4391 Journal of Physiology (1996), 490.1, pp.229-241

Regulation of L-arginine transport and nitric oxide release in superfused porcine aortic endothelial cells

R. G. Bogle, A. R. Baydoun, J. D. Pearson and G. E. Mann*

Vascular Biology Research Centre, Physiology Group, Biomedical Sciences Division, King's College, Campden Hill Road, London W8 7AH, UK

1. We have investigated whether changes in extracellular ion composition and substrate deprivation modulate basal and/or bradykinin-stimulated L-arginine transport and release of nitric oxide (NO) and prostacyclin (PGI_2) in porcine aortic endothelial cells cultured and superfused on microcarriers.

2. Saturable L-arginine transport ($K_m = 0{\cdot}14 \pm 0{\cdot}03$ mM; $V_{max} = 2{\cdot}08 \pm 0{\cdot}54$ nmol min^{-1} $(5 \times 10^6 \text{ cells})^{-1}$) was pH insensitive and unaffected following removal of extracellular Na^+ or Ca^{2+}.

3. Cationic arginine analogues, including L-lysine and L-ornithine, inhibited L-arginine transport, whilst 2-methylaminoisobutyric acid, β-2-amino-bicyclo[2,2.1]-heptane-2-carboxylic acid, L-phenylalanine, 6-diazo-5-oxo-norleucine, L-glutamine, L-cysteine and L-glutamate were poor inhibitors.

4. Deprivation of L-arginine (30 min to 24 h) reduced intracellular free L-arginine levels from $0{\cdot}87 \pm 0{\cdot}07$ to $0{\cdot}40 \pm 0{\cdot}05$ mM ($P < 0{\cdot}05$) and resulted in a 40% stimulation of L-arginine, L-lysine and L-ornithine transport.

5. L-arginine and N^G-monomethyl-L-arginine (L-NMMA), but not N^ω-nitro-L-arginine methyl ester (L-NAME), *trans*-stimulated efflux of L-[^{3}H]arginine.

6. Depolarization of endothelial cells with 70 mM K^+ reduced L-arginine influx and prevented the stimulation of transport by 100 nM bradykinin, but agonist-induced release of NO and PGI_2 was still detectable.

7. Basal rates of L-arginine transport and NO release were unaffected during superfusion of cells with a nominally Ca^{2+}-free solution. Bradykinin-stimulated L-arginine transport was insensitive to removal of Ca^{2+}, whereas agonist-induced NO release was abolished.

8. Although bradykinin-stimulated NO release does not appear to be coupled directly to the transient increase in L-arginine transport, elevated rates of L-arginine influx via system y^+ in response to agonist-induced membrane hyperpolarization or substrate deprivation provide a mechanism for enhanced L-arginine supply to sustain NO generation.

Synthesis of nitric oxide (NO) from L-arginine has been identified as a widespread mechanism involved in the regulation of the cardiovascular, immune and central nervous systems (Moncada, Palmer & Higgs, 1991; Knowles & Moncada, 1994). Vascular endothelial cells synthesize NO via a particulate Ca^{2+}–calmodulin-sensitive constitutive NO synthase (eNOS), which is activated by vasoactive agonists known to elevate intracellular Ca^{2+} (Hecker, Mülsch, Bassenge, Förstermann & Busse, 1994). Relaxation of vascular smooth muscle by NO is mediated through activation of soluble guanylate cyclase and the elevation of cyclic GMP (cGMP) (Moncada *et al.* 1991).

Under normal physiological conditions, L-arginine itself has no significant effect on blood pressure *in vivo* (Aisaka, Gross, Griffith & Levi, 1989), coronary perfusion pressure (Amezcua, Palmer, de Souza & Moncada, 1989) or the tension of isolated arterial rings (Gold, Bush & Ignarro, 1989). The K_m of eNOS for L-arginine is $< 0{\cdot}01$ mM, with maximal stimulation detected at concentrations of L-arginine between 0·03 and 0·1 mM (Palmer & Moncada, 1989; Mayer, Schmidt, Humbert & Böhme, 1989). Since reported values for intracellular L-arginine concentrations in cultured endothelial cells range between 0·1 and 0·8 mM (Gold *et al.* 1989; Baydoun, Emery, Pearson & Mann, 1990;

* To whom correspondence should be addressed.

Mitchell, Hecker, Anggard & Vane, 1990), eNOS would be expected to be saturated under normal conditions. Nevertheless, accumulating evidence from studies in cultured endothelial cells, perfused heart, intact animals and humans indicates that exogenous L-arginine can reverse inhibition of eNOS and enhance or sustain agonist-induced release of NO from the endothelium (Palmer, Ashton & Moncada, 1988; Amezcua *et al.* 1989; Aisaka *et al.* 1989). L-Arginine has also been reported to normalize impaired endothelium-dependent relaxation of resistance vessels exposed to hyperglycaemia (Poston & Taylor, 1995) or isolated from cholesterol-fed rabbits (Cooke, Andon, Girerd, Hirsch & Creager, 1991). Thus, these findings suggest that availability and transport of L-arginine can limit NO production.

We have previously reported that brief exposure of porcine aortic endothelial cells to bradykinin or ATP transiently stimulates L-arginine influx and NO production (Bogle, Coade, Moncada, Pearson & Mann, 1991). Using the same system, with endothelial cells cultured and superfused on microcarrier beads at constant flow, we have now characterized the kinetics of L-arginine transport and examined whether changes in external ionic composition modulate basal or bradykinin-stimulated L-arginine transport and NO release, assayed by measuring increases in cGMP levels in reporter LLC-PK_1 pig kidney epithelial cells (see Bogle *et al.* 1991). In order to validate the responsiveness of our endothelial cell microcarrier cultures to bradykinin, we also monitored basal and agonist-stimulated production of the vasodilator prostacyclin (PGI_2).

METHODS

Isolation and culture of porcine aortic endothelial cells

Thoracic aortae were collected from a local abattoir and placed in a pot containing 150 ml Hank's balanced salt solution (supplemented with 10 mM Hepes, 10 mM $NaHCO_3$, 100 μg ml^{-1} each of penicillin and streptomycin, 150 μg ml gentamicin and 10 μg ml^{-1} amphotericin B, pH 7·4) and stored on ice for up to 4 h before processing. Porcine aortic endothelial cells were isolated by collagenase (0·5 mg ml^{-1}) digestion and cultured in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories, UK) supplemented with penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}), fetal calf serum (10% w/v), new-born calf serum (10% w/v) and L-glutamine (4 mM). Cells were incubated at 37 °C in an atmosphere containing 5% CO_2 for 5–7 days until a confluent monolayer was obtained. Confluent monolayers were briefly exposed to trypsin–EDTA (0·05% : 0·02%), resuspended in culture medium and seeded (~4 × 10^6 cells) into siliconized 125 ml Techne stirrer flasks containing 40 ml serum-containing DMEM and 2 ml Biosilon microcarrier beads (Nunc, Roskilde, Denmark). Cells were incubated at 37 °C in 5% CO_2 and stirred intermittently at 40 r.p.m. for 2 min every 20 min using a Techne MCS-104S magnetic stirrer base. Microcarrier culture medium (20 ml) was removed every 2 days and replaced with fresh serum-containing DMEM. Cells became confluent on the microcarriers within 4–7 days with approximately 50–80 cells per bead.

