA to 20 µM Nor-NOHA or saturating concentrations of NOS 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).

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L-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 non saturable components (Figure 5A). In the range from 10 to 100 µM, the amounts of incorporated [^{3}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 (Km=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 Km 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 vs 12±3 pmol/mg/min of [^{3}H]L-citrulline (n=4) and 30±3 vs 34±3 nM of 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). It, however, abolished the effects of extracellular L-Arg and L-lysine.

Arginase expression. The arginase II mRNA was constitutively expressed in unstimulated HUVECs (Fig. 9, upper 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, lower 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 a.u, n=3).

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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 isoform 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 Km values in the range of those previously reported (Stuehr et al., 1991; Hardy and May, 2002). The demonstration of a non-freely 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 µM 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 it has been previously observed for both NOHA and Nor-NOHA in rat aorta (Vetrovsky et al., 2002; Beranova et al., 2005). At 20 µM, the Nor-NOHA-induced increase in NOS activity may rationally result from increased L-Arg availability in the non-exchangeable pool, because this NOHA derivative is not a NOS3 substrate (Mansuy and Boucher, 2004). Our observation of non-cumulative effects of Nor-NOHA and L-Arg on thrombin-activated NOS 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 hydrolyses 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. Noteworthy, 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.

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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 Km 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 polarised cells and is also transporter for L-lysine that competes with L-Arg (Broer et al., 2000). Interestingly, the Km 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 co-localised with CAT-1 in the plasma membrane microdomains 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 non-exchangeable 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 signalling and to prevent accumulation of the hypoxia-inducible factor-1 (Quintero et al., 2006). Thus, endothelial mitochondria may act as signalling 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.

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REFERENCES

Bachetti T, Comini L, Francolini G, Bastianon D, Valetti B, Cadei M, Grigolato P, Suzuki H, Finazzi D, Albertini A, Curello S and Ferrari R (2004) Arginase pathway in human endothelial cells in pathophysiological conditions. *J Mol Cell Cardiol* **37**:515-523.

Beranova P, Chalupsky K, Kleschyov AL, Schott C, Boucher JL, Mansuy D, Munzel T, Muller B and Stoclet JC (2005) Nomega-hydroxy-L-arginine homologues and hydroxylamine as nitric oxide-dependent vasorelaxant agents. *Eur J Pharmacol* **516**:260-267.

Berkowitz DE, White R, Li D, Minhas KM, Cernetich A, Kim S, Burke S, Shoukas AA, Nyhan D, Champion HC and Hare JM (2003) Arginase reciprocally regulates nitric oxide synthase activity and contributes to endothelial dysfunction in aging blood vessels. *Circulation* **108**:2000-2006.

Boucher JL, Custot J, Vadon S, Delaforge M, Lepoivre M, Tenu JP, Yapo A and Mansuy D (1994) N omega-hydroxyl-L-arginine, an intermediate in the L-arginine to nitric oxide pathway, is a strong inhibitor of liver and macrophage arginase. *Biochem Biophys Res Commun* **203**:1614-1621.

Broer A, Wagner CA, Lang F and Broer S (2000) The heterodimeric amino acid transporter 4F2hc/y+LAT2 mediates arginine efflux in exchange with glutamine. *Biochem J* **349 Pt 3**:787-795.

Buga GM, Singh R, Pervin S, Rogers NE, Schmitz DA, Jenkinson CP, Cederbaum SD and Ignarro LJ (1996) Arginase activity in endothelial cells: inhibition by NG-hydroxy-L-arginine during high-output NO production. *Am J Physiol* **271**:H1988-1998.

Closs EI, Scheld JS, Sharafi M and Forstermann U (2000) Substrate supply for nitric-oxide synthase in macrophages and endothelial cells: role of cationic amino acid transporters. *Mol Pharmacol* **57**:68-74.

Custot J, Moali C, Brollo M, Boucher J-L, Delaforge M, Mansuy D, Tenu J-P and Zimmermann J-L (1997) The New alpha-amino acid Nw-Hydroxy-nor-L-arginine: ahigh-affinity inhibitor of arginase well adapted to bind to its manganese cluster. *J Am; Chem. Soc.* **119**:4086-4087.

Daghigh F, Fukuto JM and Ash DE (1994) Inhibition of rat liver arginase by an intermediate in NO biosynthesis, NG-hydroxy-L-arginine: implications for the regulation of nitric oxide biosynthesis by arginase. *Biochem Biophys Res Commun* **202**:174-180.

David-Dufilho M, Brunet A, Privat C and Devynck MA (2001) Analysis of agonist-evoked nitric oxide release from human endothelial cells: role of superoxide anion. *Clin Exp Pharmacol Physiol* **28**:1015-1019.

David-Dufilho M, Schussler O, Pernollet MG, Brunet A, Millanvoye-Van Brussel E, Le Quan Sang KH and Rendu F (2003) Therapeutic immunoglobulin reduces Ca(2+) mobilization and von Willebrand factor secretion, and increases nitric oxide release in human endothelial cells. *Thromb Haemost* **90**:1046-1053.

