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SPECIAL REPORT

Identification of inhibitors of nitric oxide synthase that do not interact with the endothelial cell L-arginine transporter

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The effects of inhibitors of nitric oxide (NO) synthase and other cationic amino acids on unidirectional L-arginine transport were studied in porcine aortic endothelial cells cultured in microwell plates or perfused in microcarrier columns. L-Homoarginine, L-lysine and L-ornithine inhibited transport of L-arginine. The NO synthase inhibitors N^G-monomethyl-L-arginine and N^G-iminoethyl-L-ornithine also reduced L-arginine uptake, whereas N^G-nitro-L-arginine and its methyl-ester had no inhibitory effect. The ability to modulate selectively endothelial cell L-arginine transport or NO synthase activity will allow further characterization of the arginine transporter and its role in regulating NO biosynthesis.

Keywords: Endothelial cells; nitric oxide; L-arginine transport; nitric oxide synthesis inhibitors; system y^+; L-homoarginine; N^G-monomethyl-L-arginine (L-NMMA); N^G-nitro-L-arginine methylester (L-NAME); N^G-iminoethyl-L-ornithine (L-NIO); N^G-nitro-L-arginine (L-NOARG)

Introduction Synthesis of nitric oxide (NO) from L-arginine has been identified as a widespread mechanism involved in the regulation of cellular function and communication. Vascular endothelial cells generate NO from L-arginine via a Ca^{2+}/calmodulin-dependent constitutive enzyme and a cytokine-inducible Ca^{2+}-independent NO synthase not normally present in endothelial cells (see Moncada *et al.*, 1991). Analogues of L-arginine such as N^G-monomethyl-L-arginine (L-NMMA), N^G-iminoethyl-L-ornithine (L-NIO), N^G-nitro-L-arginine (L-NOARG) and N^G-nitro-L-arginine methylester (L-NAME) are potent inhibitors of the release of NO from vascular endothelial cells (Moore *et al.*, 1989; Rees *et al.*, 1990), but the time course of maximal inhibition by these analogues and their reversibility by L-arginine appear to differ.

Endothelial cells take up L-arginine by a saturable transport system y^+ which is inhibited by L-NMMA and other cationic amino acids (Mann *et al.*, 1991) and stimulated by bradykinin (Bogle *et al.*, 1991). In the present study we have examined the inhibitory potency of other arginine analogues on L-arginine uptake by porcine aortic endothelial cells.

Methods Porcine aortic endothelial cells were isolated and cultured in Dulbecco's modified Eagle's medium supplemented with penicillin 100 units ml^{-1}, streptomycin 100 $\mu g\ ml^{-1}$, 10% foetal and 10% new born calf serum and L-glutamine 4 mM. When confluent, endothelial cells were either plated into 96-well plates or transferred onto Biosilon microcarriers (Nunc, Denmark).

Confluent monolayers in 96-well plates (2×10^4 cells per well) were rinsed with HEPES-buffered Krebs solution (see Bogle *et al.*, 1991) and L-arginine uptake was measured following incubation of monolayers with 50 μM L-[^{3}H]-arginine and D-[^{14}C]-mannitol (an extracellular tracer). Transport was terminated by washing monolayers 3 times with ice-cold phosphate-buffered saline and protein was determined with the BioRad reagent. Monolayers were digested with formic acid and samples taken for scintillation counting. Recovery of D-[^{14}C]-mannitol was always $<0.1\%$. Microcarrier cultures ($\sim 5 \times 10^6$ cells per column) were perfused at 0.5 ml min^{-1} with HEPES-buffered Krebs solution and cells were exposed briefly (150 μl in 30 s) to 0.5 μM L-[^{3}H]-arginine and D-[^{14}C]-mannitol. Fractions of the column effluent were collected sequentially to determine uptake of L-arginine relative to D-mannitol (Mann *et al.*, 1991). Unlabelled L-arginine analogues (0.05–5 mM) were added individually to the incubation media or microcarrier column perfusate.

