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Characterization of Endothelial Cell Amino Acid Transport Systems Involved in the Actions of Nitric Oxide Synthase Inhibitors

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SUMMARY

N^{ω}-Substituted analogues of L-arginine have proven useful as specific inhibitors of nitric oxide formation in various biological systems. In the present study we describe the characteristics of amino acid transporters that mediate uptake of N^{ω}-methyl-L-arginine (L-NMA) and N^{ω}-nitro-L-arginine (L-NNA) into cultured porcine aortic endothelial cells. The transport of L-[^{14}C]NMA showed biphasic kinetics, with K_m values of 4 and 368 μM, and was inhibited by L-arginine, L-homoarginine, L-lysine, and L-ornithine but not by L-leucine or L-isoleucine. Similar transport kinetics (K_m values of 6 and 609 μM) and substrate specificities were obtained for L-[^{3}H]arginine uptake, indicating that L-arginine and L-NMA are transported by the same system. In contrast to L-arginine and L-NMA transport, uptake of L-[^{3}H]NNA was monophasic (K_m = 617 μM) and was inhibited by L-leucine and L-isoleucine but not by L-arginine, L-homoarginine, L-NMA, L-lysine, or L-ornithine. Uptake studies with L-[^{3}H]leucine revealed that the transport of this amino acid occurred in a manner very similar to that of L-[^{3}H]NNA transport, suggesting that the uptake of both compounds may be mediated by the same system. In additional experiments, we determined the effects of L-NMA and L-NNA on the A23187-induced accumulation of intracellular cGMP, to establish to what extent these transport systems are involved in the actions of nitric oxide synthase inhibitors. L-Lysine and L-ornithine, which both inhibited L-NMA uptake, increased the IC_{50} of L-NMA from 7.8 μM to 57 μM but did not reduce the inhibitory effects of L-NNA. In the presence of L-leucine or L-isoleucine, however, which both inhibited L-NNA uptake, the IC_{50} of L-NNA was increased from 1.2 μM to 37 μM but the inhibitory actions of L-NMA remained unaffected. These data demonstrate that the endothelial transport systems for L-arginine and L-leucine mediate the biological effects of L-NMA and L-NNA, respectively.

In response to various hormones and neurotransmitters endothelial cells release NO, which acts as a potent vasodilator through stimulation of soluble guanylyl cyclase in adjacent smooth muscle cells (for review, see Refs. 1 and 2). Biosynthesis of endothelium-derived NO is triggered by the intracellular concentration of free Ca^{2+}, which is required for the activation of constitutively expressed soluble and membrane-associated forms of NO synthase (3, 4). Consistent with the identification of the guanidino group of L-arginine as the precursor of NO (5, 6), N^{ω}-derivatized analogues of L-arginine were found to be potent inhibitors of NO formation (2). L-NMA, L-NNA, and L-NAME have been used frequently to block NO synthesis in isolated blood vessels (7–9), cultured cells (10–13), and cell-free systems (4, 14–17). Interestingly, these compounds seem to exhibit different pharmacological profiles, because the rank orders of their potencies were determined to be tissue dependent (7, 10, 18–20). Different properties of NO synthase isoforms or different cellular uptake mechanisms may account for the diverse actions of these inhibitors. In fact, L-NMA was described as a potent and irreversible inhibitor of cytokine-inducible NO synthase (21), whereas the constitutive isozymes are more effectively inhibited by the nitro derivatives of L-arginine (17, 22). Moreover, L-NNA and L-NAME inhibit the substrate-independent reduction of molecular oxygen that is catalyzed by brain NO synthase, whereas L-NMA does not affect this reaction (23, 24). These data suggest profound differences in the molecular actions of these compounds. Only little is known, however, about the transport systems mediating cellular uptake of NO synthase inhibitors. It was recently demonstrated that L-NMA but not L-NNA inhibits L-[^{3}H]arginine uptake, indicating that the uptake of L-NMA may be mediated by the endothelial L-arginine transporter (25), but the mechanism by which L-NNA enters intact cells is still unclear. We addressed this issue and used radiolabeled L-NMA and L-NNA to investigate the transport of these compounds into cultured porcine aortic endothelial cells. Using this direct approach, we found that L-NMA utilizes the L-arginine transporter, whereas uptake

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ABBREVIATIONS: NO, nitric oxide; L-NMA, N^{ω}-methyl-L-arginine; L-NNA, N^{ω}-nitro-L-arginine; L-NAME, N^{ω}-nitro-L-arginine methyl ester; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

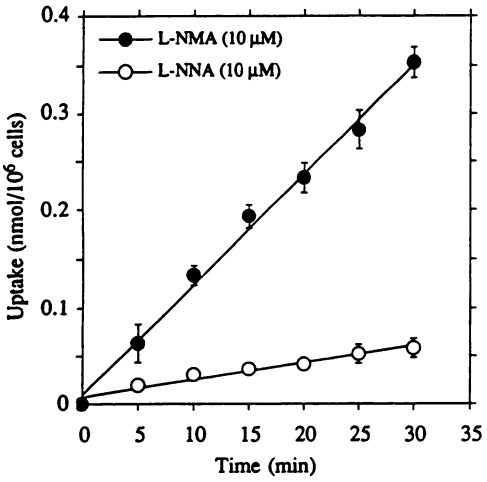

Fig. 1. Time course of L-NMA and L-NNA uptake. Endothelial cells were incubated at 37° in the presence of 10 μM L-[^{14}C]NMA or L-[^{3}H]NNA (~300,000 dpm each). At the time points indicated, cells were washed three times with 2 ml of ice-cold incubation buffer and digested with 0.1 N NaOH, and radioactivity was measured by liquid scintillation counting. Data are mean ± standard error values from three separate experiments.