Elfering SL, Sarkela TM and Giulivi C (2002) Biochemistry of mitochondrial nitric-oxide synthase. *J Biol Chem* **277**:38079-38086.

Fiermonte G, Dolce V, David L, Santorelli FM, Dionisi-Vici C, Palmieri F and Walker JE (2003) The mitochondrial ornithine transporter. Bacterial expression, reconstitution, functional characterization, and tissue distribution of two human isoforms. *J Biol Chem* **278**:32778-32783.

Gao S, Chen J, Brodsky SV, Huang H, Adler S, Lee JH, Dhadwal N, Cohen-Gould L, Gross SS and Goligorsky MS (2004) Docking of endothelial nitric oxide synthase (eNOS) to the mitochondrial outer membrane: a pentabasic amino acid sequence in the autoinhibitory domain of eNOS targets a proteinase K-cleavable peptide on the cytoplasmic face of mitochondria. *J Biol Chem* **279**:15968-15974.

Hanafy KA, Krumenacker JS and Murad F (2001) NO, nitrotyrosine, and cyclic GMP in signal transduction. *Med Sci Monit* **7**:801-819.

Hardy T and May J (2002) Coordinate regulation of L-arginine uptake and nitric oxide synthase activity in cultured endothelial cells. *Free Radicla Biol Med* **32**:122-131.

Knowles RG and Moncada S (1994) Nitric oxide synthases in mammals. *Biochem J* **298**:249-258.

Lamas S, Michel T, Brenner B and Mardsen P (1991) Nitric Oxide synthesis in endothelial cells: Evidence for a pathway inducible by TNF-alpha. *Am J Physiol* **261**:C634-C642.

Lantoine F, Iouzalen L, Devynck MA, Millanvoye-Van Brussel E and David-Dufilho M (1998) Nitric oxide production in human endothelial cells stimulated by histamine requires Ca^{2+} influx. *Biochem J* **330**:695-699.

Lantoine F, Trévin S, Bédioui F and Devynck J (1995) Selective and sensitive electrochemical measurement of nitric oxide in aqueous solution: discussion and new results. *Electroanalytical Chemistry* **392**:85-89.

Li C, Huang W, Harris MB, Goolsby JM and Venema RC (2005) Interaction of the endothelial nitric oxide synthase with the CAT-1 arginine transporter enhances NO release by a mechanism not involving arginine transport. *Biochem J* **386**:567-574.

Mann GE, Yudilevich DL and Sobrevia L (2003) Regulation of amino acid and glucose transporters in endothelial and smooth muscle cells. *Physiol Rev* **83**:183-252.

Mansuy D and Boucher JL (2004) Alternative nitric oxide-producing substrates for NO synthases. *Free Radic Biol Med* **37**:1105-1121.

McDonald KK, Zharikov S, Block ER and Kilberg MS (1997) A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric-oxide synthase may explain the "arginine paradox". *J Biol Chem* **272**:31213-31216.

Moali C, Brollo M, Custot J, Sari MA, Boucher JL, Stuehr DJ and Mansuy D (2000) Recognition of alpha-amino acids bearing various C=NOH functions by nitric oxide synthase and arginase involves very different structural determinants. *Biochemistry* **39**:8208-8218.

Nissim I, Luhovyy B, Horyn O, Daikhin Y, Nissim I and Yudkoff M (2005) The role of mitochondrially bound arginase in the regulation of urea synthesis: studies with [U-15N4]arginine, isolated mitochondria, and perfused rat liver. *J Biol Chem* **280**:17715-17724.

Noris M, Todeschini M, Cassis P, Pasta F, Cappellini A, Bonazzola S, Macconi D, Maucci R, Porrati F, Benigni A, Picciolo C and Remuzzi G (2004) L-arginine depletion in preeclampsia orients nitric oxide synthase toward oxidant species. *Hypertension* **43**:614-622.

Quintero M, Colombo SL, Godfrey A and Moncada S (2006) Mitochondria as signaling organelles in the vascular endothelium. *Proc Natl Acad Sci U S A* **103**:5379-5384.

Rouzaut A, Subira ML, de Miguel C, Domingo-de-Miguel E, Gonzalez A, Santiago E and Lopez-Moratalla N (1999) Co-expression of inducible nitric oxide synthase and arginases in different human monocyte subsets. Apoptosis regulated by endogenous NO. *Biochim Biophys Acta* **1451**:319-333.

Sala R, Rotoli BM, Colla E, Visigalli R, Parolari A, Bussolati O, Gazzola GC and Dall'Asta V (2002) Two-way arginine transport in human endothelial cells: TNF-

alpha stimulation is restricted to system y(+). *Am J Physiol Cell Physiol* **282**:C134-143.

Schussler O, Lantoine F, Devynck M-A, Glotz D and David-Dufilho M (1996) Human immunoglobulins inhibit thrombin-induced Ca^{2+} movements and nitric oxide production in endothelial cells. *J Biol Chem* **271**:26473-26476.

