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Biochimica et Biophysica Acta 1324 (1997) 133–141

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Inhibition of endothelial cell amino acid transport System y^+ by arginine analogs that inhibit nitric oxide synthase

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Received 19 August 1996; revised 10 October 1996; accepted 10 October 1996

Abstract

A variety of N^{ω}-monosubstituted L-arginine analogs are established inhibitors of nitric oxide synthase; in all cases, initial binding is competitive with the substrate L-arginine. The efficacy of such compounds in vivo will depend on their transport into the relevant nitric oxide synthase-containing cells; in fact, inhibition may actually be augmented if cellular uptake of L-arginine is also blocked by the analogs. Because vascular endothelial cells synthesize vasoactive nitric oxide under both physiological and pathophysiological conditions, we have performed inhibition analyses with novel arginine analogs to determine the substrate specificity of the primary L-arginine transport system, Na^+-independent System y^+, present in porcine pulmonary artery endothelial cells. As reported by others, no Na^+-independent System $b^{o,+}$ activity was detectable. For System y^+, Dixon plots suggest competitive inhibition and apparent K_i values, which ranged between 0.1 and 0.8 mM, were estimated for each inhibitor. Some influence of amino acid side chain structure could be detected, but in general, the data establish that this transport system accepts a broad range of arginine derivatives. Loading the cells with individual arginine analogs resulted in trans-stimulation of arginine uptake suggesting that they serve as substrates of System y^+ as well as inhibitors. These results indicate that plasma membrane transport is unlikely to be a limiting factor in drug development for nitric oxide synthase inhibitors.

Keywords: Amino acid transport; Nitric oxide; Endothelial cell; Arginine metabolism; Amino acid analog

Abbreviations: CAT, cationic amino acid transporter; HA, homoarginine; NAA, N^{ω}-amino-L-arginine; NAHA, N^{ω}-amino-L-homoarginine; NEA, N^{ω}-ethyl-L-arginine; NBA, N^{ω}-butyl-L-arginine; NIBO, N^{δ}-iminobutyl-L-ornithine; NIPO, N^{δ}-iminopropyl-L-ornithine; NIEO, N^{δ}-iminoethyl-L-ornithine; 1-AMA, N^{ω}-methylamino-L-arginine; 2-AMA, N^{ω}-amino-N^{ω}-methyl-L-arginine; 3-AMA, N^{ω}-amino-$N^{\omega'}$-methyl-L-arginine; NMHA, N^{ω}-methyl-L-homoarginine; NIEL, N^{ϵ}-iminoethyl-L-lysine; NIBL, N^{ϵ}-iminobutyl-L-lysine; MARG, *RS*-γ-methyl-DL-arginine; PPAE, porcine pulmonary artery endothelial (cells); L-NAME, N^{ω}-nitro-L-arginine methyl ester; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase

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PII S0005-2736(96)00226-X

1. Introduction

Nitric oxide (NO), originally identified as endothelium-derived relaxing factor (EDRF) [1], is synthesized from a guanidino nitrogen of L-arginine by nitric oxide synthase (NOS), a NADPH- and O_2-dependent enzyme [2]. Three isoforms of NOS have been identified [2–4]. A Ca^{2+}/calmodulin-dependent constitutive NOS within vascular endothelial cells (eNOS) is activated by blood flow (i.e. shear stress) or by various Ca^{2+} transport agonists (e.g. bradykinin) and produces NO that is an important vasorelaxant in blood pressure homeostasis [3,5–7]. Within some central and peripheral neurons, a distinct Ca^{2+}/calmodulin-dependent, constitutive NOS isoform (nNOS) produces NO that serves an incompletely characterized role in signal transduction [8]. Finally, in response to various inflammatory cytokines or lipopolysaccharide (LPS), many cell types express an inducible NOS isoform (iNOS) that is not regulated by changes in cellular Ca^{2+} levels [3]. Vascular smooth muscle and endothelial cells are among the tissues expressing high levels of iNOS following exposure to endotoxin or inflammatory cytokines. In the intact animal, iNOS-mediated overproduction of NO by vascular cells can cause marked vasorelaxation leading to clinically significant hypotensive cardiovascular shock [9].