Endothelial cells were identified by their typical cobblestone morphology when viewed by phase contrast microscopy, their ability to take up acetylated low-density lipoprotein and the absence of specific staining following incubation with anti-smooth muscle α-actin antibody (data not shown).

Superfusion of endothelial cell microcarrier cultures

As described previously (Mann, Pearson, Sheriff & Toothill, 1989*b*), endothelial cell-coated microcarriers were transferred into the barrel of a 1 ml syringe (~5 × 10^6 cells per 0·5 ml) and superfused from below at 0·5 ml min^{-1} with a Hepes-buffered physiological salt solution of composition (mM): NaCl, 131; KCl, 5·4; $CaCl_2$, 2·5; $MgCl_2$, 1; $NaHCO_3$, 25; Na_2HPO_4, 1; D-glucose, 5·5, Hepes, 20) at pH 7·4 and 37 °C. Unless stated otherwise, cells were equilibrated with the above Krebs solution for 20–30 min before measuring amino acid transport.

Sodium dependency of transport was investigated by superfusing cells with a Na^+-free solution, in which Na^+ was replaced isosmotically with choline chloride, choline bicarbonate and KH_2PO_4. In other experiments, Krebs solution was adjusted to pH 6·0, 7·0, 7·4 and 8·0 by the addition of HCl (0·5 N) or NaOH (0·5 N) to the Krebs medium. Nominally Ca^{2+}-free solutions were prepared by omission of $CaCl_2$. In experiments with 70 mM K^+, the perfusate had the following composition (mM): sodium gluconate, 145; KCl, 11·13; potassium gluconate, 58·87; $MgSO_4$, 1; $CaCl_2$, 2·5; glucose, 5·5; and Hepes, 10, to maintain a constant $[K^+]:[Cl^-]$ product and thus minimize potential changes in cell volume (see Carter, Bogle & Bjaaland, 1991).

Measurement of unidirectional tracer uptake and cellular efflux

Unidirectional amino acid transport was measured using a rapid dual tracer dilution technique, previously applied to microcarrier cultures of endothelial cells (Mann *et al.* 1989*b*). Cells were challenged for 30 s with Hepes-buffered perfusate containing an L-[^{3}H]amino acid (1·4 μCi ml^{-1}) and D-[^{14}C]mannitol (0·7 μCi ml^{-1}, extracellular tracer), and the column effluent was sampled sequentially for 90 s. A final effluent sample was collected for a further 90 s to assess tracer recoveries and amino acid efflux. As illustrated in Fig. 1, the time course of tracer uptake in successive effluent samples was quantified using the equation:

$$\text{Uptake} = \{1 - (\text{L-}[^3\text{H}]\text{amino acid}/\text{D-}[^{14}\text{C}]\text{mannitol})\}.$$

The fractional maximal amino acid uptake (U_{max}) was used to calculate influx (v) using the equation:

$$v = [-F \times \ln(1 - U_{max}) \times C_a],$$

where C_a is the perfusate amino acid concentration and F the perfusion rate in ml min^{-1}. Efflux of a labelled amino acid (or ^{3}H-metabolite) during a rapid transit through an endothelial cell column was estimated from:

$$\text{Efflux}(\%) = (1 - U_T/U_{max}) \times 100,$$

where U_T is the net amino acid uptake relative to D-[^{14}C]mannitol determined from the integrated recoveries of both tracers during a 3 min sampling period (Mann *et al.* 1989*b*).

Specificity and kinetics of unidirectional L-arginine transport

In cross-inhibition studies of L-arginine influx, endothelial cells were superfused with 10 μM L-[^{3}H]arginine in the absence or

presence of an unlabelled amino acid (1 mM). Inhibition of the initial rate of L-[^{3}H]arginine transport was calculated from:

$$\text{Inhibition (\%)} = (1 - J_i/J_c) \times 100,$$

where J_c and J_i are the influx values measured in the absence or presence, respectively, of an inhibitor amino acid. The kinetics of unidirectional L-arginine transport were measured during superfusion of cells successively with different concentrations of L-arginine (0·025–1 mM). Kinetic data were analysed using the computer program Enzfitter (Biosoft, Cambridge, UK) and were fitted to a Michaelis–Menten equation plus a non-saturable linear component.

Net L-arginine uptake and efflux in cell loading and washout experiments

In these experiments, confluent endothelial cell monolayers on microcarrier were cultured for 24 h in L-arginine-free DMEM, supplemented with L-glutamine (4 mM) and 10% fetal and 10% new-born calf serum, which had been dialysed twice against a 100-fold excess of phosphate-buffered saline using dialysis tubing with a 10000 molecular weight cut-off (Medicell International Ltd, UK). Chromatographic analysis of L-arginine-free DMEM revealed that this medium was also free of ornithine, glutamate, taurine, alanine and citrulline, normally present at low concentrations in non-dialysed calf serum (see Table 2).

Arginine-deprived cells were superfused continuously with an amino acid-free Krebs solution containing L-[^{3}H]arginine (1 μCi ml^{-1}) and D-[^{14}C]mannitol (0·1 μCi ml^{-1}). The column effluent was initially sampled at 3 s intervals for the first minute to monitor unidirectional uptake, and then at 1 min intervals for the next 15–20 min to monitor net uptake (data not shown; see Mann, Norman & Smith, 1989*a*). Net uptake of L-[^{3}H]arginine (U) was measured in successive samples from:

$$U = 1 - (\text{L-}[^3\text{H}]\text{arginine}/\text{D-}[^{14}\text{C}]\text{mannitol}).$$

Cells were challenged with bradykinin (100 nM, 2 min) or Krebs solution after 20 min continuous superfusion with Krebs solution of normal ionic composition, nominally Ca^{2+} free or containing 70 mM K^+. During an agonist challenge the column effluent was again collected at 3 s intervals to monitor rapid changes in L-[^{3}H]arginine uptake. In some experiments the perfusate was switched to an isotope-free solution, after a 20–40 min cell loading period, to monitor washout of L-[^{3}H]arginine and D-[^{14}C]mannitol in response to brief challenges (100 μl in 30 s) with L-arginine, N^G-monomethyl-L-arginine (L-NMMA), N^ω-nitro-L-arginine methyl ester (L-NAME) or D-mannitol. During washout of radiolabelled L-arginine and D-mannitol, the column effluent was sampled at 3 s intervals.

Metabolism of L-[^{3}H]arginine

Metabolism of L-[^{3}H]arginine during a single transit through an endothelial cell microcarrier column was assessed by thin layer chromatography (TLC). The column effluent was pooled into three samples: 0–30, 30–60 and 60–90 s, which were evaporated to dryness and resuspended in 50 μl of 75% ethanol, of which 20 μl were spotted on TLC plates (0·1 mm cellulose-coated plastic sheets, Merck 5577, BDH Chemicals) and eluted using *n*-butanol–acetic acid–water (12 : 3 : 5). TLC plates were scanned using a Berthold LB2760 TLC scanner to quantify the eluted ^{3}H radioactivity. Radioactivity eluted from the columns as a single peak with an R_f (relative band speed) value equivalent to that of the L-[^{3}H]arginine standard (data not shown), indicating that within a rapid single transit through the column metabolism of L-[^{3}H]arginine was negligible.