Shaul PW (2002) Regulation of endothelial nitric oxide synthase: location, location, location. *Annu Rev Physiol* **64**:749-774.

Simon A, Plies L, Habermeier A, Martine U, Reining M and Closs EI (2003) Role of neutral amino acid transport and protein breakdown for substrate supply of nitric oxide synthase in human endothelial cells. *Circ Res* **93**:813-820.

Stuehr DJ, Kwon NS, Nathan CF, Griffith OW, Feldman PL and Wiseman J (1991) N omega-hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. *J Biol Chem* **266**:6259-6263.

Suschek CV, Schnorr O, Hemmrich K, Aust O, Klotz LO, Sies H and Kolb-Bachofen V (2003) Critical role of L-arginine in endothelial cell survival during oxidative stress. *Circulation* **107**:2607-2614.

Tenu JP, Lepoivre M, Moali C, Brollo M, Mansuy D and Boucher JL (1999) Effects of the new arginase inhibitor N(omega)-hydroxy-nor-L-arginine on NO synthase activity in murine macrophages. *Nitric Oxide* **3**:427-438.

Vetrovsky P, Boucher JL, Schott C, Beranova P, Chalupsky K, Callizot N, Muller B, Entlicher G, Mansuy D and Stoclet JC (2002) Involvement of NO in the endothelium-independent relaxing effects of N(omega)-hydroxy-L-arginine and other compounds bearing a C=NOH function in the rat aorta. *J Pharmacol Exp Ther* **303**:823-830.

Wu G and Morris SM, Jr. (1998) Arginine metabolism: nitric oxide and beyond. *Biochem J* **336**:1-17.

Xu W, Kaneko FT, Zheng S, Comhair SA, Janocha AJ, Goggans T, Thunnissen FB, Farver C, Hazen SL, Jennings C, Dweik RA, Arroliga AC and Erzurum SC (2004) Increased arginase II and decreased NO synthesis in endothelial cells of patients with pulmonary arterial hypertension. *Faseb J* **18**:1746-1748.

Zani BG and Bohlen HG (2005) Transport of extracellular l-arginine via cationic amino acid transporter is required during in vivo endothelial nitric oxide production. *Am J Physiol Heart Circ Physiol* **289**:H1381-1390.

FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. Relationships between L-Arg uptake, arginases and NO synthesis in the cell. The cationic acid transporter (CAT) transports the L-Arg inside the cell where it is oxidized into NO and L-citrulline with intermediate formation of N^{ω}-hydroxy-L-Arg (NOHA) by the constitutive NOS (NOS) of cell membrane, the inducible NOS (iNOS) and the mitochondrial NOS (mtNOS). L-Arg is also converted into ornithine and urea by the cytosolic arginase I and the mitochondrial arginase II. As indicated by non-arrows (**T**), the two arginase isoforms are inhibited by high concentration of NOHA (100 µM) and by N^{ω}-hydroxy-nor-L-Arg (Nor-NOHA) in the range of 10 µM.

Fig. 2. Dose-dependent effect of NOHA and Nor-NOHA on basal arginase activity. 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 unlabelled L-Arg and 0.5 µCi/ml [^{3}H]L-Arg (10 nM). Arginase activity was assayed by counting [^{3}H]Urea and expressed as % of values from untreated cells (1.4±0.4 nmol/mg protein/min, n=4). * $p< 0.05$, ** $p< 0.01$ when compared to values of untreated cells.

Fig. 3. Dose-dependent effect of Nor-NOHA and NOHA on eNOS activity. A, basal [^{3}H]L-citrulline formation in unstimulated HUVECs. B, thrombin-activated [^{3}H]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 unlabelled L-Arg and 1 µCi/ml [^{3}H]L-Arg (20 nM), washed and stimulated with 2 U/ml thrombin for 10 min in the absence of L-Arg. Thrombin-induced [^{3}H]L-citrulline formation was calculated by subtracting the basal values determined in the absence of thrombin. Results are from 4

independent experiments. * $p< 0.05$, ** $p< 0.01$ and *** $p<0.001$when compared to values of untreated cells.

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 2U/ml thrombin in PBS-MgCa without (NOHA, Nor-NOHA) or with L-Arg. Results are means of 5-9 independent experiments. *$p<0.05$, **$p<0.01$, *** $p<0.001$ when compared to values from untreated cells.

Fig. 5. Influence of Nor-NOHA on L-Arg uptake. A, cells were untreated (Control) or treated for 30 min with 20 µM Nor- NOHA. After washing, they were incubated for 20 min at 37°C in PBS-MgCa containing various concentrations of unlabelled L-Arg and 0.5 µCi/ml [^{3}H]-L-Arg (10 nM) and for further 10 min in the absence (○) or presence of 2U/ml thrombin (●). B, data obtained as described above are presented in a double reciprocal plot. The Km and Vmax values were calculated by linear regression analysis. Results are means of 4-5 independent experiments. ***$p<0.001$ when compared to values measured without unlabelled L-Arg.