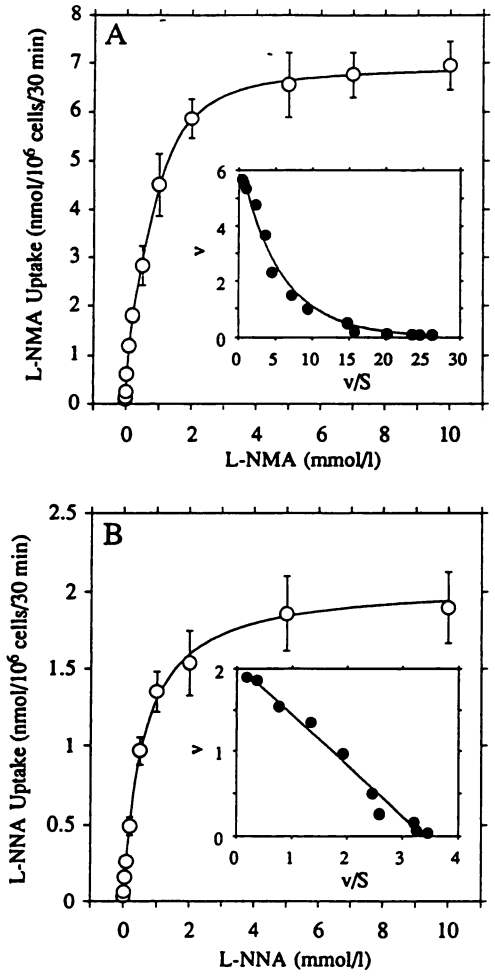

Fig. 2. Kinetics of L-NMA (A) and L-NNA (B) transport. Uptake of L-[^{14}C]NMA and L-[^{3}H]NNA (0.3 μM to 10 mM; ~300,000 dpm) was measured at 37° over a time period of 30 min. Data represent mean ± standard error values from four separate experiments. *Insets*, respective Eadie-Hofstee plots.

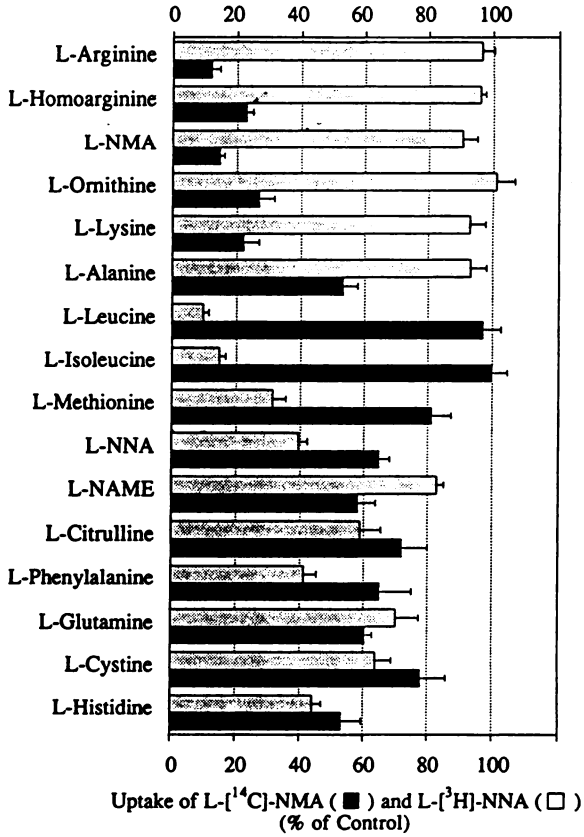

Fig. 3. Effects of amino acids on L-[^{14}C]NMA and L-[^{3}H]NNA uptake. Endothelial cells were incubated at 37° for 30 min with ~2 μM L-[^{14}C]NMA or ~3 nM L-[^{3}H]NNA (~300,000 dpm each), in the absence (control) or presence of the unlabeled amino acids (1 mM each). Data are mean ± standard error values (three to six experiments) and are expressed as percentage of control uptake.

of L-NNA is apparently mediated by the L-leucine transporter. Determination of intracellular cGMP levels as a marker for NO synthesis revealed that both transport systems are directly involved in the inhibitory actions of NO synthase inhibitors in endothelial cells.

Experimental Procedures

Materials. Tissue culture media and ingredients except fetal calf serum (SEBAK GmbH, Aidenbach, Germany) were from GIBCO/BRL GmbH (Eggenstein, Germany); plates and Petri dishes were from Costar Europe Ltd. (Badhoevedorp, The Netherlands). L-[2,3,4,5-^{3}H]Arginine, L-[2,3,4,5-^{3}H]NNA, and L-[5-^{14}C]NMA were generous gifts from Amersham International, UK. All other biochemicals were purchased from Sigma Chemical GmbH (Deisenhofen, Germany).

Cell culture. Porcine aortic endothelial cells were isolated by enzymatic treatment with 0.1% collagenase and were cultured for up to two passages in Petri dishes in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and antibiotics, as described previously (26, 27). Identity of the endothelial cells was verified by immunofluorescence (factor VIII antigen). Before the experiments, the cells were subcultured in six-well plastic plates and confluent monolayers (~10^{6} cells/dish) were used for transport and cGMP measurements.

