Interaction of L-Arginine Analogs with L-Arginine Uptake in Rat Renal Brush Border Membrane Vesicles

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ABSTRACT

The dibasic amino acid, L-arginine, is a substrate for both nitric oxide synthase (NOS) and arginase and therefore, plays an important role in cell signaling and cell growth. We examined the effects of various NOS inhibitors on L-arginine transport into rat renal brush border membrane (BBM) vesicles. L-Arginine uptake was stimulated in the presence of an inwardly directed Na⁺ gradient and an imposed inside negative potential in BBM but not basolateral membrane vesicles. In BBM vesicles, the L-arginine analogs, N-iminoethyl-L-ornithine and N⁰-monomethyl-L-arginine (L-NMMA) were potent inhibitors of L-arginine uptake (IC₅₀ of 0.48 and 0.82 mM, respectively), while N⁰-nitro-L-arginine was less active (IC₅₀ = 10 mM) and N⁰-nitro-L-arginine methyl ester (L-NAME) was inactive. The inhibition of L-arginine transport by L-NMMA was competitive in nature. L-NIO, L-NMMA as well as L-arginine and L-lysine but not N⁰-nitro-L-arginine methyl ester, trans-stimulated L-arginine uptake when preloaded into BBM vesicles. The L-arginine analogs had no effect on the transport of the neutral amino acid, L-leucine, in the same preparations. The data suggest that in addition to inhibiting NOS, the L-arginine analogs, N-iminoethyl-L-ornithine, N⁰-NMMA and to a lesser extent L-NA, also inhibit L-arginine transport across the BBM of proximal tubules.

Materials and Methods

Membrane preparation. BBM vesicles were isolated from the renal cortex of male Sprague-Dawley rats (250-300 g, Charles River, Wilmington, MA) by the Mg⁺⁺ precipitation method as described previously (Edwards et al., 1995). Briefly, renal cortices were homogenized at 4°C in 50 mM mannitol and 10 mM HEPES-Tris (pH 7.5) with a Polytron (3x30 sec at speed 6). MgCl₂ was added to a final concentration of 10 mM, and the homogenate was stirred on ice for 20 min. The homogenate was centrifuged for 10 min at 2,000 × g, and the resulting supernatant was centrifuged for 20 min at 35,000 × g. The pellet was resuspended in intravesicular buffer which consisted of 100 mM mannitol, 100 mM KCl, 10 μM valinomycin and 5 mM HEPES-Tris, pH 7.5, using a glass/Teflon homogenizer and centrifuged for 15 min at 35,000 × g. The loosely packed BBM layer was gently washed off the pellet and recentrifuged an additional three times as described above. The final BBM pellet was resuspended in intravesicular buffer at a protein concentration of 10 to 15 mg/ml and stored at room temperature until uptake studies were performed on the same day. The purity of the BBM was assessed by the enrichment of the BBM enzyme, alkaline phosphatase, the effects of various NOS inhibitors on L-arginine transport into rat renal brush border membrane (BBM) vesicles. L-Arginine uptake was stimulated in the presence of an inwardly directed Na⁺ gradient and an imposed inside negative potential in BBM but not basolateral membrane vesicles. In BBM vesicles, the L-arginine analogs, N-iminoethyl-L-ornithine and N⁰-monomethyl-L-arginine (L-NMMA) were potent inhibitors of L-arginine uptake (IC₅₀ of 0.48 and 0.82 mM, respectively), while N⁰-nitro-L-arginine was less active (IC₅₀ = 10 mM) and N⁰-nitro-L-arginine methyl ester (L-NAME) was inactive. The inhibition of L-arginine transport by L-NMMA was competitive in nature. L-NIO, L-NMMA as well as L-arginine and L-lysine but not N⁰-nitro-L-arginine methyl ester, trans-stimulated L-arginine uptake when preloaded into BBM vesicles. The L-arginine analogs had no effect on the transport of the neutral amino acid, L-leucine, in the same preparations. The data suggest that in addition to inhibiting NOS, the L-arginine analogs, N-iminoethyl-L-ornithine, N⁰-NMMA and to a lesser extent L-NA, also inhibit L-arginine transport across the BBM of proximal tubules.

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using p-nitrophenylphosphate as a substrate (Sigma kit 104). The enrichment of alkaline phosphatase in the BBM relative to the original homogenate was 11.3 ± 0.3-fold (n = 19). Protein was measured by Lowry’s method.

In a limited number of experiments, BLM vesicles were also used and were prepared by a Percoll density gradient centrifugation method previously described in detail (Edwards et al., 1997). Enrichment of the BLM marker enzyme, Na^+--K^+--ATPase and the BBM marker, alkaline phosphatase, were 15.4 ± 2.3- and 2.1 ± 1.1-fold, respectively.