Fig. 6. Dose-dependent effect of extracellular L-Arg on arginase activity. Cells were incubated for 20 min at 37°C in PBS-MgCa containing various concentrations of unlabelled L-Arg and 0.5 µCi/ml [^{3}H]-L-Arg (10 nM) and for further 10 min in the absence (○) or presence of 2U/ml thrombin (●). The arginase activity was assayed by counting [^{3}H]-Urea. Results are means of 6-8 independent experiments. Dose-dependent

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effects was indicated by $p<0.001$ in the absence and presence of thrombin. $^{**}p<0.01$ and *** $p<0.001$ when compared to values measured in the absence of unlabelled L-Arg.

Fig. 7. Effect of extracellular L-Arg on Nor-NOHA-induced inhibition of arginase. A, arginase activity in unstimulated cells. B, arginase activity in thrombin-stimulated cells. HUVECs were treated for 30 min at 37°C with 20 µM Nor-NOHA. After washing, they were incubated for 20 min in PBS-MgCa containing indicated concentrations of unlabelled L-Arg and 0.5 µCi/ml [^{3}H]L-Arg (10 nM) and for further 10 min without or with 2U/ml thrombin. Arginase activity was assayed by counting [^{3}H]Urea. Results are means of 3-5 independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, *** $p<0.001$ when compared to values measured in the absence of unlabelled L-Arg.

Fig. 8. Influence of L-lysine and L-glutamine on thrombin-activated NO release. HUVECs were incubated for 90 min either in the culture medium containing 2.4 mM glutamine (A) or in glutamine-free medium (B) and, for further 30 min without (Control, L-Arg) or with 20 µM Nor-NOHA in the absence (-) or presence (+) of 1mM L-Lysine. They were then stimulated by 2U/ml thrombin in PBS-MgCa without or with 100 µM L-Arg ± 1mM L-Lysine. Results are means of 3-6 independent experiments. $^{**}p< 0.01$ when compared to values from control cells. $^{+}$ $p< 0.05$, $^{++}$ $p< 0.01$ when compared to values measured in the absence of L-lysine under the same experimental conditions.

Fig 9. RT-PCR analysis for human arginase I and II mRNAs. Reverse transcriptase samples of arginase I (6 µl) were amplified with 40 cycles and those of arginase II (3 µl) 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 4 independent cultures.