Measurement of amino acid transport. Endothelial cells were washed with isotonic HEPES buffer, pH 7.4, containing 2.5 mM $CaCl_2$ and 1 mM $MgCl_2$ (incubation buffer) and were equilibrated for 15 min at 37° in 0.8 ml of the same buffer. Amino acid uptake was initiated by addition of 0.1 ml of a solution of the respective radioactive compound, L-[^{14}C]NMA, L-[^{3}H]NNA, L-[^{3}H]arginine, or L-[^{3}H]leucine (~300,000

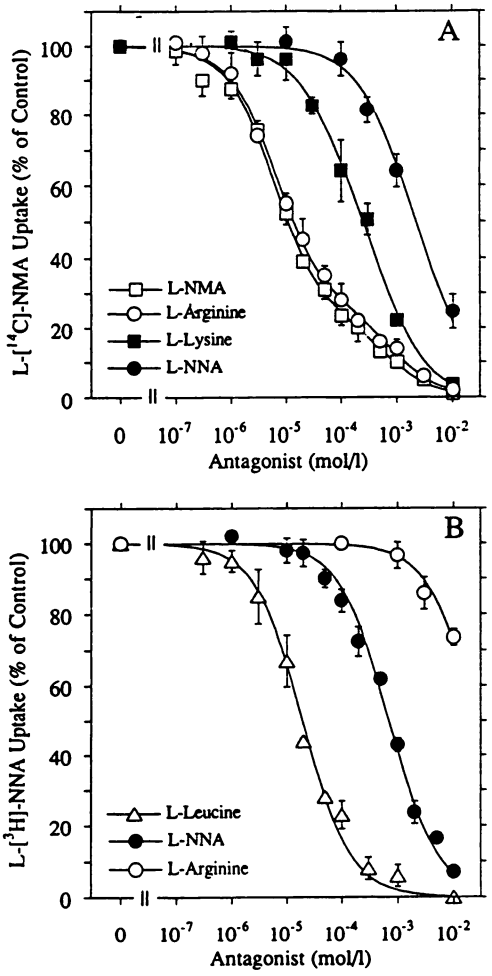

Fig. 4. Concentration-dependent inhibition by amino acids of L-[^{14}C]NMA (A) and L-[^{3}H]NNA (B) uptake. Endothelial cells were incubated at 37° for 30 min with ~2 μM L-[^{14}C]NMA or ~3 nM L-[^{3}H]NNA (~300,000 dpm each), in the absence (control) or presence of increasing concentrations of the unlabeled amino acids. Data are mean ± standard error values (four or five experiments) and are expressed as percentage of control uptake.

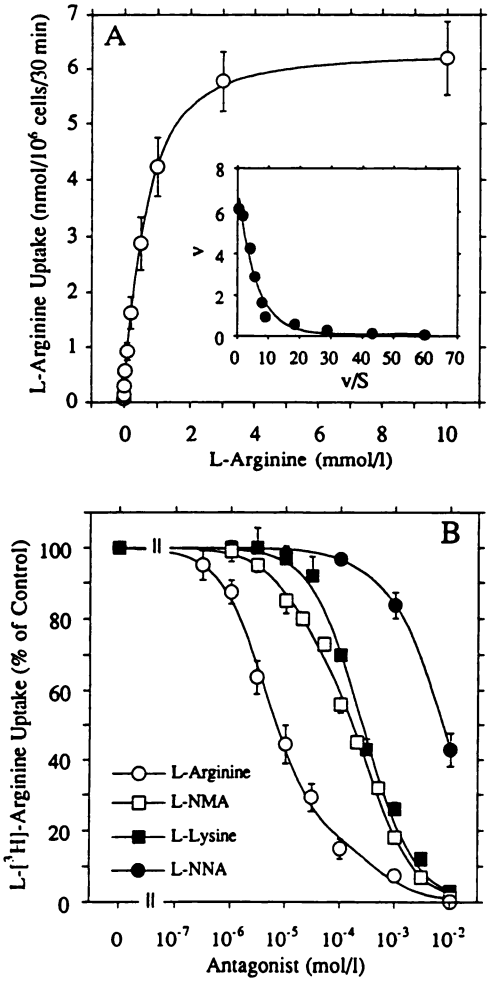

Fig. 5. Kinetics of L-arginine transport (A) and inhibition by unlabeled amino acids of L-[^{3}H]arginine uptake (B). A, Experiments were performed with L-[^{3}H]arginine concentrations ranging from 0.3 μM to 10 mM (~300,000 dpm). Data represent mean ± standard error values from five separate experiments. *Inset*, Eadie-Hofstee plot of these data. B, Endothelial cells were incubated with ~3 nM L-[^{3}H]arginine (~300,000 dpm) in the absence (control) or presence of increasing concentrations of the unlabeled amino acids. Data are mean ± standard error values (four or five experiments) and are expressed as percentage of control uptake.

dpm each), and 0.1 ml of a solution of the compounds to be tested. After 30 min, cells were washed three times with 2 ml of ice-cold incubation buffer and digested with 1 ml of 0.1 N NaOH. The alkaline solution was transferred into 4-ml plastic vials and mixed with 3 ml of a scintillation cocktail (Ultima Gold XR; Canberra/Packard), and the radioactivity was measured. In some experiments, D-[^{14}C]mannitol was additionally present during the incubation as an extracellular tracer. The recovery of D-[^{14}C]mannitol was <0.05%. For efflux measurements, cells were incubated for 30 min with the respective radioactive compounds, washed three times with 2 ml of prewarmed incubation buffer, and incubated for an additional 30 min in the absence of any amino acids. The radioactivity released into the incubation buffer was measured and compared with the initially incorporated radioactivity. Within 30 min, 15 ± 1.5% of L-[^{3}H]arginine, 12 ± 2.9% of L-[^{3}H]leucine, 21 ± 1.5% of L-[^{3}H]NNA, and 38 ± 4.9% of L-[^{14}C]NMA were released from the endothelial cells (four experiments).