Both physiological and pathophysiological NOS-mediated formation of NO are dependent on an adequate and continuing supply of L-arginine. There are two potential sources of intracellular L-arginine, de novo synthesis from other metabolites and exogenous L-arginine taken up from the bloodstream. The latter route is particularly important in the pathological overproduction of NO inpatients with septic shock [10]. With regard to exogenous L-arginine uptake, there are four known L-arginine plasma membrane transport systems. System y^+ is a sodium-independent system encoded by the CAT^1 gene family [11,12]. System y^+ transports only cationic amino acids in the absence of Na^+, but at relatively high concentrations (mM) certain neutral amino acids can serve as inhibitors in the presence of Na^+ [13,14]. Systems $B^{o,+}$, $b^{o,+}$, and y^+L are activities that transport both cationic and neutral amino acids at physiologic concentrations [15–17]. The relative expression of these four systems varies widely among different tissues and cell types [18], but porcine pulmonary artery endothelial cells have been reported to express System y^+ as a primary route for arginine transport [19].

There is considerable interest in the selective inhibition of NOS isoforms, particularly iNOS, in septic shock [9,10] and inflammatory conditions [20,21]. Although several L-arginine analogs such as N^{ω}-nitro-L-arginine methyl ester (L-NAME) and N^{ω}-methyl-L-arginine (L-NMA) have been proposed as clinically useful NOS inhibitors, these derivatives inhibit all NOS isoforms with little selectivity [2,21]. We considered that the utility of L-arginine analogs as NOS inhibitors in vivo is likely to depend on the substrate specificity and uptake rates of the corresponding transport systems present in the target cell. One of our laboratories (OWG) has synthesized a number of potentially useful arginine analogs that are potent inhibitors of the constitutive and inducible forms of NOS; some are moderately selective [22]. Therefore, the ability of these analogs to competitively inhibit L-arginine transport by System y^+ was examined in porcine pulmonary artery endothelial (PPAE) cells in primary culture. As mentioned, System y^+ is a primary transporter of cationic amino acids in these cells, and its Na^+-independent mechanism will permit bi-directional plasma membrane transport of an arginine analog, if it is an acceptable substrate. Conversely, if the analog serves as an inhibitor of System y^+, even one that has little or no translocation, it may block arginine availability for NO synthesis by the inducible form of the enzyme.

The inhibition analyses reported here revealed that the endothelial cell System y^+ activity is strongly inhibited by a broad range of arginine analogs which also serve as substrates. These results indicate that a wide spectrum of arginine analogs may limit L-arginine uptake significantly, and possibly, also deplete intracellular arginine stores by *trans*-stimulation. The data also demonstrate that transport across the plasma membrane is unlikely to be a limitation with regard to development of NOS inhibitors.

2. Materials and methods

2.1. Synthesis of arginine analogs

Arginine analogs and N^{ω}-monoalkyl arginine analogs were prepared from L-ornithine or *RS*-γ-

