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Substrate Supply for Nitric-Oxide Synthase in Macrophages and Endothelial Cells: Role of Cationic Amino Acid Transporters

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ABSTRACT

The current study was designed to investigate the importance of cationic amino acid transporters (CATs) for the L-arginine supply to nitric oxide (NO) synthases in mouse J774A.1 macrophages and human EA.hy926 endothelial cells. CAT-1 was expressed in both cell types, whereas CAT-2B was only expressed in activated macrophages. Apparent K_M values for transport of L-arginine in both cell types was consistent with the expression of the system y^+ carriers CAT-1 (and CAT-2B in macrophages). In addition, L-arginine transport was Na^+ independent and sensitive to *trans*-stimulation. A 2-h preincubation of activated macrophages in 2 mM L-lysine (which is exchanged for L-arginine by the CATs) reduced the intracellular L-arginine concentration from 2 mM to 160 μM. At the same time, nitric-oxide synthase (NOS) II activity was completely abolished. NOS II activity could be restored with extracellular L-arginine. No difference in NO production was seen between macrophages preincubated in L-arginine-containing buffer and incubated either with or without L-arginine during the 2-min NO assay. Incubation of endothelial cells in 2 mM L-lysine for up to 24 h decreased the intracellular L-arginine concentration from 3.5 mM to about 600 μM but did not reduce the NOS III activity. Our results suggest that both activated macrophages and endothelial cells have an L-arginine pool that is not freely exchangeable with the extracellular space. This pool seems to be accessible to NOS III in endothelial cells but not to NOS II in macrophages.

Macrophages and endothelial cells represent two cell types capable of synthesizing nitric oxide (NO) from L-arginine. However, NO synthesis in the two cell types differs in several aspects. Nitric-oxide synthase (NOS) II in macrophages is only expressed after induction with bacterial lipopolysaccharide (LPS) or cytokines. In contrast, NOS III in endothelial cells is constitutively expressed. The activity of NOS III is dependent on the intracellular Ca^{2+} concentration, whereas NOS II is also active at resting, unstimulated Ca^{2+} concentrations. The half-saturating concentrations (K_M) of the substrate L-arginine measured in vitro for NOS II (3–30 μM) and NOS III (3 μM) are one to two orders of magnitude below the intracellular concentrations of the amino acid measured in macrophages and endothelial cells (100–800 μM; for review, see Förstermann et al., 1994). Accordingly, endothelial NOS III activity has been shown to be largely independent of the extracellular L-arginine supply (Palmer et al., 1988). The constantly high L-arginine concentrations might, at least in part, be due to the ability of endothelial cells to recycle L-arginine from L-citrulline (Hecker et al., 1990). However, under certain pathophysiological conditions such as diabetes, hypertension, or hypercholesterolemia (Cooke et al., 1991; Creager et al., 1992; Laurant et al., 1995; Pieper and Peltier, 1995), increased plasma L-arginine levels can lead to improved vasodilation presumably due to enhanced NOS III activity in endothelial cells. The increase in NOS III activity in response to elevated extracellular L-arginine concentrations in spite of sufficiently high intracellular substrate levels has been referred to as the arginine paradox (Förstermann et al., 1994). The activity of the constantly active NOS II has previously been determined by measuring NO oxidation products, nitrite, and nitrate, accumulated over hours. In such long-term experiments, NO synthesis in macrophages has been reported to be dependent on extracellular L-arginine (Granger et al., 1990; Assreuy and Moncada, 1992; Bogle et al., 1992). The dependence on extracellular L-arginine is often explained by the high and long-lasting activity of NOS II and other L-arginine-using enzymes such as arginase. However, no data are available on the intracellular L-arginine concentrations in macrophages induced to express NOS II and incubated in L-arginine-free medium for several hours.

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[1] This work contains major parts of the theses of J.-S.S. and M.S.

ABBREVIATIONS: NO, nitric oxide; CAT, cationic amino acid transporter (h, human; m, mouse); NOS, nitric-oxide synthase (h, human; m, mouse); LPS, bacterial lipopolysaccharide; DMEM, Dulbecco's modified Eagle's medium.

The transport of L-arginine through the plasma membrane seems to occur via similar pathways in macrophages and endothelial cells. In both cell types, system y^+ has been described to be the major transport system for cationic amino acids including L-arginine (Bogle et al., 1991, 1992; Baydoun et al., 1993). In murine macrophages, LPS induction leads to a parallel increase in NOS II expression and in system y^+-mediated L-arginine transport (Sato et al., 1991; Bogle et al., 1992). System y^+ is a pH- and Na^+-independent transport system for cationic amino acids that catalyzes an exchange of basic amino acids between the intracellular and extracellular spaces (for review, see White, 1985).