HPLC analysis of endothelial cell amino acid concentrations

Confluent cell monolayers (~2×10^5 cells) were cultured for 30 min, 1 h, 6 h or 24 h in normal DMEM containing 20% serum or in L-arginine-free DMEM supplemented with 20% dialysed serum. Cells were washed twice with phosphate-buffered saline (10 ml) and harvested using trypsin–EDTA. Following centrifugation (1000 r.p.m. for 5 min), the supernatant was discarded and cell pellets were lysed with 90% methanol and the deproteinized supernatant (20 μl) analysed by reverse-phase high performance liquid chromatography using a Beckman 344 gradient HPLC system (Beckman RIIC Ltd, Bucks, UK; Baydoun *et al.* 1990). Amino acid concentrations were calculated with reference to the internal standard homoserine and the measured intracellular water space of 1 pl.

Cell number, protein synthesis and intracellular volume

Cell number was determined using a Coulter counter or haemocytometer. Protein synthesis was measured in actively replicating cells incubated with 5 μCi ml^{-1} L-[^{3}H]leucine. After 24 h, monolayers were rinsed twice with 500 μl warmed phosphate-buffered saline, exposed to 5% trichloroacetic acid for 5 min and then rinsed with 90% methanol. Radioactivity in formic acid cell digests was determined by liquid scintillation counting. The intracellular water space (1×10^{-12} l) was calculated from the uptake of a non-metabolizable sugar, 3-*O*-methyl-D-glucose (Kletzien, Pariza, Becker & Potter, 1975). Uptake of 3-*O*-methyl-D-[^{3}H]glucose (1 μCi ml^{-1}) by confluent endothelial cell monolayers reached a steady state within 60 min and was linear over substrate concentrations ranging from 2·5 to 10 mM (data not shown).

Radioimmunoassay of 6-oxo-prostaglandin $F_{1\alpha}$ and cyclic GMP

Release of prostacyclin (PGI_2) from superfused endothelial cell columns was determined by radioimmunoassay of its stable metabolite, 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-PGF$_{1\alpha}$; Needham, Cusack, Pearson & Gordon, 1987). Endothelium-derived NO release was assayed indirectly by measuring accumulation of cGMP in LLC-PK$_1$ pig kidney epithelial cells exposed to endothelial cell microcarrier column effluent. The antibody was a goat anti-cGMP antibody (MRC Clinical Research Centre, Harrow, UK) used at 1 : 15000 dilution, and cross-reactivity with other cyclic nucleotides was < 1%.

LLC-PK$_1$ cells were obtained from the European Collection of Animal Cells Cultures (Porton Down, Wiltshire, UK) and maintained in T75 or T225 tissue culture flasks containing medium M199 and 10% fetal calf serum. Cells between passage 180 and 200 were rinsed with Hepes-buffered salt solution, incubated with isobutylmethylxanthine (0·5 mM) for 10 min to inhibit cGMP breakdown by phosphodiesterases, and then exposed to column perfusate of varied ionic composition, bradykinin (100 nM), sodium nitroprusside (SNP, 100 μM) or the effluent from perfused endothelial cell microcarrier columns (Bogle *et al.* 1991). When endothelial cells were challenged with bradykinin (100 nM for 2 min), 1 min fractions of the effluent were collected into 2 cm^2 wells containing LLC-PK$_1$ cells. After 30 s, cells were extracted with 0·1 N HCl and cGMP was determined by radioimmunoassay following acetylation. We have previously validated LLC-PK$_1$ reporter cells as an assay system for endothelium-derived NO production (Bogle *et al.* 1991). Incubation of LLC-PK$_1$ cells for

5 min with increasing concentrations of SNP, which generates NO in aqueous solution, resulted in a concentration-dependent increase in cGMP accumulation. Moreover, superfusion of endothelial cells with 100 μM L-NAME, an NO synthase inhibitor, abolished increases in LLC-PK_1 cGMP levels in response to the effluent collected from bradykinin-stimulated endothelial cells. Exposure of LLC-PK_1 cells to bradykinin (10–1000 nM) for 5 min did not elevate cGMP levels, nor did incubation of LLC-PK_1 cells with a nominally Ca^{2+}-free medium alter basal or SNP-stimulated cGMP formation (data not shown).

Radioactive molecules

L-[2,3-^{3}H]arginine (50·4 Ci $mmol^{-1}$), L-[4,5-^{3}H]lysine (87·4 Ci $mmol^{-1}$), L-[2,3-^{3}H]ornithine (50 Ci $mmol^{-1}$), L-[3,4,5-^{3}H]leucine (153 Ci $mmol^{-1}$), L-[3,4-^{3}H]glutamine (58·4 Ci $mmol^{-1}$), L-[3,4-^{3}H]glutamate (54·7 Ci $mmol^{-1}$), L-[5-^{3}H]proline (5·2 Ci $mmol^{-1}$) and D-[1-^{14}C]mannitol (53·4 mCi $mmol^{-1}$) were obtained from NEN, Dreieich, Germany. L-[4-^{3}H]phenylalanine (26 Ci $mmol^{-1}$) and L-[^{3}H]serine (37 Ci $mmol^{-1}$) were obtained from Amersham International, UK. [^{125}I]-6-Oxo-$PGF_{1\alpha}$ was obtained from ICN Radiochemicals, USA and [^{125}I]-cGMP from Metachem Diagnostics, UK.

Statistics

Data are presented as the means ± S.E.M. of determinations in n different porcine aortic endothelial cell cultures. Statistical analyses were performed using Student's paired or unpaired t test and $P < 0·05$ was considered significant.