**Uptake studies.** Uptake of 3H-L-arginine was measured at 20°C by a rapid filtration method. Ten μl of BBM vesicles (90-120 μg protein) equilibrated with intravesicular buffer (100 mM mannitol, 100 mM KCl and 5 mM HEPES-Tris, pH 7.5) were rapidly mixed with 120 μl of uptake buffer consisting of 100 mM mannitol, 100 mM NaCl and 5 mM HEPES-Tris, pH 7.5 containing 3H-L-arginine (50 μM) and other constituents as described in ‘Results.’ At various time intervals, 3 ml of uptake buffer without substrate were added to the test tube and the solution was filtered through 45 μm Millipore filters. The vesicles retained on the filter were washed with an additional 2 × 3 ml of buffer and the filters were counted in a scintillation counter. Each experiment was performed using triplicate or quadruplicate determinations and corrections were made for 3H-L-arginine bound to the filters in the absence of vesicles which was always <0.1% of added counts. Uptakes are expressed as pmol per mg protein. Each experiment (n) was repeated on three to six different vesicle preparations. Statistical analysis was performed with Student’s t test or analysis of variance followed by Dunnett’s test for multiple comparisons.

**Chemicals.** 3H-L-arginine (40 Ci/mmol) was obtained from New England Nuclear. Valinomycin and unlabeled amino acids were obtained from Sigma (St. Louis, MO). The NOS inhibitors, L-NA, L-NAME, L-NIO and L-NMMA were obtained from RBI (Natick, MA). All other compounds were of the highest grade commercially available. Valinomycin was dissolved in 100% ethanol and added to the vesicle preparations in a 1:130 dilution. An equivalent volume of ethanol was added to control preparations.

**Results**

In agreement with previous studies which have examined the characteristics of L-arginine or other dibasic amino acid transport in BBM (Hilden and Sacktor, 1981), we found that imposition of an inwardly directed Na^+-gradient in the presence of an intravesicular negative potential greatly enhanced L-arginine uptake into BBM vesicles (fig. 1). In the presence of a Na^+-gradient and valinomycin, L-arginine uptake was rapid and reached a maximum at 2 min representing a 3.5-fold increase over the equilibrium value at 90 min. An inwardly directed Na^+-gradient alone caused a small overshoot of L-arginine uptake (1.7-fold), although uptake in the absence of a Na^+-gradient (KCl) did not exceed the equilibrium value. Unless stated otherwise, all further experiments were performed in the presence of an inwardly directed Na^+-gradient and a valinomycin-induced inside negative potential.

In contrast to the results obtained with BBM vesicles, L-arginine uptake into BLM vesicles was not affected by an inwardly directed Na^+-gradient in the absence or presence of an inside negative potential (fig. 1). As there was no evidence of Na^+-dependent uptake of L-arginine, BLM vesicles were not studied further.

Addition of L-NMMA (0.3 and 1 mM) to the extravesicular medium markedly inhibited L-arginine uptake at all time points studied, except at equilibrium, suggesting that L-NMMA did not affect vesicle size or integrity (fig. 2). Of the L-arginine analogs tested, L-NIO and L-NMMA were the most potent in inhibiting L-arginine uptake (fig. 3). The concentrations of these analogs required to reduce L-arginine uptake by 50% (IC<sub>50</sub>) were 0.48 ± 0.05 mM and 0.82 ± 0.29 mM for L-NIO and L-NMMA, respectively (n = 3). The IC<sub>50</sub> value for L-NA was approximately 10 mM, while L-NAME had no significant effect on L-arginine uptake over the concentration range tested. Kinetic analysis of the uptake data suggested that the inhibition of L-arginine uptake by
L-NMMA was competitive in nature (fig. 4) because the analog (at 1 mM) had no effect on the maximum uptake of L-arginine (Vmax; control, 2168 ± 237 pmol/mg min, n = 3) but significantly (P < .01) reduced the apparent affinity, Km, from 136 ± 24.0 to 304 ± 32.8 μM. In addition to L-NIO, L-NA and L-NMMA, the dibasic amino acids, lysine and ornithine, also inhibited L-arginine uptake, while citrulline and agmatine had no effect (fig. 5).

If the L-arginine analogs share the same transporter as L-arginine, then an imposed transmembrane gradient of the analog should drive the uphill transport of L-arginine (trans-stimulation). Accordingly, BBM vesicles were preloaded with buffer (control) or with 5 mM L-arginine, L-lysine, L-NIO, L-NAME or L-NMMA for 2 hr at room temperature. Uptake of 3H-L-arginine was initiated by diluting the vesicles 20-fold into uptake buffer containing a final concentration of 250 μM 3H-L-arginine. L-Arginine, L-lysine, L-NIO, L-NMMA but not L-NAME, significantly increased L-arginine uptake when compared to control (fig. 6).