At least some of the carrier proteins mediating system y^+ activity have been identified. They belong to a family of related carrier proteins for cationic amino acids [cationic amino acid transporter (CAT); for review, see Closs, 1996; MacLeod and Kakuda, 1996; Deves and Boyd, 1998]. Three of the four family members characterized so far have system y^+ properties (for review, see Closs, 1996). CAT-1 is ubiquitously expressed except for the liver (Kim et al., 1991). Based on data from immunofluorescence staining of porcine endothelial cells, a colocalization of CAT-1 with caveolin and NOS III has been suggested and it has been proposed that CAT-1 might directly provide substrate for NOS III (McDonald et al., 1997). Expression of the second system y^+ carrier, CAT-2B, seems to be either very low or absent in normal tissues. However, induction of CAT-2B expression has been reported both in vivo and in vitro (MacLeod et al., 1990; Closs et al., 1993; Stevens et al., 1996). CAT-2B has a slightly lower apparent affinity to cationic amino acids than CAT-1 and shows less *trans*-stimulation. The physiological role of CAT-2B that seems to be always expressed in addition to the constitutive CAT-1 is not apparent. Based on the coinduction of NOS II and CAT-2B, it has been speculated that CAT-2B might provide substrate for NOS II. However, there are no data demonstrating CAT-2B-dependent NOS II activity. The recently identified CAT-3 also demonstrates system y^+ activity (Hosokawa et al., 1997; Ito and Groudine, 1997). However, as CAT-3 expression seems to be restricted to the brain in adult animals, CAT-3 is unlikely to play a role for L-arginine transport in either macrophages or endothelial cells.

To determine the role of CAT-mediated L-arginine transport across the plasma membrane for substrate supply of NOS II and NOS III, we investigated the CAT expression and kinetic parameters of L-arginine transport in the murine macrophage cells J774.A1 and the human endothelial cells EA.hy926. In addition, we designed short-term experiments where NOS activity in the two cell types was measured under defined extracellular and intracellular L-arginine concentrations, thereby excluding indirect effects of L-arginine consumption or recycling on the availability of substrate for NO synthesis.

Materials and Methods

Cell Culture. The murine macrophage cell lines RAW 264.7 and J774.A1, and the rat lung fibroblast cell line RFL-6 were obtained from American Type Culture Collection (Bethesda, MD). The human endothelial cell line EA.hy926 was a gift from C.-J. S. Edgell (University of North Carolina at Chapel Hill). RFL-6 cells were grown in F-12 medium, supplemented with 4 mM glutamine and 15% fetal bovine serum. All other cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 4 mM glutamine and 10% fetal bovine serum. Cells were regularly tested for mycoplasma infection using 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; Roche Molecular Biochemicals, Mannheim, Germany). No contamination could be detected.

RFL-6 Reporter Assay. All cells were grown to confluence in six-well plates (diameter 3.5 cm). Twelve hours before the assay, macrophages were induced to express NOS II by adding 1 μg/ml bacterial LPS to the culture medium. Up to 24 h before the assay, endothelial cells were cultured in DMEM free of cationic amino acids and supplemented with 10% dialyzed fetal bovine serum and either 2 mM L-arginine or 2 mM L-lysine. Before the assay (30 min to 3.5 h), endothelial cells and macrophages were washed twice in Locke's solution (composition: 154 mM NaCl; 5.6 mM KCl; 2 mM $CaCl_2$; 1 mM $MgCl_2$; 10 mM HEPES; 3.6 mM $NaHCO_3$; 5.6 mM glucose) and preincubated in Locke's solution containing the indicated concentrations of L-arginine or L-lysine at 37°C with two to three buffer changes. The supernatant was then replaced by Locke's solution containing the same concentration of L-arginine or L-lysine, respectively, and supplemented with 20 U/ml superoxide dismutase (Roche Molecular Biochemicals, Mannheim, Germany). RFL-6 cells were washed twice in Locke's solution and incubated in Locke's solution supplemented with 600 μM 3-isobutyl-1-methylxanthine (Serva, Heidelberg, Germany) for 30 min at 37°C.

For measurement of NO production, cells were washed three times with ice-cold Locke's solution and incubated for 2 min at 37°C in Locke's solution containing either 2 mM L-arginine or no cationic amino acids and supplemented with 300 μM 3-isobutyl-1-methylxanthine, 20 U/ml superoxide dismutase and, where indicated, 10 μM Ca^{2+}-ionophore A23187. The supernatants were then transferred to RFL-6 cells. After a 2-min incubation of the RFL-6 cells at 37°C, the reaction was stopped by aspirating the medium, adding 1 ml of ice-cold sodium acetate buffer (20 mM, pH 4.0) and rapidly freezing the samples with liquid nitrogen. The cGMP content of each sample was determined by radioimmunoassay as described previously (Ishii et al., 1991).

L-Arginine to L-Citrulline Conversion Assay. Endothelial cells or LPS-induced macrophages (1 μg/ml for 12 h) grown to confluence in cell culture dishes (diameter 14.5 cm) were washed twice in PBS and then lysed by sonication in 25 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, 3 μg/ml aprotinin, 10 μg/ml leupeptin, and 35 μg/ml phenylmethylsulfonyl fluoride. To the lysates from endothelial cells 3-([cholamidopropyl]-dimethylammonio)-1-propanesulfonate was added to a final concentration of 20 mM. The lysates were rotated at 4°C for 1 h and then spun for 30 min at 100,000*g*. The protein content of the supernatants was determined by the Bradford assay (Bio-Rad, Munich, Germany). The conversion of citrulline to arginine was assayed by adding lysate (4–20 μg protein) to a reaction mixture containing 50 mM Tris-HCl pH 7.4, 6 μM tetrahydrobiopterin, 2 μM flavin adenine dinucleotide, 2 μM flavin adenine mononucleotide, 2 mM reduced nicotinamide adenine dinucleotide phosphate, 1.25 mM $CaCl_2$, and 1 to 100 μM [^{14}C]L-arginine (ICN Biochemicals, Eschwege, Germany). For endothelial cell lysates 0.1 μM calmodulin was also added. The reactions were stopped after a 15- or 30-min incubation at 37°C by the addition of 2 volumes of ice-cold methanol. The samples were dried and resuspended in 10 μl of water. L-arginine and L-citrulline were separated by thin-layer chromotography (Polygram SIL N-HR; Macherey-Nagel, Düren, Germany) developed in chloroform/methanol/ammonium hydroxide/water (0.5:4.5:2:1) (v/v/v/v).