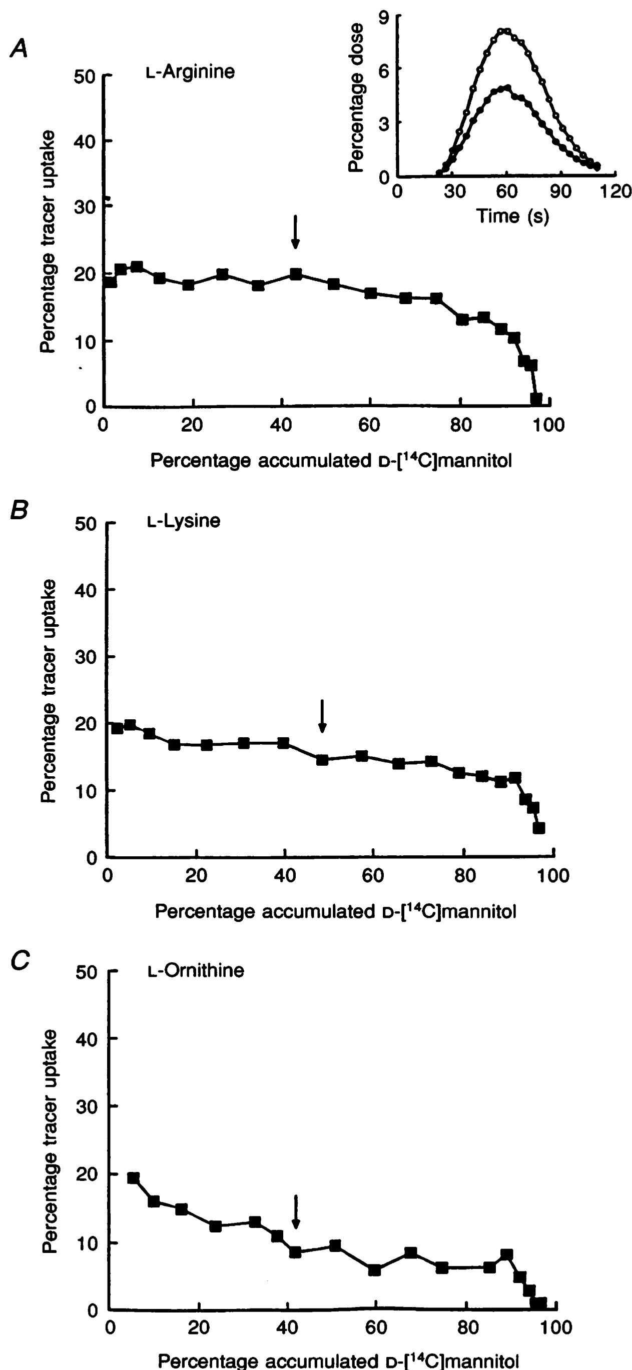

Figure 1. Unidirectional transport of cationic amino acids

Rapid (20 s) uptake of ^{3}H-labelled cationic amino acids was measured relative to D-[^{14}C]mannitol during a single transit of both tracers through an endothelial cell microcarrier column. Uptake was weighted for the accumulated recovery of D-[^{14}C]mannitol (extracellular reference tracer) in each of the successive effluent samples, and arrows denote the peak effluent radioactivity of D-[^{14}C]mannitol. Inset, dilution profiles for L-[^{3}H]arginine (●, lower trace) and D-[^{14}C]mannitol (○, upper trace) expressed as a percentage of the radiotracer doses injected into the inflow of the microcarrier column. Data are from single experiments representative of at least 6 others.

RESULTS

Endothelial cell uptake and efflux of L-arginine and other amino acids

The inset in Fig. 1 shows effluent dilution profiles for L-[^{3}H]-arginine and D-[^{14}C]mannitol (extracellular tracer) following a 30 s challenge of endothelial cells with both tracers. The lower recovery of L-[^{3}H]arginine relative to D-[^{14}C]mannitol reflects endothelial cell uptake of L-arginine. As shown in Fig. 1, unidirectional uptake (20 s) of the cationic amino acids L-arginine, L-lysine and L-ornithine was followed by tracer efflux. Significant uptakes were also measured for neutral and acidic amino acids and L-proline (Table 1), and efflux of ^{3}H (or labelled metabolite) associated with L-arginine, L-leucine, L-glutamine and L-serine was enhanced during superfusion of cells with 50 μM unlabelled substrate.

Transport of L-arginine was unaffected by changes in extracellular pH or removal of Na^+ (data not shown), and unless stated otherwise experiments were performed in the presence of Na^+.

Kinetics and specificity of L-arginine transport

The kinetics of L-arginine transport were examined under conditions of constant flow (0·5 ml min^{-1}). Although transport was saturable at plasma concentrations, a non-saturable component was detected at higher extracellular L-arginine concentrations (Fig. 2). The mean kinetic parameters determined from five different experiments were: $K_m = 0{\cdot}14 \pm 0{\cdot}03$ mM, $V_{max} = 2{\cdot}08 \pm 0{\cdot}54$ nmol min^{-1} (5 × 10^6 cells)$^{-1}$ and a non-saturable component $K_D = 4{\cdot}5 \pm 1{\cdot}1$ μl min^{-1} (5 × 10^6 cells)$^{-1}$. We have reported previously that L-arginine transport was stereospecific and inhibited by cationic arginine analogues, including L-homoarginine, L-lysine, L-ornithine and the NO synthase (NOS) inhibitors L-NMMA and N^{ω}-iminoethyl-L-ornithine (L-NIO) (Bogle *et al.* 1992). We have extended these inhibition experiments and found that transport of 10 μM L-arginine was not inhibited significantly by 1 mM of the neutral amino acid analogues, 2-methylaminoisobutyric acid (MeAIB, 4 ± 4%, $n = 3$), β-2-amino-bicyclo[2,2.1]-heptane-2-carboxylic acid (BCH, 3 ± 4%, $n = 3$) and 6-diazo-5-oxo-norleucine (DON, 3 ± 4%, $n = 5$), and the naturally occurring amino acids L-cysteine (10 ± 7%, $n = 5$), L-glutamine (13 ± 4%, $n = 5$), L-phenylalanine (16 ± 9%, $n = 5$) and L-glutamate (5 ± 5%, $n = 3$). L-Canavanine, a structural analogue of L-arginine known to inhibit NO formation by vascular endothelial cells (Palmer *et al.* 1988; Schmidt *et al.* 1988), caused a significant inhibition (49 ± 12%, $n = 5$, $P < 0{\cdot}05$).

L-Arginine deprivation alters membrane transport and intracellular L-arginine levels

Confluent endothelial cell microcarrier cultures were incubated for 30 min, 1 h, 6 h or 24 h in DMEM containing normal serum or L-arginine-free DMEM supplemented with 20% dialysed serum. Arginine deprivation for up to 24 h had no significant effect on cell volume or number (data not shown). Moreover, incorporation of L-[^{3}H]leucine into endothelial cell protein was similar ($P > 0{\cdot}05$) in

Table 1. Unidirectional transport of L-arginine and other amino acids by porcine aortic endothelial cells cultured and superfused on microcarriers

Amino acid	Isotope solution † (μM)	Control tracer uptake (%)	Percentage tracer efflux	
			Control	+ 50 μM amino acid
Cationic				
L-Arginine	0·5	19 ± 1	16 ± 2	37 ± 4*
L-Lysine	0·3	21 ± 3	23 ± 5	n.d.
L-Ornithine	0·5	21 ± 2	48 ± 3	n.d.
Large neutral				
L-Leucine	0·4	31 ± 3	26 ± 3	50 ± 3*
L-Phenylalanine	0·4	42 ± 2	17 ± 1	20 ± 5
L-Glutamine	0·4	40 ± 3	5 ± 3	67 ± 4*
Short-chain				
L-Serine	0·8	39 ± 3	18 ± 3	60 ± 2*
Acidic				
L-Glutamate	0·4	16 ± 2	14 ± 2	n.d.
Imino				
L-Proline	0·2	24 ± 4	61 ± 5	n.d.

Cells were challenged with an L-[^{3}H]amino acid and D-[^{14}C]mannitol in the absence or presence of 50 μM unlabelled substrate. Tracer uptake and efflux were measured simultaneously (see Methods); control efflux values were calculated during superfusion of cells in the absence of 50 μM substrate. Data are the means ± S.E.M. of measurements in 6–14 different cell cultures. * $P < 0{\cdot}05$, unpaired t test compared with control efflux. † Amino acid concentration in the isotope injectate. n.d., not determined.