Measurement of endothelial cGMP levels. Endothelial cells were washed twice with isotonic HEPES buffer, pH 7.4, containing 2.5 mM $CaCl_2$ and 1 mM $MgCl_2$ and were preincubated for 15 min at 37° in 1.4 ml of the same buffer containing 1 mM isobutylmethylxanthine and 1 μM indomethacin, as described previously (26, 27). The incubation was started by addition of 0.1 ml of a solution of the compound to be tested and was stopped after 4 min by removal of the incubation medium and addition of 1 ml of 0.01 N HCl. Within 1 hr, intracellular cGMP was completely released into the supernatant and was measured by radioimmunoassay.

Data analysis. Data obtained from saturation or inhibition experiments were analyzed with a Hewlett-Packard work station (series 9000, model 310) using the GIPMAX nonlinear least-squares computer curve-fitting program (28). All data were given equal weight and were fitted to a one- or two-site model. Statistical significance of the difference between the two models was tested by comparing their residual variances of fits to the data by a partial F test. All K_m, K_i, and IC_{50} values were derived from individual concentration-response curves and are expressed as geometric means with 95% confidence limits, calculated as the product of the standard error × Student's t value; V_{max} values were calculated as arithmetic means ± standard errors.

Results

Time course of L-NMA and L-NNA transport. Uptake of L-NMA and L-NNA into cultured porcine aortic endothelial cells was linear over at least 30 min. Within this time, uptake of L-NMA and L-NNA (10 μM each) was 353 ± 15 pmol/10^{6}

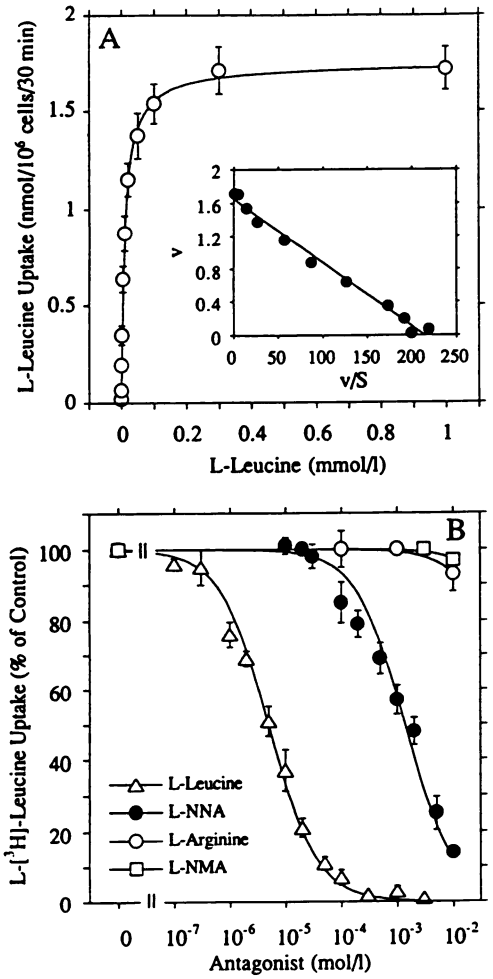

Fig. 6. Kinetics of L-leucine transport (A) and inhibition by unlabeled amino acids of L-[^{3}H]leucine uptake (B). A, Experiments were performed with L-leucine concentrations ranging from 0.1 μM to 1 mM (~300,000 dpm). Data points represent mean ± standard error values from four separate experiments. *Inset*, Eadie-Hofstee plot of these data. B, Endothelial cells were incubated with ~3 nM L-[^{3}H]leucine (~300,000 dpm) in the absence (control) or presence of increasing concentrations of the unlabeled amino acids. Data are mean ± standard error values (four or five experiments) and are expressed as percentage of control uptake.

cells and 58 ± 2 pmol/10^6 cells, respectively (Fig. 1). Similarly, a constant uptake rate was observed when only tracer concentrations of L-[^{14}C]NMA (~1 μM) or L-[^{3}H]NNA (~3 nM) were used (data not shown). In all additional experiments uptake was measured over a time period of 30 min.

Kinetics of L-NMA and L-NNA transport. In kinetic studies, saturable uptake of L-NMA and L-NNA was found at concentrations ranging from 0.3 μM to 10 mM (Fig. 2). As evident from Eadie-Hofstee plots and from computer modeling of the raw data (see Experimental Procedures), uptake of L-NMA was biphasic, with two clearly separated affinity states. The K_m values and 95% confidence limits were 6 (4–9) μM and 815 (425–1555) μM for the high and low affinity states, respectively, and the corresponding V_{max} values (mean ± standard error) were 0.16 ± 0.03 and 6.1 ± 0.67 nmol/10^6 cells/30 min. Transport of L-NNA, however, followed simple Michaelis kinetics, with a K_m of 617 (363–1050) μM and a V_{max} of 2.0 ± 0.33 nmol/10^6 cells/30 min.

Inhibition by unlabeled amino acids of L-[^{14}C]NMA and L-[^{3}H]NNA uptake. The effects of various amino acids and L-arginine derivatives (1 mM each) were studied to investigate the substrate specificity of the transport systems accounting for the uptake of L-NMA and L-NNA. As shown in Fig. 3, the uptake of L-[^{14}C]NMA was inhibited by L-arginine (12.1% of control), L-homoarginine (23.0%), L-NMA (14.8%), L-ornithine (27.0%), L-lysine (22.2%), and L-alanine (53.3%) but remained unaffected in the presence of L-leucine (97.2%) or L-isoleucine (99.2%). On the other hand, uptake of L-[^{3}H]NNA was effectively blocked by L-leucine (9.7%) and L-isoleucine (14.8%) but was not affected by L-arginine (96.6%), L-homoarginine (96.0%), L-NMA (90.5%), L-ornithine (101.0%), L-lysine (93.0%), or L-alanine (93.3%). A number of other compounds did not clearly differentiate between the two putative transport systems (L-methionine, L-NNA, L-NAME, L-citrulline, L-phenylalanine, L-glutamine, L-cystine, and L-histidine) or were completely inactive (L-threonine, L-tryptophan, L-tyrosine, L-valine, and L-serine; data not shown).