Analysis of Intracellular Amino Acid Content. Immediately after transfer of the supernatants to RFL-6 cells (see above), macrophages and endothelial cells were washed three times in ice-cold Locke's solution and lysed in 500 μl of methanol/0.5 M boric acid, pH 7.7, 9:1 (v/v). γ-Aminobutyric acid was added as the internal standard (1 nmol/36 μl). Cell debris was sedimented at 14,000*g* for 5 min, and 36 μl of the supernatant was used for precolumn derivatization with 9-fluorenylmethyl-oxycarbonylchloride. Amino acid derivatives were separated on a Superspher 60 RP-8 250–4 HPLC column

(Merck, Darmstadt, Germany) using a three-solvent gradient as detailed previously (Closs et al., 1997a). Cells grown in parallel cultures and treated identically to the cells in the RFL-6 assay were trypsinized and counted, and the cell volume was determined using the CASY 1 cell counter and analyzer system (Schärfe System, Reutlingen, Germany).

Measurement of L-Arginine Transport. Cells grown to confluence in 24-well plates (diameter 1 cm) were washed twice with Locke's solution containing a defined concentration of L-arginine and then incubated in the same solution at 37°C for 30 min to allow for equilibration. Then the Locke's solution was replaced with the same solution containing [^{3}H]L-arginine (5 μCi/ml) and the cells were incubated at 37°C for the times indicated. At the end of the incubation period, the cells were immediately transferred on ice, washed three times with ice-cold Locke's solution, and lysed in 90% methanol. The radioactivity in the lysates was determined by liquid scintillation counting. For efflux studies, cells were loaded with 100 μM L-arginine (5 μCi/ml) at 37°C for 60 min. The cells were then washed three times with ice-cold Locke's solution, transferred in Locke's solution containing either no cationic amino acids or 1 mM L-lysine, and incubated for 2.5 min at 37°C. The radioactivity of the supernatants was determined by liquid scintillation counting.

Ribonuclease Protection Analyses. The probes used for the mouse CAT isoforms were described previously (Simmons et al., 1996). Probes for the human CAT isoforms were obtained by subcloning short cDNA fragments of hCAT-1 (bp 1242–1447; Albritton et al., 1993), hCAT-2A (bp 1222–1388; Closs et al., 1997b), and hCAT-2B (bp 1258 −1391; Closs et al., 1997b). A probe for mouse NOS II containing a 160-bp fragment of the 5′ untranslated region of the NOS II cDNA (Lyons et al., 1992) was a gift from C.R. Lyons (Department of Internal Medicine, University of New Mexico, Albuquerque, NM). The probes for human NOS III and mouse and human β-actin were described previously (Kleinert et al., 1996, 1998).

Total RNA was isolated from macrophages and endothelial cells using the method of Chomczynki and Sacchi (1987). Ribonuclease protection analyses were performed with 20 μg of RNA/sample using [α-^{32}P]UTP-labeled probes as described (Closs and Mann, 1999).

Results

CAT and NOS Expression in LPS-Induced J774.A1 Macrophages and EA.hy926 Endothelial Cells. RNase protection analyses demonstrated expression of mCAT-1 in both LPS-treated (1 μg/ml) and untreated macrophages (Fig. 1A). mCAT-1 expression doubled within 6 h of LPS induction and returned to control level after 18 h as assessed by quantitative analysis using a phosphoimager. The expression of mCAT-2B increased continuously up to 4-fold within 18 h of LPS induction (Fig. 1B). Neither LPS-induced nor control cells expressed mCAT-2A (data not shown). The strongest expression of NOS II mRNA was detected from 6 to 9 h after LPS induction (Fig. 1C). The increase in NOS II mRNA was about 5-fold. Eighteen hours after induction, only a very weak signal for NOS II mRNA was detected comparable to that of uninduced cells.

RNase protection analyses with probes specific for all three hCATs demonstrated the expression of hCAT-1 in EA.hy926 cells (Fig. 2). In contrast, no expression of hCAT-2A or hCAT-2B could be detected (data not shown). NOS III was constitutively expressed in EA.hy926 cells (data not shown).