Table 2. Effects of L-arginine deprivation on intracellular amino acid concentrations in porcine aortic endothelial cells

Amino acid	Control medium	Control cell	Cell : medium ratio	24h deprived cell	Cell : medium ratio
Essential					
Arginine	0·33 ± 0·20	0·87 ± 0·07	2·64	0·40 ± 0·05*	—
Histidine	0·16 ± 0·10	0·37 ± 0·06	2·31	0·44 ± 0·07	2·75
Isoleucine	0·71 ± 0·06	2·13 ± 0·21	3·00	2·54 ± 0·30	3·60
Leucine	0·66 ± 0·44	2·06 ± 0·16	3·12	2·62 ± 0·29	3·97
Lysine	0·66 ± 0·50	1·68 ± 0·20	2·55	1·83 ± 0·29	2·77
Methionine	0·16 ± 0·01	0·52 ± 0·04	3·25	0·65 ± 0·08	4·06
Phenylalanine	0·35 ± 0·30	1·08 ± 0·09	3·10	1·33 ± 0·15	3·80
Threonine	0·66 ± 0·60	2·08 ± 0·21	3·15	4·26 ± 0·66*	6·45
Tryptophan	0·08 ± 0·01	0·24 ± 0·02	3·00	0·28 ± 0·03*	3·50
Valine	0·75 ± 0·07	2·14 ± 0·19	2·85	1·21 ± 0·14*	1·61
Total	4·5 ± 0·2	12·8 ± 1·16	2·80	14·9 ± 1·81	3·57
Non-essential					
Alanine	0·18 ± 0·01	1·20 ± 0·08	6·67	2·17 ± 0·32*	—
Asparagine	—	n.d.	—	0·51 ± 0·09*	—
Aspartate	0·08 ± 0·02	4·56 ± 0·66	57·00	2·52 ± 0·38*	29·80
Citrulline	0·04 ± 0·01	0·11 ± 0·02	2·75	0·09 ± 0·03	—
Glutamate	0·17 ± 0·02	30·5 ± 5·8	179·00	20·3 ± 4·2	—
Glutamine	8·28 ± 0·37	19·9 ± 2·2	2·40	34·5 ± 5·4*	4·20
Glycine	0·41 ± 0·03	5·55 ± 0·75	13·50	11·4 ± 2·5*	27·80
Ornithine	0·09 ± 0·02	0·41 ± 0·04	4·56	0·48 ± 0·08	—
Serine	0·37 ± 0·03	2·03 ± 0·18	5·49	3·00 ± 0·55	8·11
Taurine	0·03 ± 0·00	3·05 ± 0·66	102·00	0·67 ± 0·17*	—
Tyrosine	0·32 ± 0·10	1·08 ± 0·11	3·38	1·70 ± 0·23*	5·31
Total	10 ± 0·5	56·1 ± 7·5	5·61	77·0 ± 14	8·13

Intracellular amino acid levels (mmol l^{-1}) denote the means ± S.E.M. of measurements in 8 different cell cultures. *$P < 0{\cdot}05$ *vs.* control cells, Student's unpaired *t* test. Dialysed L-arginine-free DMEM was also free of alanine, citrulline, glutamate, ornithine and taurine. n.d., not detected.

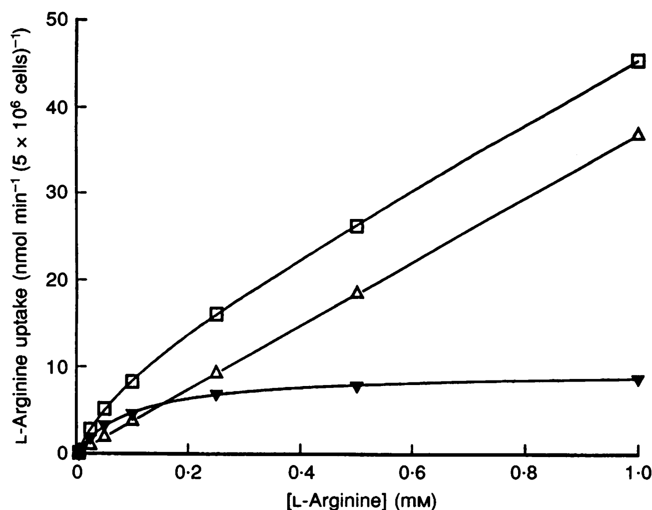

Figure 2. Kinetics of L-arginine transport

L-Arginine influx was measured in endothelial cell microcarrier cultures perfused at 0·5 ml min^{-1} with a Krebs solution containing L-arginine (0·025–1 mM). Kinetic data were best fitted by a Michaelis–Menten equation plus a linear non-saturable component. A representative experiment is shown, and saturable L-arginine transport (▼) was derived by subtraction of the non-saturable component (△) from the total influx (□) measured at each substrate concentration. Similar results were obtained in 4 other experiments.

control cells (10349 ± 155 c.p.m.) and cells deprived of L-arginine for 24 h (10064 ± 299 c.p.m., $n = 10$).

The total essential and non-essential intracellular amino acid concentrations in control cells were $12{\cdot}8 \pm 1{\cdot}2$ and $56{\cdot}1 \pm 6{\cdot}7$ mM, respectively (Table 2). These did not change significantly in L-arginine-deprived cultures, but there were significant changes in the levels of individual amino acids. Intracellular L-lysine and L-ornithine concentrations were unaffected, whereas L-arginine levels decreased within 30 min (from 0·87 to 0·4 mM) and remained depressed over 24 h. The decrease in intracellular L-arginine was paralleled by a stimulation of unidirectional L-arginine, L-lysine and L-ornithine transport (Fig. 3). In contrast, concentrations of L-valine, L-aspartate, L-glutamate and taurine decreased, whereas those of L-threonine, L-glutamine and glycine increased (Table 2).

Trans-stimulation of L-arginine efflux in cell loading and washout experiments

When tracer efflux was monitored during superfusion of cells with an isotope-free solution (Fig. 4), after preloading with L-[^{3}H]arginine and D-[^{14}C]mannitol for 20–40 min, D-[^{14}C]mannitol washout was fitted by a single exponential with a time constant of $0{\cdot}7 \pm 0{\cdot}06$ min ($n = 3$), consistent with washout occurring from an extracellular compartment (see Mann *et al.* 1989*a*). Washout of L-[^{3}H]arginine was best fitted by two exponentials, suggesting that exit of this tracer was occurring from at least two pools. The fast time constant of $0{\cdot}8 \pm 0{\cdot}04$ min ($n = 3$) was similar to that for D-[^{14}C]mannitol, whilst the slower time constant of $7{\cdot}28 \pm 0{\cdot}44$ min reflects L-arginine washout from a pool inaccessible to D-mannitol.

When loaded cells were challenged with L-arginine (Fig. 4, inset) or the cationic NOS inhibitor L-NMMA, which inhibits L-arginine entry (Bogle *et al.* 1992*a*), accelerated ^{3}H efflux was observed, whereas the neutral NOS inhibitor L-NAME had no effect (Fig. 4).