Fig. 4 shows the concentration-dependent inhibition by some selected amino acids of L-[^{14}C]NMA (Fig. 4A) and L-[^{3}H]NNA (Fig. 4B) uptake. The rank order of potency for inhibition of L-[^{14}C]NMA transport was L-arginine ≈ L-NMA > L-lysine > L-NNA. Interestingly, L-arginine and L-NMA showed biphasic inhibition curves, whereas L-lysine and L-NNA inhibited L-[^{14}C]NMA uptake in a monophasic reaction. In contrast to L-[^{14}C]NMA uptake, L-[^{3}H]NNA transport was inhibited in a monophasic manner by all amino acids investigated, with a rank order of potency of L-leucine > L-NNA > L-arginine. These data clearly indicate that uptake of L-NMA is mediated by the L-arginine transporter and uptake of L-NNA is mediated by the L-leucine transporter system.

Characterization of L-arginine and L-leucine transport. We investigated the kinetics and substrate specificity of L-arginine and L-leucine uptake, to further characterize these transport systems. Similarly to the uptake of L-NMA, transport of L-arginine was biphasic, with K_m values and 95% confidence limits of 6 (2–15) and 609 (297–1248) μM (Fig. 5A). The V_{max} values (mean ± standard error) for the high and low affinity uptake were 0.4 ± 0.09 and 6.0 ± 0.41 nmol/10^6 cells/30 min, respectively. Fig. 5B shows that uptake of L-[^{3}H]arginine was inhibited by L-arginine and L-NMA in a biphasic manner, whereas L-lysine and L-NNA showed monophasic inhibition curves. The rank order of potency for inhibition of L-[^{3}H]arginine transport was L-arginine > L-NMA > L-lysine > L-NNA.

Fig. 6A shows that endothelial cells contain a saturable monophasic transport system for L-leucine, exhibiting a K_m of 5 (2–11) μM and a V_{max} of 1.7 ± 0.39 nmol/10^6 cells/30 min. Uptake of L-[^{3}H]leucine was antagonized by L-NNA, but the NO synthase inhibitor exhibited a much lower potency than the natural substrate of the transporter (Fig. 6B). L-Arginine and L-NMA (up to 10 mM) did not interfere with the uptake of L-leucine.

Comparison between L-arginine and L-leucine transport systems. Table 1 summarizes the affinity constants of various amino acids, as obtained in inhibition experiments with L-[^{3}H]arginine, L-[^{14}C]NMA, L-[^{3}H]leucine, and L-[^{3}H]NNA as radiolabeled markers for the respective transport systems. Almost identical affinity constants for L-arginine and L-NMA were obtained when the L-arginine transporter was labeled with either L-[^{3}H]arginine or L-[^{14}C]NMA. The respective K_i values

TABLE 1

Affinities (K_i values) of various amino acids for L-arginine and L-leucine transporters

Uptake of L-[^{14}C]NMA (~2 μM), L-[^{3}H]arginine, L-[^{3}H]NNA, and L-[^{3}H]leucine (~3 nM each) was measured in the presence of increasing concentrations of various unlabeled amino acids. K_i values were derived from at least three individual inhibition curves, fitted to either one- or two-site models (see Experimental Procedures), and are expressed as geometric means, with 95% confidence limits in parentheses.

Amino acid	K_i			
	L-Arginine transporter labeled with		L-Leucine transporter labeled with	
	L-[^{14}C]NMA	L-[^{3}H]Arginine	L-[^{3}H]NNA	L-[^{3}H]Leucine
	μM			
L-Arginine	4 (2–8) 368 (152–891)	6 (2–15) 609 (297–1,248)	>10,000	>10,000
L-NMA	6 (4–9) 815 (425–1,555)	17 (12–22) 460 (284–745)	>10,000	>10,000
L-Lysine	346 (98–1,228)	220 (115–399)	>10,000	>10,000
L-Ornithine	224 (61–820)	384 (286–516)	>10,000	>10,000
L-NNA	~2,000	~7,000	617 (363–1,050)	655 (556–772)
L-Leucine	>10,000	>10,000	22 (8–68)	5 (2–11)
L-Isoleucine	>10,000	>10,000	24 (16–35)	65 (55–76)
L-NAME	~2,000	>10,000	~7,000	~7,000
D-Arginine	~3,000	~3,000	ND*	ND
D-Leucine	ND	ND	78 (26–230)	118 (65–214)

* ND, not determined.