L-Arginine Transport in Macrophages and Endothelial Cells. The transport of L-arginine was studied in J774.A1 macrophages induced for 18 h with LPS (1 μg/ml) and in EA.hy926 endothelial cells. Uptake of 1 mM L-arginine was linear for up to 10 and 6 min in J774.A1 and EA.hy926 cells, respectively (data not shown). Confluent cells in 24-well plates were equilibrated for 30 min at 37°C with defined L-arginine concentrations (30 μM to 10 mM). Uptake of [^{3}H]L-arginine (same concentration as used in the preincubation) was then measured for 4 min (J774A.1 cells) or 2.5 min (EA.hy926 cells). Eadie-Hofstee plots of the data obtained for J774.A1 macrophages suggested that L-arginine transport is mediated by both a low (K_M = 5.7 ± 1 mM)- and a high (K_M = 304 ± 87 μM)-affinity transport system in these cells (Fig. 3A). In contrast, only one high-affinity component could be detected in EA.hy926 cells (K_M = 68 ± 4 μM, Fig. 3B). The V_{max} values were 4 ± 0.6 and 1.6 ± 0.8 nmol/10^6 cells/min for the low- and high-affinity transport component,

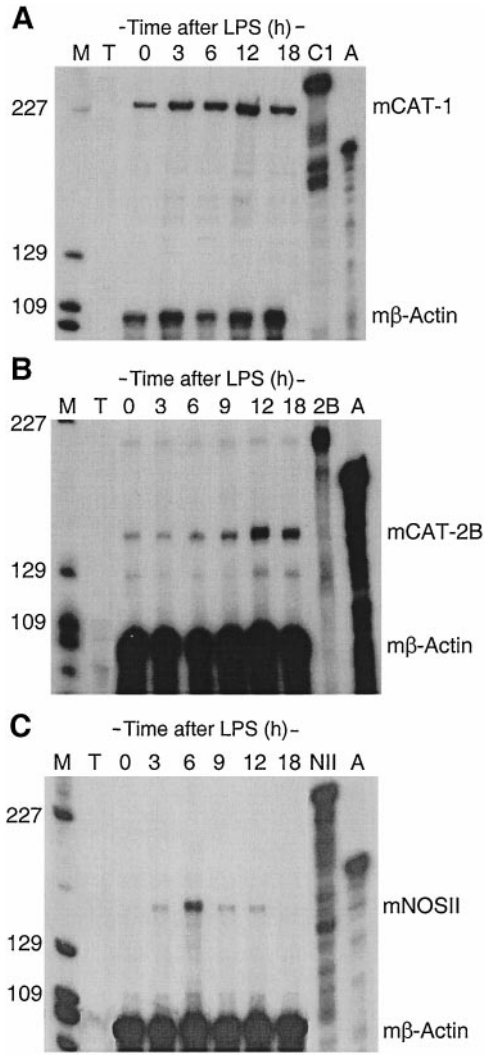

Fig. 1. Expression of mCAT-1, mCAT-2B, and NOS II in J774.A1 macrophages. Ribonuclease protection analyses using specific probes for mCAT-1 (A), mCAT-2B (B), and mNOS II (C). Mouse β-actin mRNA was also analyzed as an internal control. Total mRNA was prepared either from untreated J774.A1 macrophages or from cells treated with 1 μg/ml LPS for the time indicated. The RNAs were hybridized with cRNA probes specific for either mCAT-1, mCAT-2B, or mNOS II, and for mβ-actin. After RNase treatment, the protected RNA fragments (mCAT-1, 237 nucleotides; mCAT-2B, 148 nucleotides; mNOS II, 160 nucleotides; and mβ-actin, 109 nucleotides) were separated on 6% denaturing polyacrylamide gels. M: DNA size marker [pGl2-Basic (Promega), restricted with *Hin*fI]. T: *t*-RNA used as a negative control. C1, 2B, NII, A: undigested probes for mCAT-1 (256 nucleotides), mCAT-2B (212 nucleotides), mNOS II (260 nucleotides), and mβ-actin (187 nucleotides), respectively.

respectively, in J774.A1 cells and 1.35 ± 0.5 nmol/10^6 cells/min in EA.hy926 cells.

In both J774.A1 macrophages and EA.hy926 endothelial cells, transport of 100 μM L-arginine was independent of the presence of Na^+ ions as demonstrated by replacement of NaCl with choline chloride in the uptake buffer (Fig. 4A). In addition, in both cell types L-arginine efflux from cells preloaded with [^{3}H]L-arginine (0.1 mM, 60 min) was strongly stimulated by substrate at the *trans* side of the membrane (Fig. 4B). In the presence of 1 mM extracellular L-lysine, 5.4 and 7.2 times more L-arginine could be detected in the supernatants of J774.A1 and EA.hy926 cells, respectively, as compared with cells incubated in buffer containing no cationic amino acids. Similar results were obtained with 1 mM L-arginine in the extracellular buffer (data not shown).

Substrate Supply of NOS in Macrophages and Endothelial Cells. To determine the dependence of macrophage and endothelial cell NO synthesis on extracellular and intracellular L-arginine concentrations, we designed experiments where NOS activity was measured in short-term experiments (2 min) under defined extracellular and intracellular L-arginine concentrations using the RFL-6 reporter cell assay. The intracellular L-arginine concentrations were calculated from the L-arginine content of the cells measured by HPLC, the cell volume, and the cell number. Both the expression of CAT-1 (and CAT-2B in macrophages) and the characteristics of L-arginine transport in J774.A1 macrophages and EA.hy926 endothelial cells suggested that L-arginine transport in the two cell types is mediated by system y^+-like transport systems that exchange basic amino acids. Therefore, cells were incubated in high concentrations of extracellular L-lysine to deplete the intracellular L-arginine. Indeed, intracellular L-arginine concentrations dropped significantly after incubating J774.A1 macrophages (Fig. 5) and EA.hy926 endothelial cells (Fig. 7) in 0.3 to 2 mM L-lysine. However, significant residual L-arginine not depletable by additional incubation of the cells in L-lysine-containing buffer could