Ionic dependency of bradykinin-stimulated L-arginine transport and NO and PGI_2 release

Net uptake of L-arginine was monitored during continuous superfusion of cells with L-[^{3}H]arginine and D-[^{14}C]mannitol. The initial uptake of L-[^{3}H]arginine in these cell loading experiments was similar to the unidirectional uptake measured in rapid tracer dilution experiments (Fig. 1). When endothelial cells were challenged with 100 nM bradykinin in the presence of 5·4 mM K^+, net uptake of L-[^{3}H]arginine increased rapidly and then declined gradually to prestimulus values (Fig. 5). Exposure to 70 mM K^+ reduced both unidirectional (data not shown) and net L-arginine uptake and prevented the stimulatory effect of bradykinin on L-arginine transport (Table 3). In the same experiments, basal levels of PGI_2 release and cGMP in reporter LLC-PK_1 cells (assay for endothelial cell NO production) were unchanged by elevated K^+. Bradykinin-stimulated PGI_2 release was also unaffected by elevated K^+, whereas bradykinin-stimulated NO release was reduced marginally ($P < 0{\cdot}05$, Table 3).

Superfusion of cells with a nominally Ca^{2+}-free solution for 20 min had no significant effect on basal or bradykinin-

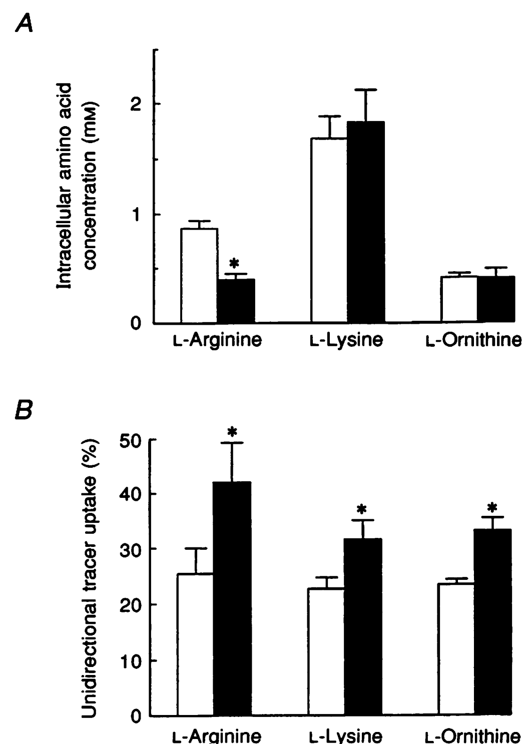

Figure 3. Effects of L-arginine deprivation on transport and intracellular cationic amino acid concentrations

A, effects of 24 h L-arginine deprivation on intracellular concentrations of L-arginine, L-lysine and L-ornithine. Data denote the means ± S.E.M. of measurements in 8 different cell cultures. *B*, effect of L-arginine deprivation on transport of 10 μM L-arginine, L-lysine and L-ornithine. Data denote the means ± S.E.M. of measurements in 3 cell cultures. * $P < 0{\cdot}05$, Student's unpaired *t* test. □, control; ■, L-arginine deprived.

stimulated L-arginine transport (Table 3). Basal cGMP levels in reporter LLC-PK_1 cells and basal prostacyclin (PGI_2) release were not altered by removal of extracellular Ca^{2+}, but bradykinin-induced NO release was abolished and PGI_2 production was significantly reduced (Table 3).

Effects of sodium nitroprusside on L-arginine transport

To determine whether acute release of NO by the endothelium or raised intracellular cGMP levels modulate agonist-induced increases in L-arginine transport, cells were superfused with sodium nitroprusside (SNP), which spontaneously liberates NO in aqueous solution and increases cGMP levels in porcine aortic endothelial cells (Schini, Boulanger, Regoli & Vanhoutte, 1990). When cells were challenged with SNP (100 μM, 2 min), during continuous superfusion with radiolabelled L-arginine and D-mannitol, net uptake of L-arginine was unaltered (inset, Fig. 5). This 2 min stimulation period with SNP was based on the 2 min challenge of endothelial cells with bradykinin.

DISCUSSION

Endothelial cell transport of L-arginine has been attributed largely to the Na^+-independent cationic amino acid system y^+ (Mann *et al.* 1989; Bogle *et al.* 1991, 1992; Bussolati, Sala, Astorri, Rotoli, Dall'Asta & Gazzola, 1993; Greene, Pacitti & Souba, 1993; Schmidt, Klatt & Mayer, 1993; Sobrevia, Cesare, Yudilevich & Mann, 1995), although the reported characteristics of L-arginine transport vary considerably in cells isolated from different vascular beds.

In the present study, L-arginine transport by porcine aortic endothelial cells exposed to constant flow was saturable at plasma concentrations, Na^+-independent, unaffected by changes in extracellular Ca^{2+} or pH, sensitive to *trans*-stimulation and selectively inhibited by cationic arginine analogues. All these properties are characteristic of system y^+ (White, 1985), recently cloned from murine fibroblasts and expressed in *Xenopus* oocytes (Kim, Closs, Albritton & Cunningham, 1991). Additional murine homologues of system y^+ (MCATs; murine cationic amino

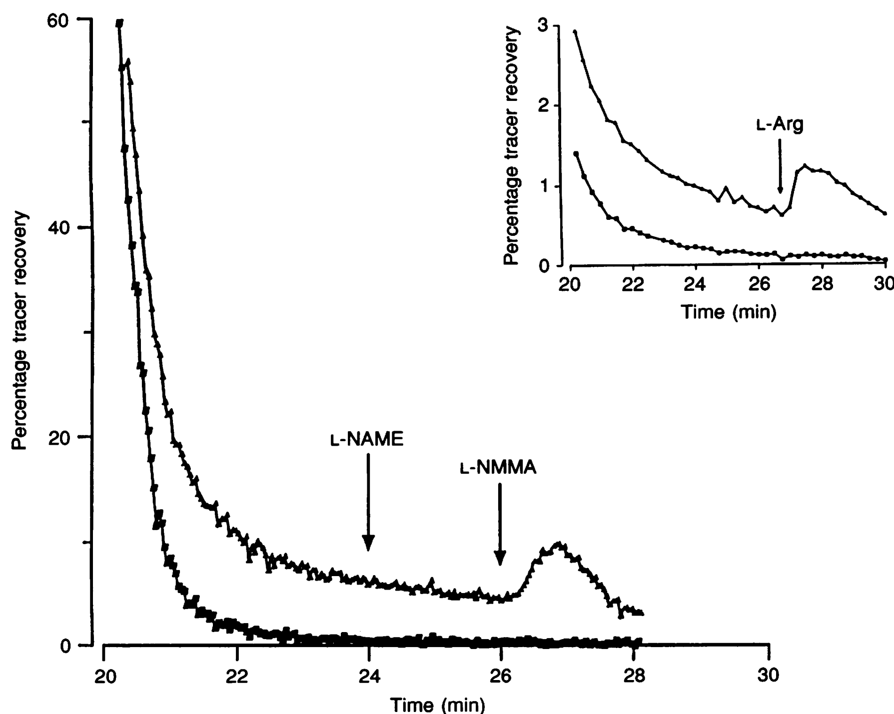

Figure 4. *Trans*-stimulation of L-arginine efflux by L-arginine and L-NMMA but not by L-NAME

L-Arginine-deprived (24 h) endothelial cells were superfused with an amino acid-free Krebs solution containing L-[^{3}H]arginine (▲) and D-[^{14}C]mannitol (■). After a 20 min cell loading period (data not shown), the perfusate was switched to an isotope-free Krebs solution and tracer washout was monitored by sampling the effluent at 3 s intervals. During the washout phase cells were challenged with a bolus injection (50 μl over 5 s) of either L-arginine (10 mM, inset) or L-NAME (20 mM) and then L-NMMA (20 mM). Washout profiles for L-[^{3}H]arginine (or ^{3}H-labelled metabolite) and D-[^{14}C]mannitol were unaffected following a challenge with 20 mM D-mannitol (data not shown). Data are representative of 3 experiments.