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Figure 1

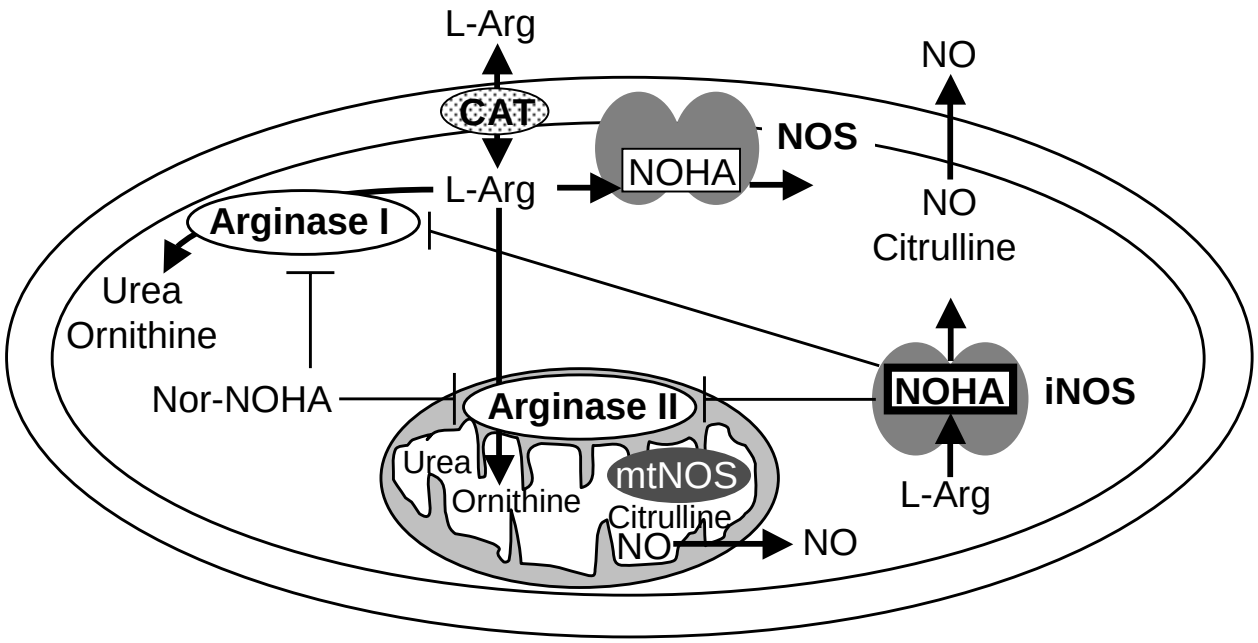

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Figure 2

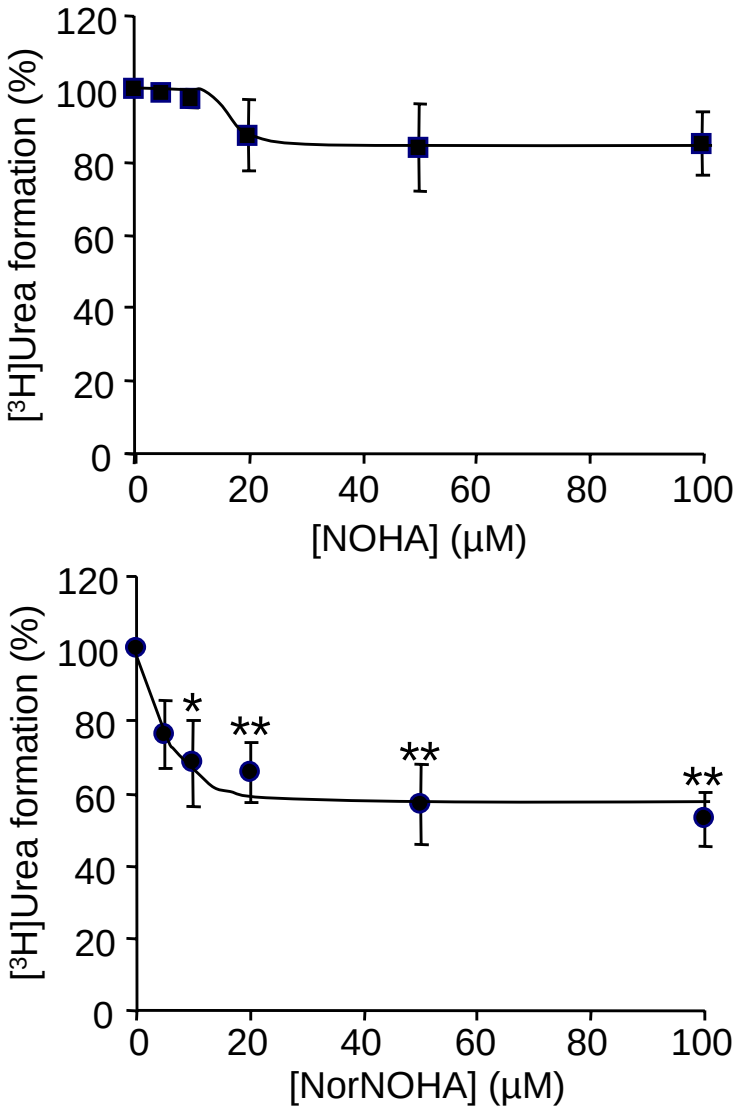