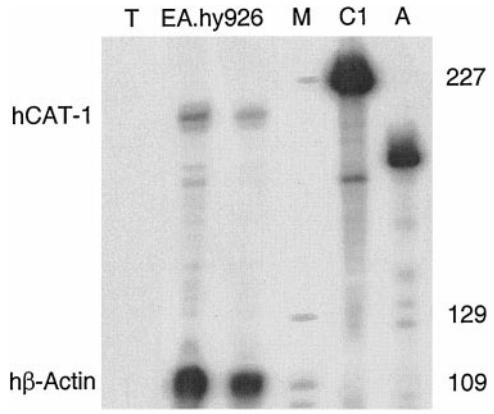

Fig. 2. Expression hCAT-1 in EA.hy926 endothelial cells. Ribonuclease protection analyses using specific probes for hCAT-1 and human β-actin. Total mRNA was prepared from EA.hy926 endothelial cells and hybridized with cRNA probes specific for hCAT-1 and hβ-actin. After RNase treatment, the protected RNA fragments (hCAT-1, 201 nucleotides; hβ-actin, 109 nucleotides) were separated on 6% denaturing polyacrylamide gels. T: *t*-RNA. M: DNA size marker. C1: undigested probes for hCAT-1 (215 nucleotides). A: undigested probe for hβ-actin (187 nucleotides).

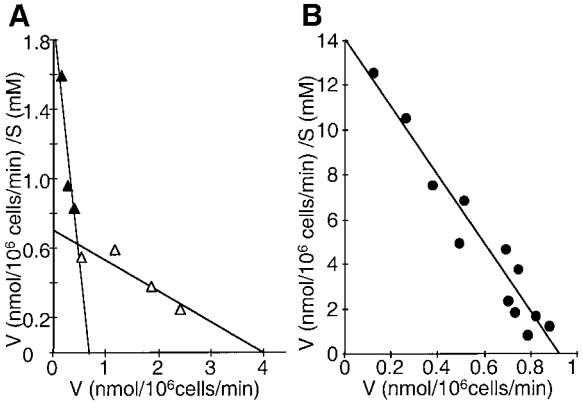

Fig. 3. L-arginine uptake in LPS-induced J774.A1 macrophages and EA.hy926 endothelial cells as a function of extracellular L-arginine concentrations. Eadie-Hofstee plots of [^{3}H]L-arginine uptake in J774.A1 macrophages (A) and EA.hy926 endothelial cells (B). Closed and open symbols represent components of L-arginine transport with high and low affinity, respectively. The macrophages had been induced with LPS (1 μg/ml, 18 h) before the uptake assay. To cells equilibrated with a defined concentration of unlabeled L-arginine (30 μM-10 mM) in Locke's solution, [^{3}H]L-arginine was added and the cells were incubated for 4 min (J774) or 2.5 min (EA.hy) at 37°C. Cells were then washed carefully and lysed in 90% methanol, and the radioactivity of the lysates was determined.

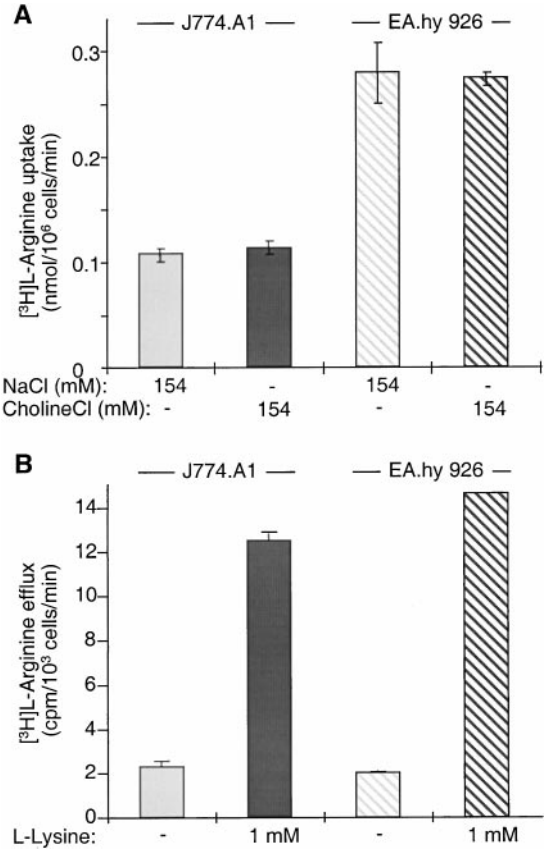

Fig. 4. Na^+-independence and *trans*-stimulation of L-arginine transport in LPS-induced J774.A1 macrophages and EA.hy926 endothelial cells. [^{3}H]L-arginine uptake (A) or efflux (B) was measured from J774.A1 macrophages (solid columns) or EA.hy926 endothelial cells (hatched columns). The macrophages had been induced with LPS (1 μg/ml, 18 h) before the experiments. A, cells were incubated for 4 min (J774) or 2.5 min (EA.hy) in Locke's solution containing 100 μM [^{3}H]L-arginine (5 μCi/ml) and either 154 mM Na^+ or 154 mM choline at 37°C. The L-arginine taken up in the cell was determined by liquid scintillation counting (means ± S.E., n = 6). B, [^{3}H]L-arginine efflux from cells preloaded with [^{3}H]L-arginine (100 μM, 5 μCi/ml for 60 min). After loading, cells were washed carefully and then incubated in Locke's solution with (dark columns) or without (light columns) 1 mM L-lysine for 2.5 min at 37°C. The radioactivity of the supernatant was determined (means ± S.E., n = 6).