Table 3. Ionic dependency of bradykinin-stimulated L-arginine transport and nitric oxide and prostacyclin release

	[^{3}H]arginine uptake (% net)		cGMP production (% control)		6-oxo-PGF$_{1\alpha}$ (pmol min^{-1})	
Condition	Basal	BK stimulated	Basal	BK stimulated	Basal	BK stimulated
Control	12 ± 3	32 ± 2*	100 ± 11	259 ± 50**	0·4 ± 0·1	45 ± 13**
+70 mM K$^+$	8 ± 3†	11 ± 4	100 ± 7	173 ± 34*	1·6 ± 0·9	60 ± 5**
−Ca^{2+}	18 ± 5	28 ± 5*	100 ± 12	100 ± 21	0·7 ± 0·3	22 ± 11*

Cells were superfused with Krebs solution of normal ionic composition (5·4 mM K$^+$), nominally Ca^{2+} free or containing 70 mM K$^+$. Net L-arginine transport was monitored during continuous loading with L-[^{3}H]arginine and D-[^{14}C]mannitol. Changes in L-arginine transport and release of NO and prostacyclin (6-oxo-PGF$_{1\alpha}$) were then measured in response to a 2 min challenge with the appropriate Krebs solution (control) or bradykinin (BK; 100 nM). PGI$_2$ release was measured by radioimmunoassay of 6-oxo-PGF$_{1\alpha}$ in the column effluent and NO release by radioimmunoassay of cGMP production in reporter LLC-PK$_1$ cells (see Methods). Basal values for cGMP in control conditions, +70 mM K$^+$ and −Ca^{2+} were 1·97 ± 0·28, 0·72 ± 0·31 and 2·53 ± 0·46 pmol (10^6 cells)$^{-1}$, respectively. Data denote the means ± S.E.M. of measurements in 3–6 different cell cultures. ** $P < 0·01$, * $P < 0·05$ bradykinin stimulated *vs.* respective basal values, Student's paired *t* test; † $P < 0·05$, +70 mM K$^+$ *vs.* control, Student's unpaired *t* test.

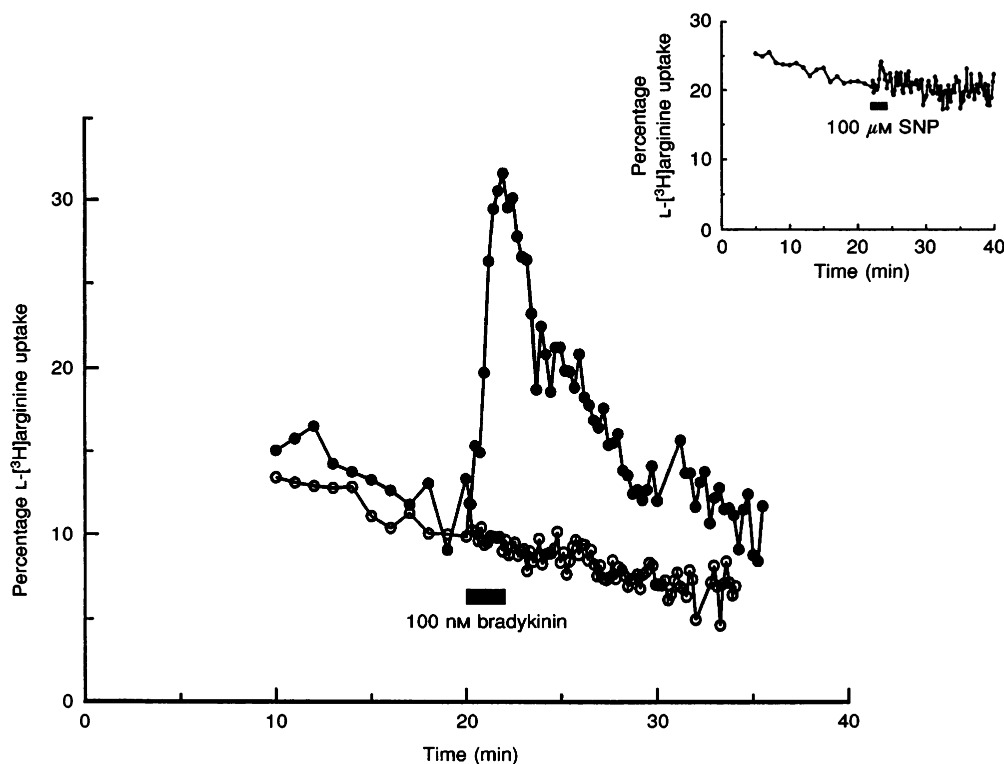

Figure 5. Inhibition of bradykinin-stimulated L-arginine transport in endothelial cells depolarized with 70 mM K$^+$

Endothelial cell microcarrier cultures were perfused with Krebs solution containing either 5·4 mM K$^+$ (●) or 70 mM K$^+$ (○) and L-[^{3}H]arginine and D-[^{14}C]mannitol. After 20 min loading, cells were challenged with bradykinin (100 nM, 2 min). Inset: effects of sodium nitroprusside (SNP, 100 μM for 2 min) on net uptake of L-[^{3}H]arginine during superfusion of cells with Krebs containing 5·4 mM K$^+$. Data are representative of 3–6 different experiments.

acid transporters) have also been cloned (Closs, Lyons, Kelly & Cunningham, 1993). The selectivity and kinetic properties of system y^+ (MCAT-1) are indistinguishable from MCAT-2B, but this isoform has only been identified in activated macrophages. The K_m value that we determined for saturable L-arginine transport in porcine aortic endothelial cells (0·14 mM) is similar to that reported for MCAT-1 ($K_m = 0{\cdot}14–0{\cdot}25$ mM), rather than the very low affinity MCAT-2A isoform ($K_m > 4$ mM) identified in hepatocytes (Closs *et al.* 1993). However, since L-arginine transport is sensitive to changes in membrane potential (Bussolati *et al.* 1993; Kavanaugh, 1993; Sobrevia *et al.* 1995), classification of transporters on the basis of differences in K_m alone may be inadequate.

Our kinetic experiments were performed over a wide range of substrate concentrations, but we found no evidence of Na^+-dependent, high-affinity ($K_m < 100$ μM) or Na^+-independent, low-affinity ($K_m > 4$ mM) components of L-arginine transport, as described in porcine pulmonary artery endothelial cells (Greene *et al.* 1993). Schmidt *et al.* (1993) have found two Na^+-independent processes involved in L-arginine accumulation by porcine aortic endothelial cell monolayers, but the kinetic constants ($K_m = 6$ and 600 μM) were determined over a 30 min incubation period and thus do not reflect initial rates of influx. We conclude that porcine aortic endothelial cells transport L-arginine mainly via system y^+ at physiological concentrations, though uptake can also occur via an apparently non-saturable pathway (also found in monolayers cultured under conventional static conditions, R. G. Bogle & G. E. Mann, unpublished data), which can contribute significantly when L-arginine concentrations are elevated. Further experiments are needed to determine whether this latter process is carrier mediated.