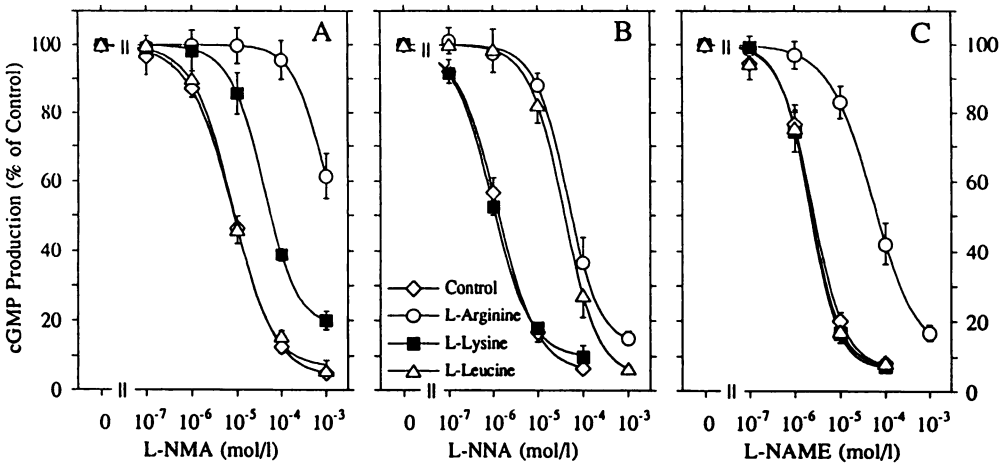

Fig. 7. Effects of L-NMA (A), L-NNA (B), and L-NAME (C) on endothelial cGMP levels. Endothelial cells were preincubated at 37° with increasing concentrations of the respective antagonist in the absence (control) or presence of 10 mM L-arginine, L-lysine, or L-leucine. After 15 min, 1 μM A23187 was added and the cells were incubated for an additional 4 min. cGMP accumulation was measured as described in Experimental Procedures. Data are mean ± standard error values (four or five experiments) and are expressed as percentage of controls measured in the absence of antagonists.

were 4–17 μM and 368–815 μM for the high and low affinity uptake, respectively. L-Lysine and L-ornithine inhibited L-[^{3}H]arginine and L-[^{14}C]NMA transport in a monophasic manner, with K_i values between 220 and 384 μM. In contrast to the basic amino acids L-arginine, L-NMA, L-lysine, and L-ornithine, L-NNA and the branched chain amino acids L-leucine and L-isoleucine exhibited much lower affinities for the L-arginine transporter than for the L-leucine transporter. Neither of these systems apparently mediates the uptake of L-NAME (K_i > 2 mM). D-Arginine (K_i ~ 3 mM) and D-leucine (K_i ~ 0.1 mM) were less active than their L-isoforms, but the stereoselectivity of the L-arginine transporter (~600-fold) was much higher than that of the L-leucine transporter (~20-fold).

Inhibition of NO biosynthesis by L-arginine derivatives. Formation of NO in response to the calcium ionophore A23187 was determined as accumulation of intracellular cGMP (26, 29). As shown in Fig. 7, A23187-stimulated cGMP formation was inhibited in a concentration-dependent manner by L-NMA (Fig. 7A), L-NNA (Fig. 7B), and L-NAME (Fig. 7C). The IC_{50} value for L-NMA was 7.8 μM under control conditions and was increased by >7-fold (to 57 μM) in the presence of 10 mM L-lysine. An even more pronounced effect was observed in the presence of 10 mM L-arginine (IC_{50} for L-NMA > 1 mM), whereas L-leucine, which does not interfere with L-arginine transport, did not antagonize the inhibitory effect of L-NMA. Fig. 7B shows that the effect of L-NNA (IC_{50} = 1.2 μM) was reduced in the presence of 10 mM L-leucine (IC_{50} = 37 μM) but remained unaffected in the presence of L-lysine. Although L-arginine does not block the uptake of L-NNA, it antagonized the effect of the inhibitor (IC_{50} = 44 μM). As shown in Fig. 7C, the IC_{50} of L-NAME was increased by L-arginine (2.4–50 μM) but not by L-lysine or by L-leucine. In all these experiments, L-ornithine and L-isoleucine exhibited effects very similar to those of L-lysine and L-leucine, respectively (data not shown).

Discussion

In the present study we characterized endothelial amino acid transporters involved in the uptake of NO synthase inhibitors. Our data are consistent with previous suggestions that different transport systems may be involved in the cellular actions of L-NMA and N^{ω}-nitro derivatives of L-arginine (7). In a recent paper it was demonstrated that L-NMA but not L-NNA or L-NAME inhibited the transport of L-[^{3}H]arginine into endothelial cells (25). These data indicate that different mechanisms account for the uptake of these compounds, but the unequivocal identification of the respective transport systems requires di-

rect uptake studies. Because radiolabeled NO synthase inhibitors became available recently, we investigated the uptake of L-[^{14}C]NMA and L-[^{3}H]NNA into endothelial cells. We found that the kinetics of L-NMA transport closely resembled those of L-arginine, whereas L-NNA was a substrate for the L-leucine transporter. The uptake of L-NMA and L-arginine was biphasic, with K_m values of ~6 μM and ~600 μM for the high and low affinity sites, respectively. Such biphasic kinetics, with almost identical affinity constants, were also found for the transport of L-arginine in *Cyanobacterium anabaena* (30), whereas only monophasic uptake mechanisms were described in most other cells and tissues, with K_m values ranging from 70 μM to 1.7 mM (31–35). L-[^{14}C]NMA uptake and L-[^{3}H]arginine uptake were inhibited by L-lysine and L-ornithine in a monophasic manner, with K_i values of ~300 μM, indicating that these amino acids interact only with the low affinity site of the L-arginine transporter.

The characteristics of L-NNA transport were completely different from those of L-arginine transport, because L-NNA uptake was strictly monophasic and effectively inhibited by L-leucine. Furthermore, the K_m for L-[^{3}H]NNA transport was almost identical to the K_i for inhibition of L-[^{3}H]leucine uptake. These data demonstrate that L-NNA is a substrate of the L-leucine transporter. In accordance with a previous study using human umbilical vein endothelial cells (36), we observed a rather low stereoselectivity of leucine uptake. The affinity constant for L-leucine we obtained with porcine aortic endothelial cells (5 μM) was 15–50-fold lower than those previously described for endothelial cells cultured from cerebral capillaries (37) or human umbilical veins (36).