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Figure 3

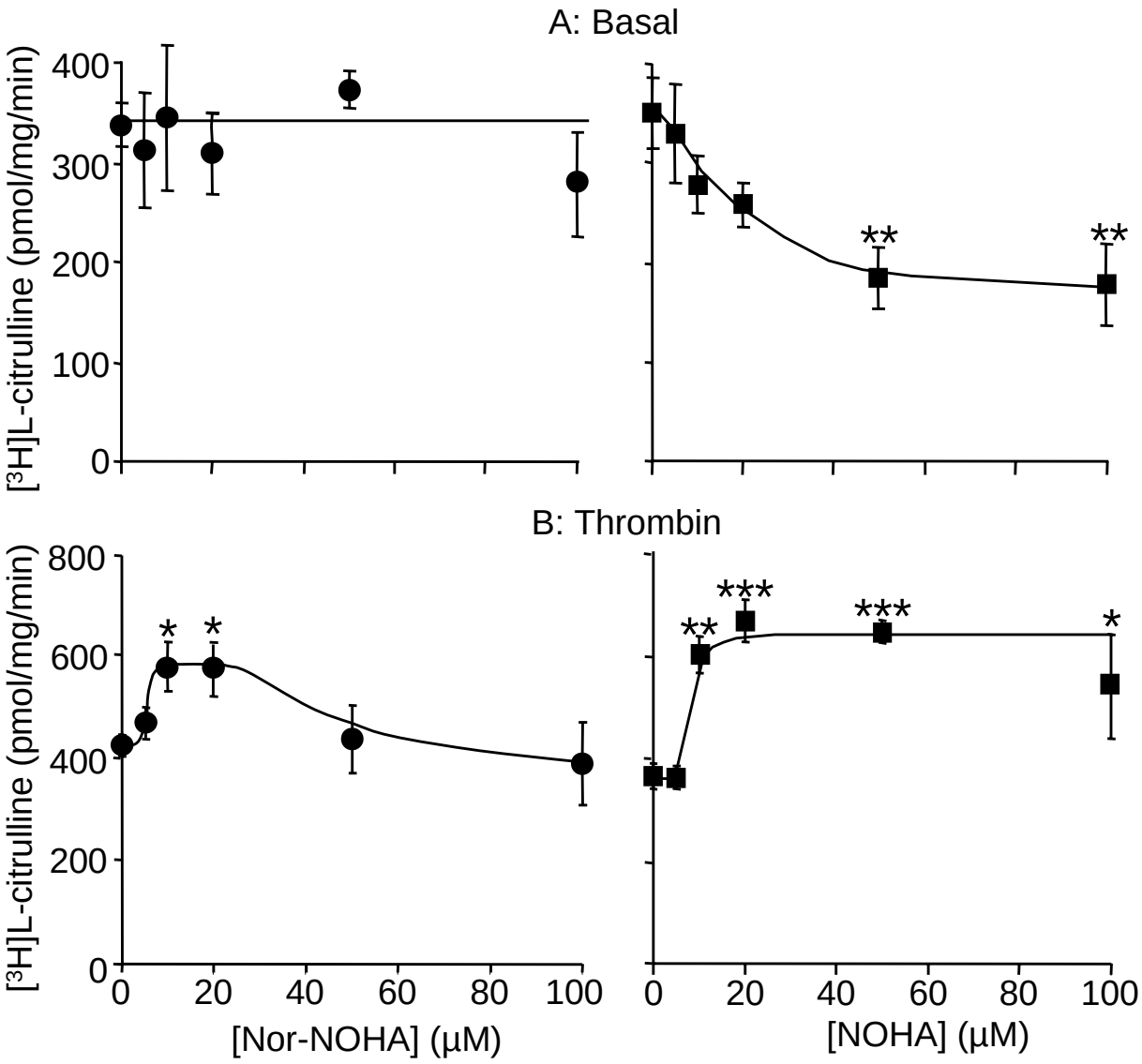

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Figure 4

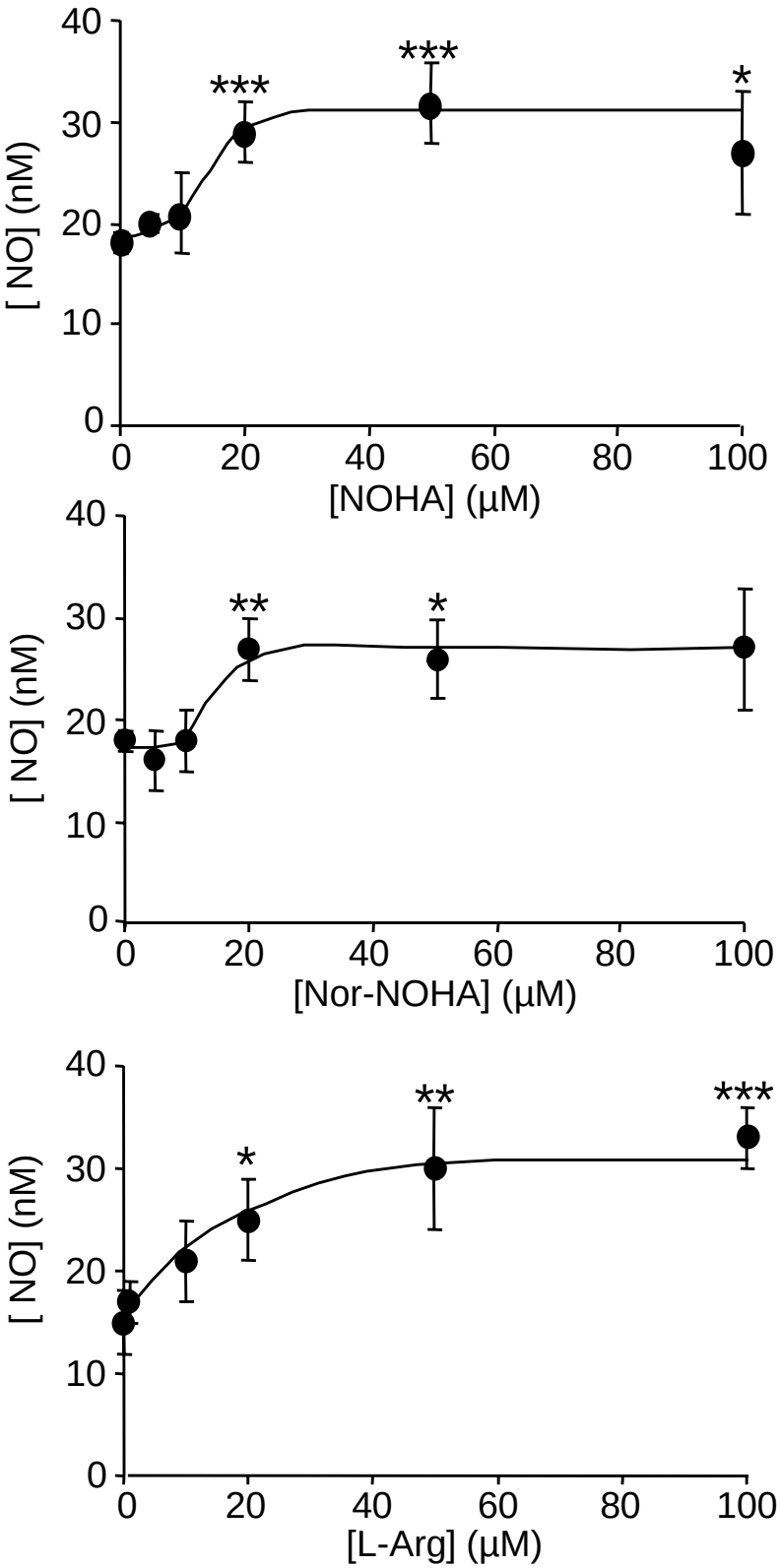