methyl-DL-ornithine by the general method of Corbin and Reporter [22]. Briefly, the Cu^{2+}-complex of ornithine or substituted ornithine was reacted under alkaline conditions with the appropriate *S*-methyl thiopseudouronium iodide (i.e., R-NH-$C(SCH_3)NH_2^+I^-$, where R=H for arginine and γ-methyl-arginine derivatives, and R=CH_3-, C_2H_7- and C_4H_9- for N^ω-methyl, N^ω-ethyl, N^ω-propyl, and N^ω-butyl derivatives). After removing Cu^{2+} with Chelex resin, the resulting product was separated from unreacted ornithine or substituted ornithine by crystallization as the flavianic acid salt [23,24] or by chromatography on Dowex 50 resin (E.B. Campbell and O.W. Griffith, unpublished data). Homoarginine analogs were prepared similarly using L-lysine in place of L-ornithine. N^ω-Amino-L-arginine was prepared from N^ω-nitro-L-arginine as described previously [25,26]. N^ω-Amino-L-homoarginine was prepared from L-lysine and *S*-methyl thiosemicarbazide, essentially as described for the N^ω-alkyl derivatives. Similarly, N^ω-methylamino, L-arginine, N^ω-amino-$N^{\omega'}$-methyl-L-arginine, and N^ω-amino, N^ω-methyl-L-arginine were prepared from appropriate substituted *S*-methyl thiosemicarbazides and L-ornithine. N^δ-iminoethyl-L-ornithine (L-NIO) and its higher homologs were prepared by reaction of the appropriate *O*-methyl imidate with L-ornithine as described [27]; imidates were prepared by treating alkylnitriles with methanolic HCl. N^ω-Iminoethyl-L-lysines were prepared similarly from L-lysine.

All compounds were >95% pure by HPLC and gave the expected NMR and/or elemental analyses. Structures of the analogs and the abbreviations used throughout this report are given in Fig. 1. Note that all analogs contain a strongly basic side chain and therefore are cationic at pH 7.4.

2.2. Endothelial cell isolation and culture

Porcine pulmonary artery endothelial (PPAE) cells were obtained from the main pulmonary artery of 6- to 7-month-old pigs and placed in primary culture as described previously [28]. The primary cultures were grown in 60-mm diameter plastic culture dishes pretreated with fibronectin (Sigma Chemical Co., St. Louis, MO) in Minimum Essential Medium alpha (MEM-alpha) (Gibco), 10% fetal bovine serum, and antimicrobials (100 U/ml penicillin and 100 μg/ml streptomycin). The cultures were grown in a humidified atmosphere of 5% CO_2/95% air at 37°C. For transport assays, the cells were transferred to fibronectin-coated 24-well trays and maintained for 48 h in RPMI-1640 medium supplemented with 2 mM L-glutamine (Gibco), 4% fetal bovine serum, and antimicrobials at 37°C.

2.3. Transport measurements

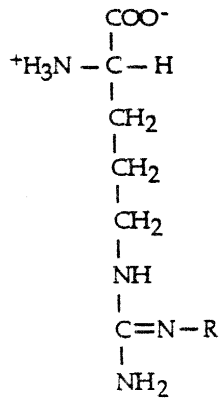
Amino acid transport of radiolabelled L-arginine was measured by a modification [29] of the method originally described by Gazzola et al. [30]. The cells were washed twice for 15 min each with 2 ml of Na^+-free, choline-containing Krebs–Ringer phosphate (choline KRP) buffer at pH 7.4 and 37°C. These incubations remove residual culture medium and minimize possible *trans*-effects by allowing partial depletion of the intracellular amino acid pool. The uptake buffers (0.25 ml per well) were prepared from choline KRP buffer containing the indicated concentrations of L-[^{3}H]arginine (Amersham Corp.) and, where noted, unlabeled L-arginine analogs. Transport assays were initiated by addition of uptake buffer to the cells at 37°C, and 30 s later transport was stopped by washing the cells four times with ice-cold choline KRP buffer (2 ml/well). The cells were allowed to dry, then the cellular protein was precipitated with trichloroacetic acid [29]. The resulting soluble extract was removed for determination of radioactivity by scintillation counting and the protein content of each well was determined by a modified Lowry method [29]. Data were calculated with software that incorporated standard statistical analyses, and the results are reported as pmol L-arginine transported per mg protein per unit time, or as percent of control uptake. All experiments represent the average of at least four assays, and each experiment was repeated with several different preparations of endothelial cells to ensure reproducibility.