always be detected. The K_M of NOS II from J774.A1 macrophages for L-arginine was determined to be 4.7 ± 1.3 μM by the conversion of L-arginine to L-citrulline using crude extracts of J774.A1 cells. Therefore, despite residual intracellular L-arginine concentrations of 160 to 300 μM, at least 35-fold above the K_M of NOS II determined in vitro, NOS II activity in J774.A1 macrophages was dramatically decreased after a 3.5-h preincubation in buffer without cationic amino acids or with 0.3 or 2 mM L-lysine (Fig. 5A). NOS activity of J774.A1 cells preincubated in 2 mM L-lysine was reduced to 6 to 7% of control. To elucidate if the NOS II in macrophages can only use extracellular L-arginine, a second set of experiments was carried out with cells preincubated for 30 min in either 2 mM L-arginine or 2 mM L-lysine, then washed carefully and incubated for the NO assay (2 min) in either 2 mM L-arginine or in the absence of cationic amino acids (Fig. 5B). A 30-min incubation in 2 mM L-lysine was sufficient to reduce NO synthesis in J774.A1 macrophages significantly in spite of intracellular L-arginine concentrations of about 300 μM. Supplementation of cells preincubated in 2 mM L-lysine with 2 mM L-arginine for 2 min during the NO assay led to a complete recovery of NO synthesis. However, also cells preincubated with 2 mM L-arginine, but without access to extracellular L-arginine during the NO assay, showed an undiminished NO synthesis. We next determined intracellular L-arginine concentrations and NOS activity of J774.A1 macrophages exposed during the preincubation period (2 h) and the NO assay period (2 min) to 0 to 400 μM L-arginine in Locke's solution supplemented with 400 to 0 μM L-lysine to give a total of 400 μM cationic amino acids. A good correlation between intracellular and extracellular L-arginine concentrations was found, with the intracellular concentrations being about 10-fold higher than the extracellular concentrations (Fig. 6). However, when plotting the data, the regression line crossed the intracellular L-arginine axis at 230 μM, demonstrating a threshold level of L-arginine that was not dependent on extracellular concentrations of the amino acid. The NOS activity detected in cells incubated at an extracellular L-arginine concentration of 1.5 μM or below was only 5% of those incubated in 400 μM L-arginine. Maximal NO synthesis was reached in cells incubated in extracellular L-arginine concentrations as low as 25 μM (data not shown).

NO production in the EA.hy926 cells was dependent on the presence of a Ca^{2+} ionophore (Fig. 7). To manipulate the intracellular L-arginine concentrations, we incubated the cells for 24 h in DMEM without cationic amino acids supplemented with dialyzed fetal bovine serum and either 2 mM L-arginine or 2 mM L-lysine. A longer incubation with either amino acid led to a decrease in NOS activity and to cell mortality, most likely due to the lack of the respective cationic amino acids for protein synthesis. Before the NO assay, the cells were then washed carefully and incubated for 2 h at 37°C (with three buffer changes) in Locke's solution containing the same concentration of L-arginine or L-lysine as used in the 24-h incubation. During the NO assay, no extracellular L-arginine was provided to the L-lysine-treated cells, whereas the L-arginine-preincubated cells were further incubated in 2 mM L-arginine. In contrast to the NOS II in macrophages,