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Figure 5

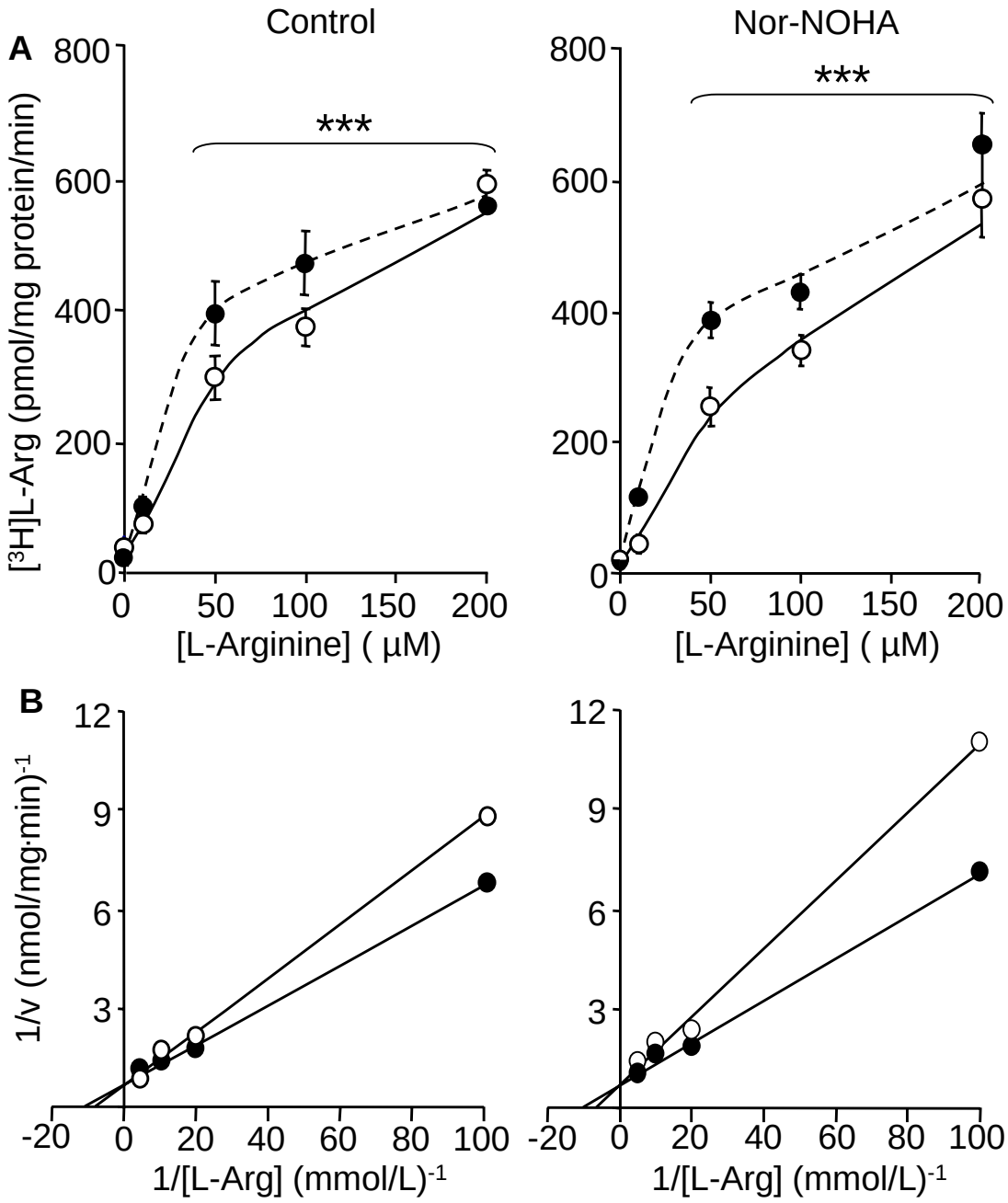

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Figure 6

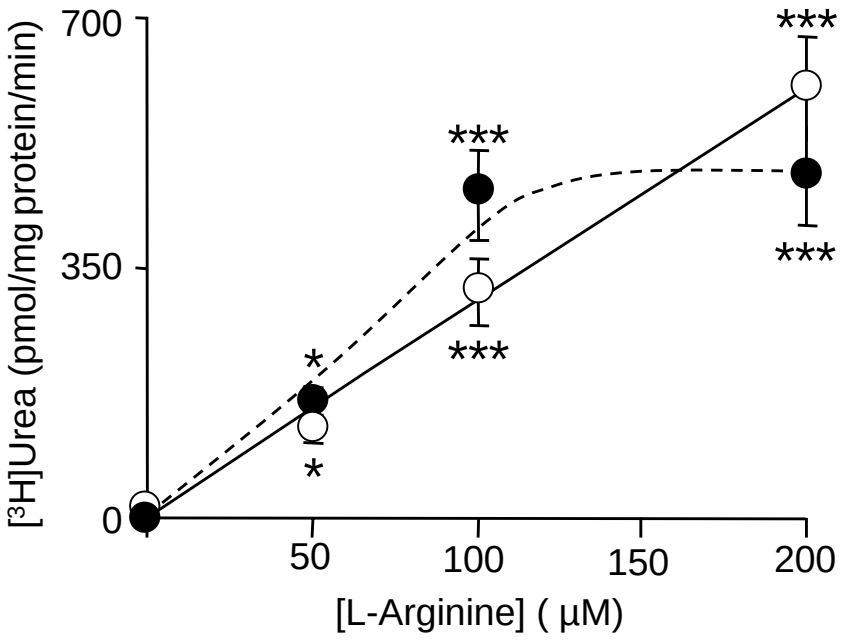

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Figure 7