We screened numerous amino acids for their ability to interact with the endothelial L-arginine and L-leucine transport systems. The basic amino acids L-arginine, L-lysine, and L-ornithine inhibited only L-[^{3}H]arginine and L-[^{14}C]NMA uptake, whereas the branched chain compounds L-leucine and L-isoleucine selectively antagonized L-[^{3}H]leucine and L-[^{3}H]NNA transport. These data suggest that L-arginine and L-leucine are transported into endothelial cells by the y^+ and L systems, respectively, which are present in most mammalian cells (for review, see Refs. 31 and 38). Both transport systems apparently mediate the biological effects of L-NMA and L-NNA in endothelial cells. L-Lysine and L-ornithine specifically antagonized the inhibition by L-NMA of A23187-induced cGMP accumulation, whereas L-leucine and L-isoleucine attenuated only the inhibitory effects of L-NNA. In contrast to the high selectivity of these amino acids in antagonizing the actions of L-NMA or L-NNA, the effects of L-arginine were quite different. The substrate of NO synthase not only was more potent than L-lysine and L-ornithine in antagonizing the inhibitory actions of L-NMA but also attenuated the effects of L-NNA and L-NAME, although it did not interfere with the uptake of these compounds. These data clearly indicate that L-arginine not only inhibits uptake of L-NMA but also antagonizes the inhibitory effects of all investigated NO synthase inhibitors by a second mechanism. This additional mechanism could involve competition between L-arginine and its N^{ω}-derivatized analogues for the substrate binding site of NO synthase and would explain why, similarly to our results, L-arginine not only reduced the inhibitory effect of L-NMA on NO formation in co-cultures of endothelial and smooth muscle cells (11) but also antagonized the effects of L-NMA, L-NNA, and L-NAME on NO production in phagocytic cells (19).

In conclusion, our data demonstrate that the membrane transporters for L-arginine and L-leucine are essentially involved in the endothelial L-arginine/NO pathway and in the actions of NO synthase inhibitors. Tissue-specific expression and/or properties of these transport systems may at least partially explain the different pharmacological profiles described for L-arginine analogues. Future studies, including the characterization of these transporters in other tissues, should provide additional insights into the mechanisms of action of NO synthase inhibitors.

Acknowledgments

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References

1. Ignarro, L. J. Biosynthesis and metabolism of endothelium-derived nitric oxide. *Annu. Rev. Pharmacol. Toxicol.* **30**:535–560 (1990).
2. Moncada, S., R. M. J. Palmer, and E. A. Higgs. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43**:109–142 (1991).
3. Mayer, B., and E. Böhme. Ca^{2+}-dependent formation of an L-arginine-derived activator of soluble guanylyl cyclase in bovine lung. *FEBS Lett.* **256**:211–214 (1989).
4. Pollock, J. S., U. Förstermann, J. A. Mitchell, T. D. Warner, H. H. H. W. Schmidt, M. Nakane, and F. Murad. Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. USA* **88**:10480–10484 (1991).
5. Palmer, R. M. J., D. S. Ashton, and S. Moncada. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature (Lond.)* **333**:664–666 (1988).
6. Schmidt, H. H. H. W., H. Nau, W. Wittfoht, J. Gerlach, K.-E. Prescher, M. M. Klein, F. Niroomand, and E. Böhme. Arginine is a physiological precursor of endothelium-derived nitric oxide. *Eur. J. Pharmacol.* **154**:213–216 (1988).
7. Rees, D. D., R. M. J. Palmer, R. Schulz, H. F. Hodson, and S. Moncada. Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.* **101**:746–752 (1990).
8. Rees, D. D., R. M. J. Palmer, H. F. Hodson, and S. Moncada. A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. *Br. J. Pharmacol.* **96**:418–424 (1989).
9. Moore, P. K., O. A. Al-Swayeh, N. W. S. Chong, R. A. Evans, and A. Gibson. L-N^{G}-Nitroarginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br. J. Pharmacol.* **99**:408–412 (1990).
10. Gross, S., D. Stuehr, K. Aisaka, E. Jaffe, R. Levi, and O. Griffith. Macrophage and endothelial cell nitric oxide synthesis: cell-type selective inhibition by N^{G}-aminoarginine, N^{G}-nitroarginine and N^{G}-methylarginine. *Biochem. Biophys. Res. Commun.* **170**:96–103 (1990).
11. Johns, R., M. Peach, J. Linden, and A. Tichotsky. N^{G}-Monomethyl L-arginine inhibits endothelium-derived relaxing factor-stimulated cyclic GMP accumulation in cocultures of endothelial and vascular smooth muscle cells by an action specific to the endothelial cell. *Circ. Res.* **67**:979–985 (1990).
12. Zembowicz, A., M. Hecker, H. Macarthur, W. C. Sessa, and J. R. Vane. Nitric oxide and another potent vasodilator are formed from N^{G}-hydroxy-L-arginine by cultured endothelial cells. *Proc. Natl. Acad. Sci. USA* **88**:11172–11176 (1991).
13. Ishii, K., H. Sheng, T. D. Warner, U. Förstermann, and F. Murad. A simple and sensitive bioassay method for detection of EDRF with RFL-6 rat lung fibroblasts. *Am. J. Physiol.* **261**:H598-H603 (1991).
14. Stuehr, D., N. Kwon, S. Gross, B. Thiel, R. Levi, and C. Nathan. Synthesis of nitrogen oxides from L-arginine by macrophage cytosol: requirement for inducible and constitutive components. *Biochem. Biophys. Res. Commun.* **161**:420–426 (1989).
15. Mayer, B., K. Schmidt, P. Humbert, and E. Böhme. Biosynthesis of endothelium-derived relaxing factor: a cytosolic enzyme in porcine aortic endothelial cells Ca^{2+}-dependently converts L-arginine into an activator of soluble guanylyl cyclase. *Biochem. Biophys. Res. Commun.* **164**:678–685 (1989).
16. Klatt, P., K. Schmidt, G. Uray, and B. Mayer. Multiple catalytic functions of brain nitric oxide synthase: biochemical characterization, cofactor requirement and the role of N^{ω}-hydroxyl-L-arginine as an intermediate. *J. Biol. Chem.* **268**:14781–14787 (1993).
17. Förstermann, U., H. H. H. W. Schmidt, J. S. Pollock, H. Sheng, J. A. Mitchell, T. D. Warner, M. Nakane, and F. Murad. Isoforms of nitric oxide synthase: characterization and purification from different cell types. *Biochem. Pharmacol.* **42**:1849–1857 (1991).
18. Knowles, R. G., M. Palacios, R. M. J. Palmer, and S. Moncada. Kinetic