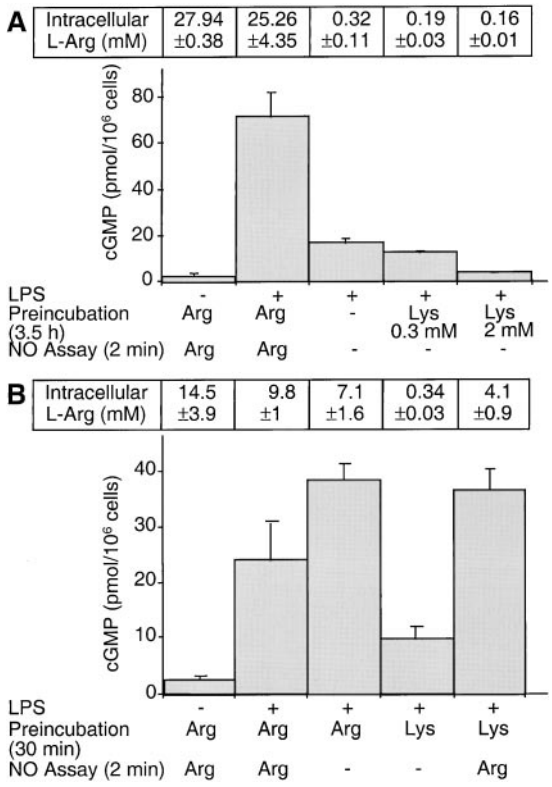

Fig. 5. Reduced NO production in J774.A1 macrophages preincubated in L-lysine. RFL-6 reporter cell assay with J774.A1 macrophages. Confluent cells in six-well plates were induced with LPS (1 μg/ml for 12–18 h) where indicated. A, cells were washed with Locke's solution and then preincubated for 3.5 h in Locke's solution containing either 2 mM L-arginine, no cationic amino acids, 0.3 mM L-lysine, or 2 mM L-lysine. The cells were then washed three times with ice-cold Locke's solution and for the NO assay, incubated during 2 min at 37°C in Locke's solution with 2 mM L-arginine or without cationic amino acids as indicated. The supernatants were then transferred to confluent RFL-6 reporter cells in six-well plates and incubated for 2 min at 37°C. Columns show intracellular cGMP concentrations of the reporter cells determined by radioimmunoassay after subtraction of the basal cGMP content of the RFL-6 cells (2.7 ± 0.9 pmol/10^6 cells) (means ± S.E., n = 4). The numbers above each column refer to intracellular L-arginine concentrations in the macrophages. B, experiments were performed as in (A) except that the preincubation of the macrophages was for 30 min in Locke's solution with 2 mM L-arginine or 2 mM L-lysine. The incubation for the NO assay was for 2 min in Locke's solution with 2 mM L-arginine or without cationic amino acids. The average cell volume (1099 ± 90 fl) did not change during the different incubations. Columns represent means ± S.E. (n = 4). The basal cGMP content of the RFL-6 cells (2.6 ± 0.21 pmol/10^6 cells) has been subtracted.

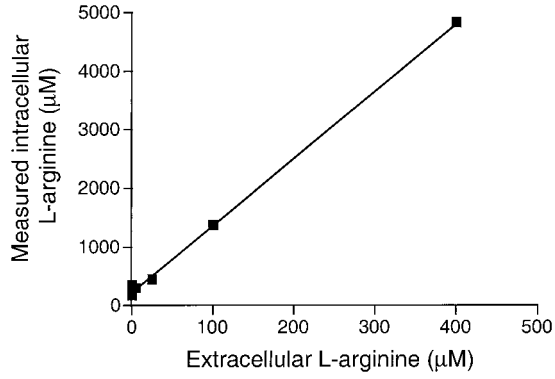

Fig. 6. Correlation between extracellular and intracellular L-arginine concentrations in J774.A1 macrophages. Plot of intracellular L-arginine concentrations measured in J774.A1 macrophages versus the extracellular L-arginine concentrations in the incubation buffer. Cells were exposed for 2 h (with three buffer changes) to 0 to 400 μM L-arginine in Locke's solution supplemented with 400 to 0 μM L-lysine to give a total of 400 μM cationic amino acids. After the incubation, cells were washed with ice-cold Locke's solution and lysed in 90% methanol, and the L-arginine content was determined by HPLC.

NOS III activity in EA.hy926 endothelial cells could not be altered by extracellular L-lysine (Fig. 7). Using crude extracts (100,000 g supernatant) of EA.hy926 cells in an arginine to citrulline conversion assays the K_M of NOS III for L-arginine was determined to be 3.4 ± 0.2 μM.

Discussion

The experiments presented were designed to study the substrate supply of NO synthases in macrophages and endothelial cells. Analysis of the CAT mRNA expression in LPS-induced J774A.1 mouse macrophages and human EA.hy926 endothelial cells revealed a constitutive expression of CAT-1 in both cell types. In contrast, CAT-2B expression was only found in macrophages. Similar to earlier results with mouse RAW 264.7 macrophages (Closs et al., 1993), LPS treatment strongly increased mCAT-2B expression in J774.A1 macrophages. The peak of CAT-2B expression was only reached after 12 to 18 h when NOS II expression was already back to the levels of uninduced cells, indicating a differential regulation of the expression of the two genes.

In agreement with the expression of CAT-1 and CAT-2B, transport of L-arginine was Na^+-independent and sensitive to *trans*-stimulation in both J774.A1 macrophages and EA.hy926 endothelial cells. The apparent K_M values for L-arginine obtained for the murine cells conform with K_M values obtained for mCAT-1 and mCAT-2B expressed in Xenopus oocytes (Closs et al., 1993). The K_M values for L-arginine transport in the human endothelial cells were about 3 to 4 times lower than in mouse macrophages correlating with the expression of hCAT-1 that also shows a higher apparent affinity than mCAT-1 or mCAT-2B when expressed in Xenopus oocytes (Closs et al., 1997b). As the low-affinity mCAT-2A could not be detected in either cell type, the transport component with low affinity for L-arginine found in J774.A1 macrophages is likely to derive from a yet unidentified carrier for L-arginine.