3. Results

3.1. Inhibition of NOS isoforms by arginine analogs

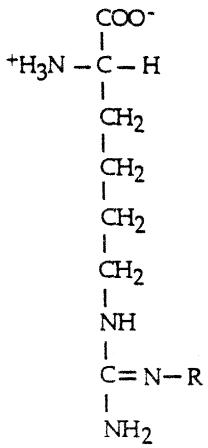
Previous studies have elucidated the major structural constraints of arginine analog binding to NOS

I. N^{ω}-substituted arginines



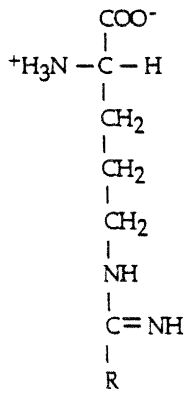
R= NH_2, N^{ω}-amino-L-arginine
R= C_2H_5, N^{ω}-ethyl-L-arginine
R= C_3H_7, N^{ω}-propyl-L-arginine
R= C_4H_9, N^{ω}-butyl-L-arginine

II. N^{ω}-substituted homoarginines



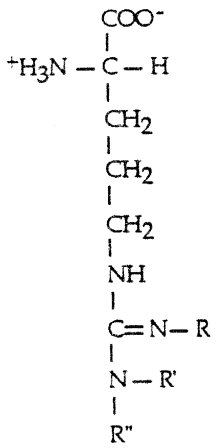
R= H, L-homoarginine
R= CH_3, N^{ω}-methyl-L-homoarginine
R= NH_2, N^{ω}-amino-L-homoarginine

III. iminoornithines



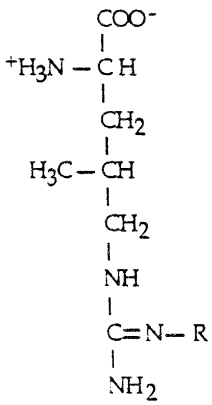
R= CH_3, N^{δ}-iminoethyl-L-ornithine
R= C_2H_5, N^{δ}-iminopropyl-L-ornithine
R= C_3H_7, N^{δ}iminobutyl-L-ornithine

IV. N^{ω}-disubstituted arginines



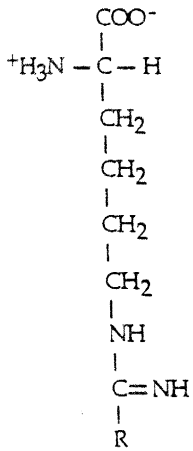
R= H, R'=NH_2, R"=CH_3,
N^{ω}-methylamino-L-arginine
R= H, R'=CH_3, R"=NH_2,
N^{ω}-amino-N^{ω}-methyl-L-arginine
R= CH_3, R'=H, R"=NH_2,
N^{ω}-amino-$N^{\omega'}$-methyl-L-arginine

V. γ-methyl derivatives



R= H, *RS*-γ-methyl-DL-arginine
R=CH_3, *RS*-γ-methyl-N^{ω}-methyl-DL-arginine

VI. substituted lysines



R= CH_3, N^{ε}-iminoethyl-L-lysine
R= C_3H_7, N^{ε}-iminobutyl-L-lysine

Fig. 1. Structures of arginine analogs used a transport inhibitors. Stereochemically pure derivatives were prepared as described in the text.

[2]. Briefly, derivatives of L-arginine and L-homoarginine are bound with high affinity (K_m or $K_i < 50$ μM) if they include an α-amino and α-carboxyl group, and are not substituted on both guanidino nitrogens. If they are monosubstituted on a guanidino nitrogen, the N^{ω}-substituent can be no larger than

n-propyl [31]. Binding remains strong if one guanidino -NH_2 is replaced by small n-alkyl groups (L-NIEO and its homologs) ([32]; K. Narayanan and O.W. Griffith, unpublished data). Substitution of one guanidino nitrogen with an amino group (L-NAA and related compounds) results in a potent inhibitor of all NOS isoforms [24,26,33], whereas substitution with both an amino and a methyl group yields compounds with some selectivity of inhibition [22]. Methyl substitution onto the L-ornithine backbone of arginine analogs is tolerated to different degrees by nNOS, eNOS, and iNOS; replacement of γ-H by a γ-CH_3 group increases eNOS inhibition relative to nNOS and iNOS inhibition [34]. Use of these analogs to block NO formation in vivo requires that they are either: (1) effectively translocated by plasma membrane transporters and subsequently directly inhibit NOS, or (2) inhibit transport with or without translocation, and serve to limit arginine availability to the cell.