L-[2,3-^{3}H]-arginine and D-[1-^{14}C]-mannitol were obtained from NEN (Germany), unlabelled amino acids, L-NAME and L-NOARG from Sigma and L-NMMA, D-NMMA and L-NIO from Wellcome, Beckenham, U.K.

Results Uptake of L-arginine by endothelial cell monolayers was linear for up to 20 min, hence, all measurements were made after a 15 min incubation period. L-Arginine uptake at 50 μM was 153 ± 27 nmol mg^{-1} protein h^{-1} (mean ± s.e.; $n = 4$). Transport was saturable over the concentration range of 1–100 μM, although at higher concentrations a non-saturable component became apparent (data not shown). Uptake of L-arginine (50 μM) was inhibited by L-homoarginine (1 mM; $52 \pm 4\%$; $n = 3$) but not by D-arginine (1 mM) or analogues selective for amino acid transport systems L (β-2-amino-bicyclo-[2,2.1]-heptane-2-carboxylic acid), N (6-diazo-5-oxo-L-norleucine) or A (2-methylaminoisobutyric acid). L-Arginine uptake was inhibited in a concentration-dependent manner by L-NMMA or L-NIO (Figure 1a) but was not inhibited by L-NOARG or L-NAME (Figure 1b). Although L-NOARG and L-NAME significantly ($P < 0.05$; unpaired t test) elevated the uptake of L-arginine, this effect did not appear to be dose-dependent and may reflect *trans*-stimulation of arginine entry (Mann *et al.*, 1991).

The effect of these NO synthase inhibitors on L-arginine uptake was investigated further in porcine aortic endothelial cells cultures and perfused on microcarrier beads. Under these conditions rapid (15 s) L-arginine transport was partially saturable (Mann *et al.*, 1991), sodium-independent (data not shown) and inhibited by 1 mM L-lysine, L-ornithine or L-homoarginine (Figure 2). Uptake of L-arginine (0.5 μM) was also inhibited by L-NMMA (1 mM; $57 \pm 6\%$) or L-NIO (1 mM; $41 \pm 4\%$) whereas L-NOARG (1 mM; $8 \pm 2\%$) and L-NAME (1 mM; $2 \pm 3\%$) were either weak or inactive inhibitors (Figure 2). D-NMMA and D-arginine were not inhibitors of L-arginine uptake (Figure 2).

Discussion Our results demonstrate that L-NOARG and L-NAME are poor inhibitors of L-arginine transport whereas

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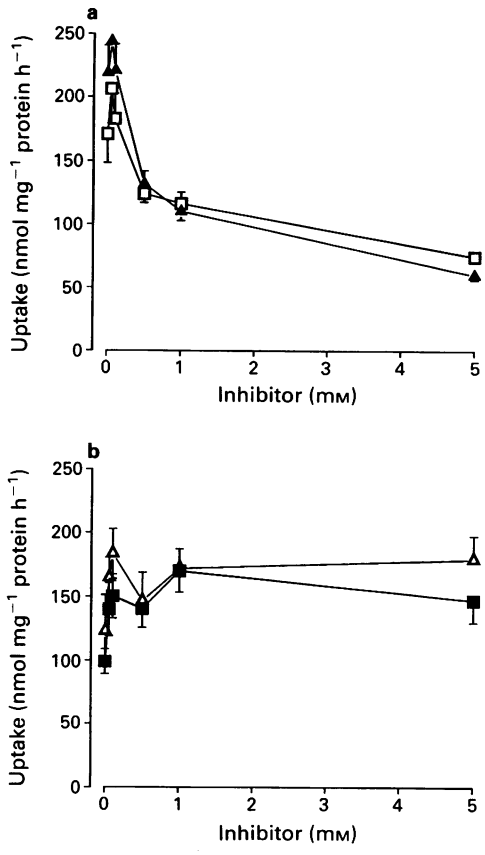

Figure 1 Effects of NO synthase inhibitors on L-arginine transport in endothelial cell monlayers. Inhibition of 50 μM L-[^{3}H]-arginine uptake by (a) N^G-monomethyl-L-arginine (□) or N^G-iminoethyl-L-ornithine (▲), (b) N^G-nitro-L-arginine (■) or N^G-nitro-L-arginine methyl ester (△). Values are mean of 15 replicates derived from three experiments using different batches of endothelial cells; vertical bar show s.e.