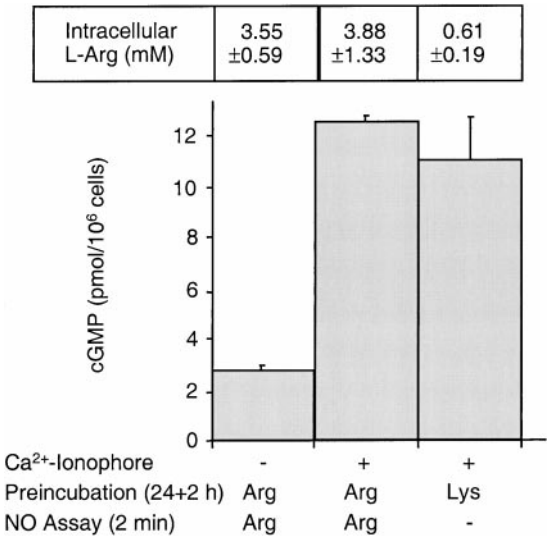

Fig. 7. Unaltered NO production in EA.hy926 endothelial cells after incubation in L-lysine. RFL-6 reporter cell assay with EA.hy926 endothelial cells. Experiments were performed as described in Fig. 5A. Cells were preincubated for 24 h in cationic amino acid-free DMEM supplemented with dialyzed fetal bovine serum and either 2 mM L-arginine or 2 mM L-lysine. The cells were then washed and incubated in Locke's solution containing the same concentration of L-arginine or L-lysine for another 2 h with two buffer changes. For the NO assay, cells were incubated for 2 min in Locke's solution containing, where indicated, 10 μM Ca^{2+} ionophore A23187 and either 2 mM L-arginine or no cationic amino acids. The average cell volume (694 ± 16 fl) did not change during the particular incubations. Columns represent means ± S.E. (n = 4). The basal cGMP content of the RFL-6 cells (3.1 ± 1.5 pmol/10^6 cells) has been subtracted.

With 1 and 0.1 mM extracellular [^{3}H]L-arginine, equilibrium in both macrophages and endothelial cells was reached after 30 and 60 min, respectively (data not shown), indicating a rapid exchange of basic amino acids between the extracellular space and an intracellullar L-arginine pool (termed pool I). In addition, efflux of [^{3}H]L-arginine was accelerated by extracellular substrate (L-arginine or L-lysine) in both cell types, and intracellular L-arginine concentrations dropped rapidly when cells were incubated in 2 mM L-lysine or vice versa. However, even a long-term incubation of the cells in 2 mM L-lysine-containing buffer (with several buffer changes) could not deplete the cells completely of intracellular L-arginine, suggesting a second L-arginine pool that is not freely exchangeable with the extracellular space via system y^+ carriers such as CAT-1 and CAT-2B (termed intracellular pool II). Additional support for the existence of pool II comes from plotting intracellular versus extracellular L-arginine concentrations where a pool of about 230 μM intracellular L-arginine was found to be independent of extracellular L-arginine. The drastically diminished NO synthesis in J774.A1 macrophages after incubation in 0.4 or 2 mM L-lysine indicates that NOS II has no access to L-arginine in the intracellular pool II. In contrast, L-arginine in both the intracellular pool I (that is freely exchangeable with the extracellular space) and in the extracellular space could be used equally well for NO synthesis by the macrophages. Access of NOS II to pool I was shown by an undiminished NO synthesis in L-arginine-preloaded macrophages that had no access to extracellular L-arginine during the NO assay. The unimpaired NO synthesis in L-lysine-depleted cells exposed to L-arginine during the NO assay demonstrates the fast replenishment of pool II by extracellular L-arginine. Studies investigating the CAT expression, L-arginine transport, and substrate supply of NOS II in RAW 264.7 macrophages showed similar results (data not shown). Taken together, NO synthesis in macrophages seems only to be dependent on extracellular L-arginine when the intracellular L-arginine pool I cannot provide sufficient substrate.

An intracellular L-arginine pool II that could not be depleted by incubation in high concentrations of L-lysine was also found in EA.hy926 endothelial cells. The NO synthesis in these cells was unchanged after L-lysine depletion, indicating that NOS III does have access to substrate in pool II. The intracellular L-arginine concentrations after L-lysine depletion were higher in endothelial cells than in macrophages. This could be due to the low activity of L-arginine-consuming enzymes such as NOS or arginase or to a high activity of L-arginine-recycling enzymes that might replenish pool II. However, L-lysine depletion of freely available L-arginine should not be a function of L-arginine consumption or synthesis, but should only depend on the rate of exchange of cationic amino acids between the intracellular and extracellular spaces. This exchange was found to be very similar in endothelial cells and macrophages. Therefore, treatment of EA.hy926 cells with 2 mM L-lysine for up to 24 h must have been sufficient to deplete pool I in these cells. Indeed, an incubation longer then 24 h in either 2 mM L-lysine or 2 mM

L-arginine led to a loss of cell viability, indicating a depletion of L-arginine or L-lysine, respectively, for crucial biosynthetic pathways such as protein synthesis. As L-lysine, that also carries a positive charge, could not drive out L-arginine from pool II, this pool cannot be simply explained by an unspecific binding of L-arginine to negatively charged cell components. The intracellular L-arginine pool II might be a membrane-encircled compartment. However, it could also consist of L-arginine-binding and/or -recycling proteins capable of passing L-arginine directly on to NOS III. It is interesting to speculate that an impaired access to pool II might underlie the L-arginine paradox, where under pathophysiological situations such as diabetes, hypertension, or hypercholesterolemia, endothelial NO production can be increased by extracellular L-arginine in spite of sufficiently high intracellular L-arginine concentrations.

In conclusion, our results suggest the existence of at least two L-arginine pools in macrophages and endothelial cells, pool I that is freely exchangeable with the extracellular L-arginine (or other cationic amino acids) via the system y^+ carriers CAT-1 and CAT-2B and pool II that is not freely exchangeable with the extracellular space. NOS II in macrophages can only use L-arginine from pool I. In contrast, NOS III in endothelial cells seems to also have access to pool II. It is therefore likely that the CAT-mediated L-arginine transport plays a more important role for the substrate supply of NOS II in macrophages than for NOS III in endothelial cells.

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