3.2. Transport of L-[^{3}H]arginine in porcine pulmonary artery endothelial cells

Our interest was to characterize the analog inhibition of Na^+-independent System y^+ transport because this transporter can function in a bi-directional mode and permit equilibration of the substrate across the plasma membrane. The data shown in Fig. 2 confirm that PPAE cells express little or no leucine-inhibitable Na^+-independent L-arginine transport by System $b^{o,+}$ [19]. However, as a precaution in all remaining experiments, System y^+ activity was assayed as the saturable sodium-independent uptake of 50 μM L-[^{3}H]arginine in the presence of 10 mM L-leucine in order to absolutely exclude uptake by System $b^{o,+}$.

3.3. L-Arginine kinetics for System y^+

Prior to determining the K_i values for analog inhibition, a kinetic analysis of System y^+-mediated transport of L-[^{3}H]arginine by PPAE cells was performed at concentrations from 0.075 to 20 mM. A double-reciprocal plot of these data, after subtraction of the nonsaturable transport component, is presented in Fig. 3. Best-fit analysis for either one or two components indicate the presence of a single Na^+-independent system confirming the earlier inhibition analyses ([19], Fig. 2). The apparent K_m value was 0.39 mM and the observed maximal velocity was 5500 pmol (mg protein)$^{-1}$ 30 s^{-1}.

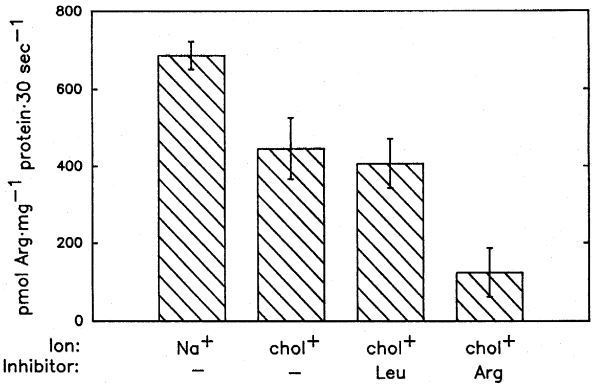


Fig. 2. Analysis of the transport of 50 μM L-[^{3}H]arginine by porcine pulmonary artery cells. The uptake of 50 μM L-[^{3}H]arginine was assayed for 30 s at 37°C. The difference between the uptake in the presence of sodium and choline represents the Na^+-dependent System $B^{o,+}$ activity. No difference between the uptake in the presence of choline and choline plus 10 mM L-leucine, representing Na^+-independent System $b^{o,+}$ activity, was observed. The difference between the uptake in choline plus 10 mM L-leucine and in choline plus 10 mM L-arginine was taken as the Na^+-independent System y^+ activity. The data presented are the averages of four determinations ± standard deviations.

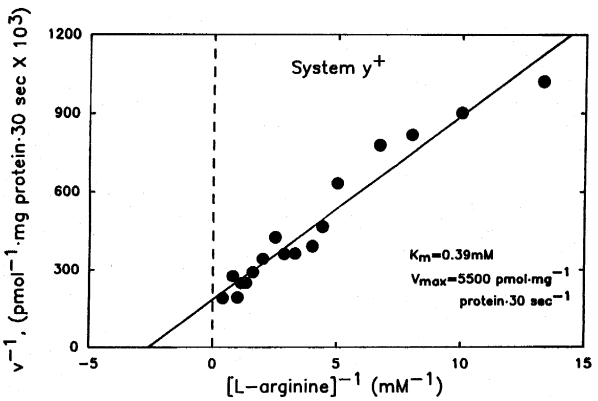


Fig. 3. Kinetic analysis of Na^+-independent L-arginine uptake. Porcine pulmonary artery endothelial cells were assayed for Na^+-independent L-arginine uptake between the concentrations of 0.075 and 20 mM. The transport assays were performed for 30 s at 37°C. The nonsaturable component was subtracted prior to calculation of the apparent kinetic constants.