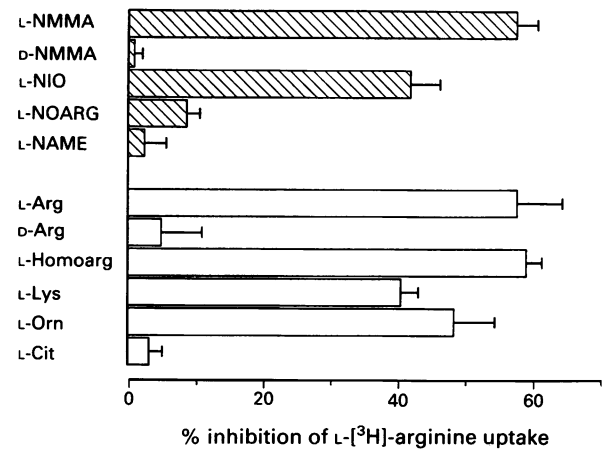

Figure 2 Effects of NO synthase inhibitors and other arginine analogues on rapid L-arginine transport in perfused endothelial cell microcarrier cultures. Cells were challenged in the absence of (J_c) and then the presence (J_i) of arginine analogues (1 mM) and the % inhibition of L-[^{3}H]-arginine uptake (0.5 μM) was calculated from $(1 - J_i/J_c) \times 100$. Values are mean of $n = 3-5$ experiments from different batches of endothelial cells, horizontal bars show s.e. For abbreviations, see text.

L-NMMA and L-NIO inhibit transport substantially, thus identifying inhibitors of NO synthase that do not block entry of L-arginine. Other cationic amino acids including L-lysine, L-ornithine and L-homoarginine, which do not inhibit the constitutive form of NO synthase (see Moncada *et al.*, 1991) also reduced L-arginine transport. The fraction of L-arginine transport insensitive to inhibition by arginine analogues may reflect entry via a second carrier with a lower substrate affinity.

Differences between the pharmacological profiles of NO synthase inhibitors have been described previously (Rees *et al.*, 1990), and it is possible that this is due to differences in the mechanisms by which they enter endothelial cells. In neutrophils and J774 cells inhibition of NO synthase has been shown to occur more rapidly with L-NIO than L-NMMA, and the effects of L-NIO were prevented following concomitant incubation with L-arginine (McCall *et al.*, 1991). This latter study also suggested that uptake of different arginine analogues may be mediated by different transport systems. Our findings are consistent with this idea, since the analogues that inhibited L-arginine transport did so reversibly and are most likely to compete with L-arginine for entry via the y^+ transporter.

The lack of effect of L-NOARG and L-NAME as inhibitors of endothelial cell L-arginine transport suggests that these compounds do not enter the cell via system y^+. Diffusion may account for the uptake of the lipophilic molecule L-NAME, however such a mechanism is unlikely to account for the entry of L-NOARG. The pathways by which these analogues are transported into endothelial cells can only be further elucidated by studies using radiolabelled L-arginine analogues.

It is likely that the major proportion of endotheial NO synthase activity is particulate (Förstermann *et al.*, 1991) and may be localised close to or at the plasma membrane (Boje & Fung, 1990). Close coupling between the L-arginine transporter and NO synthase could account for the regulation of NO release as well as for the ability of circulating L-arginine to reverse the inhibition of NO synthase by L-NMMA. Our results offer the potential to modulate L-arginine transport or NO synthase activity selectively. Recent cloning of NO synthase (Bredt *et al.*, 1991) and system y^+ (Kim *et al.*, 1991; Wang *et al.*, 1991) will allow application of immunocytochemical techniques to co-localise NO synthase and system y^+ in normal and diseased vascular endothelium.

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