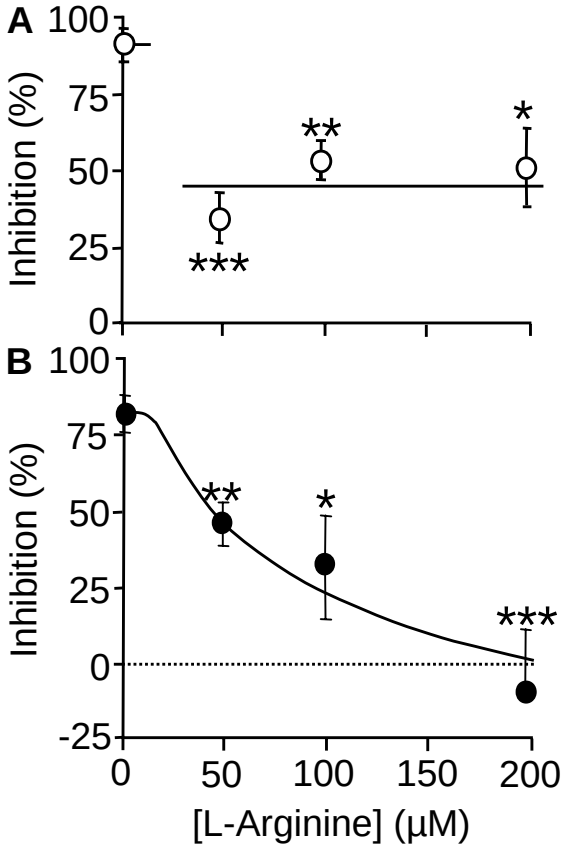

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Figure 8

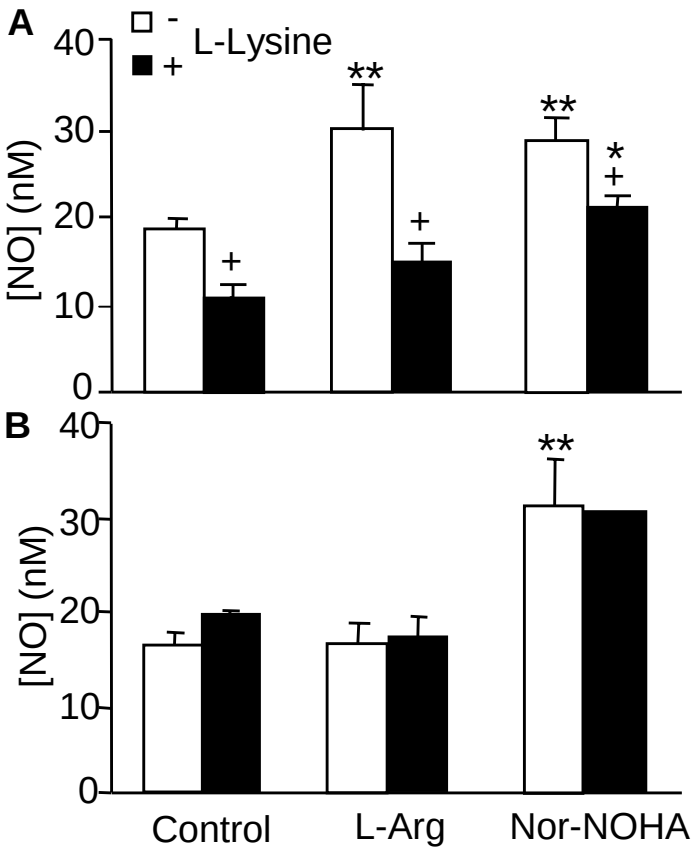

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Figure 9