3.4. Determination of the K_i values for the L-arginine analogs

The transport rate of 0.025, 0.05, or 0.20 mM L-[^{3}H]arginine was measured in the presence of varying concentrations of selected arginine analogs. As representative examples of a strong, moderate, or weak inhibitor of System y^+ activity, Dixon plots [35] for L-homoarginine (HA), N^{ω}-amino-L-arginine (L-NAA), and N^{δ}-iminoethyl-L-ornithine (L-NIEO) are shown in Fig. 4. Similar graphic analysis was performed for each of the inhibitors. A summary of the estimated K_i values obtained for the arginine analogs studied is provided in Table 1. The K_i values varied between 0.15 and 0.78 mM, not too dissimilar from the K_m value of 0.39 mM for L-arginine itself. Higher homologs of L-arginine, homoarginine (HA), N^{ω}-amino-L-homoarginine (NAHA), and N^{ω}-iminoethyl-L-lysine (NIEL), were the most potent inhibitors of System y^+. In contrast, iminoalkyl analogs of L-arginine (NIEO, NIPO, and NIBO) were the weakest of the inhibitors tested, but even their K_i values were less than 0.8 mM. Interestingly, N^{ω}-iminobutyl-L-lysine (NIBO) was not nearly

Table 1
K_i Values for inhibition of Na^+-independent system y^+ transport by arginine analogs

Inhibitors	Apparent K_i (mM)
NAA	0.14
NEA	0.30
NBA	0.30
HA	0.24
NAHA	0.14
NMHA	0.50
NIEO	0.78
NIPO	0.65
NIBO	0.75
1-AMA	0.43
2-AMA	0.44
3-AMA	0.25
MARG	0.49
NIEL	0.15
NIBL	0.70

Uptake of 0.025, 0.05, or 0.20 mM L-[^{3}H]arginine was measured at inhibitor concentrations over the range of 0–5 mM for each arginine analog. Assays were performed for 30 s at 37°C as described in Section 2. The apparent K_i values were estimated by computer analysis of Dixon plots. Fig. 4 shows three representative examples.