characteristics of nitric oxide synthase from rat brain. *Biochem. J.* **269**:207–210 (1990).
19. McCall, T. B., M. Feelisch, R. M. J. Palmer, and S. Moncada. Identification of *N*-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells. *Br. J. Pharmacol.* **102**:234–238 (1991).
20. Martin, G., M. Bolofo, and H. Giles. Inhibition of endothelium-dependent vasorelaxation by arginine analogues: a pharmacological analysis of agonist- and tissue-dependence. *Br. J. Pharmacol.* **105**:643–652 (1992).
21. Olken, N. M., K. M. Rusche, M. K. Richards, and M. A. Marletta. Inactivation of macrophage nitric oxide synthase activity by N^G-methyl-L-arginine. *Biochem. Biophys. Res. Commun.* **177**:828–833 (1991).
22. Dwyer, M. A., D. S. Bredt, and S. H. Snyder. Nitric oxide synthase: irreversible inhibition by L-N^G-nitroarginine in brain *in vitro* and *in vivo*. *Biochem. Biophys. Res. Commun.* **176**:1136–1141 (1991).
23. Heinzel, B., M. John, P. Klatt, E. Böhme, and B. Mayer. Ca^{2+}/calmodulin-dependent formation of hydrogen peroxide by brain nitric oxide synthase. *Biochem. J.* **281**:627–630 (1992).
24. Pou, S., W. S. Pou, D. S. Bredt, S. H. Snyder, and G. M. Rosen. Generation of superoxide by purified brain nitric oxide synthase. *J. Biol. Chem.* **267**:24173–24176 (1992).
25. Bogle, R. G., S. Moncada, J. D. Pearson, and G. E. Mann. Identification of inhibitors of nitric oxide synthase that do not interact with the endothelial cell L-arginine transporter. *Br. J. Pharmacol.* **105**:768–770 (1992).
26. Schmidt, K., B. Mayer, and W. R. Kukovetz. Effect of calcium on endothelium-derived relaxing factor formation and cGMP levels in endothelial cells. *Eur. J. Pharmacol.* **170**:157–166 (1989).
27. Schmidt, K., E. R. Werner, B. Mayer, H. Wachter, and W. R. Kukovetz. Tetrahydrobiopterin-dependent formation of endothelium-derived relaxing factor (nitric oxide) in aortic endothelial cells. *Biochem. J.* **281**:297–300 (1992).
28. Wiemer, G. A., A. Wellstein, D. Palm, H. M. von Hattingberg, and D. Brockmeier. Properties of agonist binding at the β-adrenoceptor of the rat reticulocyte. *Naunyn-Schmiedebergs Arch. Pharmacol.* **321**:11–19 (1982).
29. Martin, W., D. G. White, and A. H. Henderson. Endothelium-derived relaxing factor and atriopeptin II elevate cyclic GMP levels in pig aortic endothelial cells. *Br. J. Pharmacol.* **93**:229–239 (1988).
30. Herrero, A., and E. Flores. Transport of basic amino acids by the dinitrogen-fixing cyanobacterium *Anabaena* PCC 7129. *J. Biol. Chem.* **265**:3931–3935 (1990).
31. White, M. The transport of cationic amino acids across the plasma membrane of mammalian cells. *Biochim. Biophys. Acta* **822**:355–374 (1985).
32. Wang, H., M. Kavanaugh, R. North, and D. Kabat. Cell-surface receptor for ecotropic murine retroviruses is a basic amino-acid transporter. *Nature (Lond.)* **352**:729–731 (1991).
33. Kim, J., E. Closs, L. Albritton, and J. Cunningham. Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. *Nature (Lond.)* **352**:725–728 (1991).
34. Sweiry, J., M. Munoz, and G. Mann. *Cis*-inhibition and *trans*-stimulation of cationic amino acid transport in the perfused rat pancreas. *Am. J. Physiol.* **261**:C506-C514 (1991).
35. Bogle, R. G., A. R. Baydoun, J. D. Pearson, S. Moncada, and G. E. Mann. L-Arginine transport is increased in macrophages generating nitric oxide. *Biochem. J.* **284**:15–18 (1992).
36. Mann, G. E., J. D. Pearson, C.-J. Sheriff, and V. J. Toothill. Expression of amino acid transport systems in cultured human umbilical vein endothelial cells. *J. Physiol. (Lond.)* **410**:325–339 (1989).
37. Hughes, C. C. W., and P. L. Lantos. Uptake of leucine and alanine by cultured cerebral capillary endothelial cells. *Brain Res.* **480**:126–132 (1989).
38. Christensen, H. Distinguishing amino acid transport systems of a given cell or tissue. *Methods Enzymol.* **173**:576–616 (1989).

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