The inhibitory effects of the L-arginine analogs on 3H-L-arginine uptake appeared to be specific because these compounds had no effect on the Na+ dependent transport of the neutral amino acid, leucine, in the same vesicle preparations (fig. 7).

**Discussion**

The renal proximal tubule BBM contains at least seven different transport systems that mediate the reabsorption of L-amino acids and which differ in their substrate specificities (Silbernagl, 1985). We have confirmed the presence of an uptake mechanism for the dibasic amino acid, L-arginine, in...
BBM but not BLB vesicles that is energized by an inwardly directed Na\(^{+}\) gradient and an inside negative potential (Hilden and Sacktor, 1981). This system has affinity not only for L-arginine but also for other dibasic amino acids such as lysine and ornithine (fig. 5). Furthermore, we have shown that the NOS inhibitors, L-NMMA, L-NIO and to a lesser extent, L-NA, also have affinity for the dibasic amino acid transport system. This is based on the observations that these L-arginine analogs cis-inhibited, in a concentration-dependent manner, the uptake of L-arginine and trans-stimulated L-arginine uptake when preloaded into BBM vesicles. Kinetic analysis of the uptake data in the presence of L-NMMA revealed that the interaction of this analog with L-arginine uptake was competitive in nature. The effects of these analogs were specific for L-arginine transport because they did not affect the transport of the neutral amino acid, L-leucine, in the same vesicle preparations. In contrast to the effects of L-NMMA, L-NIO and L-NA, the other tested NOS inhibitor, L-NAME, had no effect on L-arginine or L-leucine uptake over the concentration range tested.

The characteristics of L-arginine and other amino acid transport into cells appear to be highly tissue specific. In porcine endothelial cells, L-arginine uptake was inhibited to approximately the same extent by L-NMMA and L-NIO, while L-NA was less active and L-NAME was inactive (Bogle et al., 1992b; Schmidt et al., 1993). In contrast, both L-NA and L-NAM inhibited L-arginine uptake in human platelets and neutrophils (Chen and Mehta, 1996). The interactions of the NOS inhibitors with L-arginine uptake observed in our study are virtually identical to those seen in porcine endothelial cells (Bogle et al., 1992b; Schmidt et al., 1993) with one major difference. Schmidt et al. (1993) found that L-NA inhibited the transport of L-leucine in porcine endothelial cells and concluded that L-NA and L-leucine share the same transporter. In the present experiments, we found that L-NA as well as the other L-arginine analogs had no effect on L-leucine uptake into renal BBM vesicles. The reason(s) for the differing results are not known. However, tissue-specific expression of different transporters is the likely reason for the observed differences. For example, in endothelial cells the transport of L-arginine and L-leucine are largely Na\(^{+}\)- independent and transported by the y\(^{-}\) and L systems, respectively (Schmidt et al., 1993). In renal BBM vesicles, L-arginine and L-leucine transport are largely Na\(^{+}\)-dependent (Silbernagl, 1985) and likely transported by the B\(^{\text{H}}\) + and A system, respectively (see Bertran et al., 1994). The mechanism by which L-NAME enters the cell in all these tissue remains to be determined. All these differing results reinforce the notion that amino acid transport in general and L-arginine transport in particular is a tissue-specific process mediated by distinct membrane transporters.

A number of studies have demonstrated the importance of L-arginine uptake in determining the activity of various L-arginine-dependent metabolic pathways. For example, in rat kidney the cellular uptake of L-arginine may be rate-limiting in the formation of urea and ornithine by arginase in some nephron segments (Levillain et al., 1994). Decreased availability of L-arginine may limit NO-dependent cell injury, cell proliferation and pathological matrix synthesis in renal disease (Narita et al., 1995; Peters and Noble, 1997). Furthermore, numerous studies in various cell types have demonstrated that the availability of extracellular L-arginine is rate-limiting for the generation of NO (Bogle et al., 1992a; Schott et al., 1993; Durante et al., 1996). Indeed, some have suggested that the primary effect of L-arginine analogs such as L-NAME and L-NA on the production of NO in some cells relates to the ability of these analogs to inhibit L-arginine transport rather than to inhibit NOS activity (Chen and Mehta, 1996). Finally, the different pharmacological profiles of NOS inhibitors in terms of potency and onset of action may be related not only to their rate of entry into cells and their direct effects on NOS but also to their ability to inhibit L-arginine uptake (Bogle et al., 1992b).

In summary, we have demonstrated that the L-arginine analogs, L-NMMA, L-NIO, L-NA but not L-NAME, inhibit the transport of L-arginine in rat proximal tubule BBM vesicles. The diverse characteristics of L-arginine transport in various tissues and the differential inhibition of this transport activity by L-arginine analogs suggests that it may be feasible to design L-arginine transport inhibitors that would be tissue and/or cell specific.

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References


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