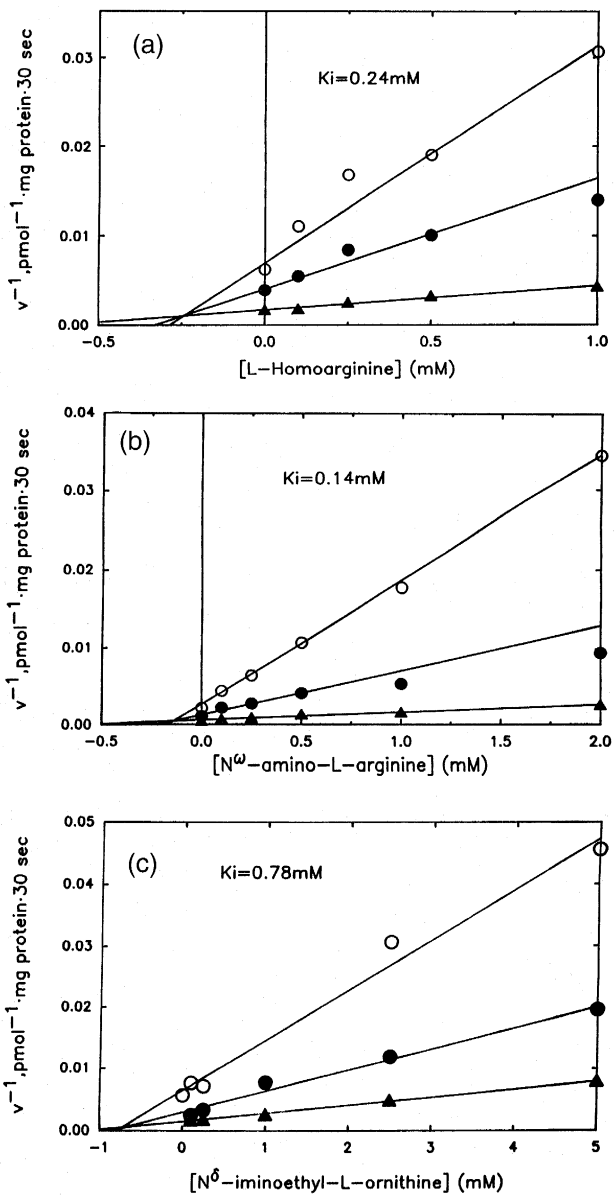


Fig. 4. Representative Dixon plots for three inhibitors of L-arginine transport by System y^+ in porcine pulmonary artery endothelial cells. The Na^+-independent transport of L-arginine at 0.025 (○), 0.05 (●), and 0.2 (▲) mM was performed in the presence of inhibitors over a range of concentrations from 0–5 mM. The uptake was measured for 30 s at 37°C. The Na^+-independent uptake rates were corrected by subtracting the nonsaturable component. Homoarginine (HA, panel a), N^{ω}-amino-L-arginine (NAA, panel b), and N^{δ}-iminoethyl-L-ornithine (NIEO, panel c) were chosen to show the range of K_i values observed. For the remainder of these values see Table 1.

as effective as the shorter ethyl derivative (Table 1). Among the analogs tested, N^{ω}-aminomethyl-L-arginine (1-AMA), N^{ω}-amino-$N^{\omega'}$-methyl-L-arginine (2-AMA), and N^{ω}-amino-N^{ω}-methyl-L-arginine (3-AMA) were intermediate in strength; the 3-AMA analog was slightly more effective than its isomers.

3.5. Arginine analogs are System y^+ substrates

To determine if the arginine analogs tested for inhibition were also translocated, *trans*-stimulation of Na^+-independent L-[^{3}H]arginine uptake was assayed. PPAE cells were incubated for 1 h in the presence of 5 mM arginine analog prior to measurement of 50 μM arginine uptake for 30 s. The analogs selected (NAA, NIEO, 1-AMA, and 3-AMA) were chosen to represent a spectrum of K_i values obtained, ranging from 0.14 mM for NAA to 0.78 mM for NIEO. *Trans*-stimulation by HA has been reported by others for a number of different cell types [14]. As shown in Table 2, each of the four analogs tested caused an increase in arginine transport consistent with the interpretation that the analogs were themselves substrates. Translocation of these analogs demonstrates that they will have the potential to decrease NO production by three mechanisms, indirectly by blocking arginine uptake across the plasma membrane, depletion of intracellular arginine by *trans*-stimulation, and directly at the NOS enzymes.

Table 2
Trans-stimulation of arginine uptake by individual analogs

Analog	Arginine uptake	Percent of control
None	152 ± 10	100
NAA	337 ± 10	222
NEIO	258 ± 7	170
1-AMA	265 ± 15	174
3-AMA	272 ± 31	179

PPAE cells were incubated in Na^+-containing Krebs–Ringer bicarbonate buffer with or without 5 mM of the indicated arginine analog for 1 h at 37°C. Following two rapid rinses in Na^+-free choline KRP, the uptake of 50 μM L-[^{3}H]arginine was measured for 30 s at 37°C as described in Section 2. The results are the averages ± S.D. of at least three assays, and the entire experiment was performed with two sets of cells to ensure qualitative reproducibility.

4. Discussion

The present data demonstrate that System y^+ mediates Na^+-independent arginine transport into porcine pulmonary artery endothelial cells with an apparent K_m for L-arginine transport of 390 μM, a value comparable to those reported by others [14,19,37–40]. Schmidt et al. [37] reported biphasic kinetics for L-arginine uptake by PPAE cells and concluded there were two transport systems with apparent K_m values of 6 and 600 μM. Our kinetic analysis used a substrate concentration range starting at 75 μM and thus, would not have identified an activity with a K_m of less than 10 μM. Because the kinetic analysis of Schmidt et al. [37] was performed in Na^+-containing medium only, no assignment to System y^+ can be made with certainty. Interestingly, they reported that leucine was not an effective inhibitor of either component and lysine inhibited ($K_i = 200$ μM) only one of the activities.

With regard to arginine analogs that are known to serve as NOS inhibitors, several groups have demonstrated that N^{ω}-methyl-L-arginine (L-NMA) competes with L-arginine for transport, but the NOS-inhibitory nitro derivatives, N^{ω}-nitro-L-arginine (L-NNA) and N^{ω}-nitro-L-arginine methyl ester (L-NAME), do not [36,37,41–43]. The nitro derivatives appear to be transported by System L, an activity that mediates the Na^+-independent uptake of neutral, not cationic, amino acids. From the present studies it is clear that derivatives that retain a cationic guanidinium or amidine group serve as competitive inhibitors for System y^+. Initial observations for inhibition of arginine transport by these analogs were confirmed by Dixon plots used to determine K_i values for inhibition of Na^+-independent System y^+. The K_i values ranged from 0.14 to 0.78 mM, a factor of two above and below the K_m for L-arginine.

The present findings elucidate previous observations regarding inhibition of NOS isoforms by these analogs and now permit a more detailed interpretation. For example, Rouhani et al. [22] reported that N^{ω}-amino-$N^{\omega'}$-methyl-L-arginine (3-AMA) is a weak inhibitor of the acetylcholine-induced relaxation of rat aortic rings, whereas N^{ω}-aminomethyl-L-arginine (1-AMA) and N^{ω}-amino-N^{ω}-methyl-L-arginine (2-AMA) are measurably better inhibitors. On the other hand, all three analogs are essentially equivalent in

their ability to inhibit isolated mouse nNOS. Although one interpretation of those results was that eNOS and nNOS differed in their recognition of these arginine analogs, it also was possible that 3-AMA was simply not transported as effectively as either 1-AMA or 2-AMA. In fact, as shown here, the K_i value for 3-AMA inhibition of System y^+ was less than those for either 1-AMA or 2-AMA, and its ability to cause *trans*-stimulation of arginine transport was as effective as 1-AMA. Therefore, the poor inhibition of aortic ring relaxation by 3-AMA is more likely to reflect its binding affinity to eNOS itself.

Overall, our transport inhibition studies characterize System y^+ as having a broad specificity. The data show that structural modifications of the arginine side chain already known to be useful in designing partially selective NOS inhibitors are acceptable inhibitors and substrates of the transporter. Our data also provide new insight into the structural constraints on substrates of System y^+. Like all NOS isoforms, System y^+ accepts both L-arginine and L-homoarginine analogs, tolerates replacement of a guanidinium -NH_2 by -CH_3 or higher alkyl groups, accepts N^{ω}-amino and alkyl substituents, and permits γ-methyl substitution of the L-ornithine sidechain. In fact, the data suggest that System y^+ has a broader specificity than any of the NOS isoforms. For example, N^{ω}-butyl-L-arginine is recognized by System y^+, but is a poor inhibitor of all NOS isoforms ([31]; K. Narayanan and O.W. Griffith, unpublished data). Given the apparently ubiquitous distribution of System y^+ activity in mammalian cells, this important result indicates that the ability of arginine analogs to serve as NOS inhibitors is unlikely to be limited by plasma membrane transport.

Acknowledgements

This work was supported by grants to M.S.K. (DK-28374), O.W.G. (DK-37116), and E.R.B. (HL-52136) from the National Institutes of Health.

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