Inhibition studies of L-arginine transport in porcine aortic endothelial cells have previously established that transport is stereoselective and inhibited significantly by cationic arginine analogues, including the eNOS inhibitors L-NMMA and L-NIO (Bogle *et al.* 1992; Schmidt *et al.* 1993). The lack of inhibition of L-arginine influx by a series of neutral or acidic amino acids (this study) or by N^{ω}-nitro-L-arginine (L-NNA) and its methyl ester L-NAME (Bogle *et al.* 1992) suggests that these amino acids are not transported effectively by system y^+, and indicates that saturable L-arginine transport in porcine aortic endothelial cells is not mediated by less selective transport systems, known to mediate entry of neutral and cationic amino acids (van Winkle, 1988; Devés, Chaves & Boyd, 1992). This conclusion is further supported by the finding that efflux of L-[^{3}H]arginine (or ^{3}H-labelled metabolite) was *trans*-stimulated by L-arginine and the cationic eNOS inhibitor L-NMMA but unaffected by the neutral eNOS inhibitor L-NAME.

Our results also provide the first evidence that system y^+ transport activity is stimulated in endothelial cells deprived of L-arginine. The omission of L-arginine from the culture medium, together with serum dialysis, mimics the experimental conditions adopted in previous studies of NO production by superfused porcine aortic endothelial cells (Palmer *et al.* 1988; Bogle *et al.* 1991). Given the substrate specificity of system y^+ (present paper; Bogle *et al.* 1992; Sobrevia *et al.* 1995), it seems unlikely that the absence of alanine, citrulline, glutamate and taurine would directly influence transport of L-arginine, L-ornithine and L-lysine via system y^+. Moreover, depletion of ornithine (normally only 90 μM in non-dialysed serum) had no effect on intracellular concentrations of either L-ornithine or L-lysine. In contrast, the decrease in intracellular L-arginine levels was paralleled by an increase in transport (10 μM) of L-arginine, L-lysine and L-ornithine, contrary to expectations for a carrier that is *trans*-stimulated, suggesting that depletion of intracellular L-arginine signals an adaptive upregulation of system y^+ activity. The 35–40% increase in L-arginine transport may actually reflect a proportionately larger increase via system y^+, since the non-saturable component of L-arginine entry contributes less at these lower substrate concentrations (see Fig. 2). Adaptive responses in system y^+ activity have already been identified in human umbilical vein endothelial cells exposed to hyperglycaemia (Sobrevia, Yudilevich & Mann, 1994). In these experiments the increase in endothelial system y^+ activity was prevented following treatment of cells with the protein synthesis inhibitor cycloheximide.

Interestingly, the concentrations of several amino acids, but mainly L-glutamine, were elevated significantly after 24 h L-arginine deprivation (Table 2), presumably reflecting increased amino acid transport and/or an enhanced rate of protein degradation (Gazzola, Dall'Asta & Guidotti, 1981). This increase in L-glutamine levels may be of relevance since L-glutamine has been suggested to depress NO release by inhibiting intracellular conversion of L-citrulline into L-arginine (Sessa, Hecker, Mitchell & Vane, 1990). Recent studies have, however, concluded that L-glutamine acts by directly inhibiting L-citrulline entry across the plasma membrane (Wu & Meininger, 1993). Although Wu & Meininger (1993) have proposed that recycling of L-citrulline to L-arginine may maintain sufficient levels of L-arginine during prolonged NO synthesis, we have reported that this cycle does not sustain maximal NO synthesis in a murine macrophage cell line J774 activated with bacterial lipopolysaccharide (Baydoun, Bogle, Pearson & Mann, 1994).

We then investigated whether the transient stimulation of L-arginine transport and synthesis of NO and PGI_2, induced by bradykinin (Bogle *et al.* 1991), was related to altered membrane potential. Agonists such as bradykinin or ATP, or changes in flow, hyperpolarize the endothelial plasma membrane and enhance NO production (Busse, Fichtner, Lückhoff & Kohlhardt, 1988; Nakache & Gaub, 1988; Schilling, 1989; Colden-Stanfield, Schilling, Possani & Kunze, 1990). Basal and bradykinin-stimulated influx of

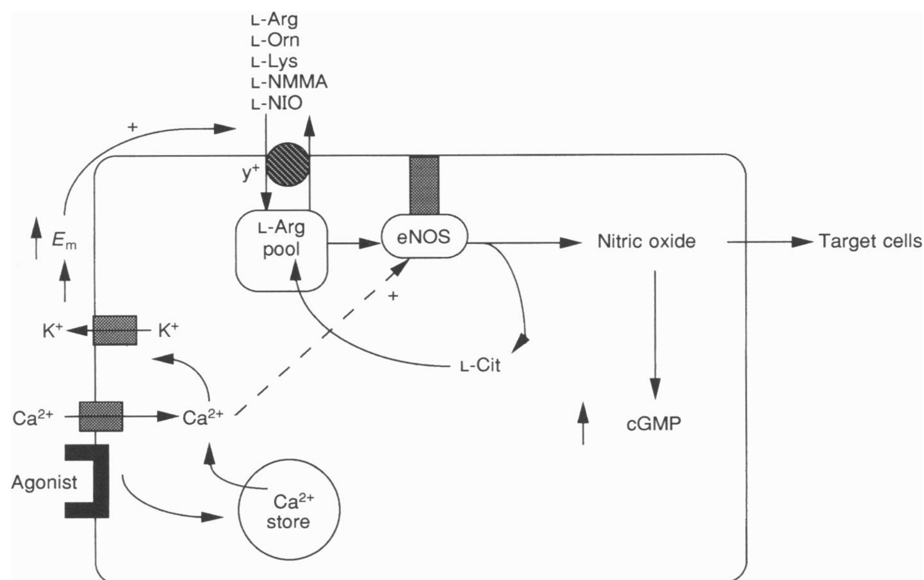

Figure 6. Scheme illustrating the possible relationship between L-arginine transport via system y^+ and NO release in vascular endothelial cells

Stimulation of endothelial cells with agonist elevates intracellular Ca^{2+}resulting in activation of particulate eNOS, conversion of L-arginine (L-Arg) into NO and L-citrulline (L-Cit) and opening of Ca^{2+}-activated K^+ channels. The latter results in hyperpolarization of the cell membrane ($\uparrow E_m$) (Schilling, 1989; Busse *et al.* 1989; Colden-Stanfield *et al.* 1990), which increases the driving force for L-arginine entry into the endothelial cell via system y^+ (Bussolati *et al.* 1993; Kavanaugh, 1993). Entry of L-arginine is inhibited by other cationic arginine analogues, including the eNOS inhibitors L-NMMA and L-NIO.

L-arginine was reduced in the presence of 70 mM K^+. This confirms and extends earlier reports that the steady-state distribution of L-arginine in fibroblasts and endothelial cells is increased by membrane hyperpolarization (Bussolati *et al.* 1989, 1993), and is consistent with the recent finding that the same is observed in oocytes expressing MCAT-1 (Kavanaugh, 1993). Bradykinin-stimulated PGI_2 release was unaffected by 70 mM K^+, demonstrating that bradykinin binding to its receptor was not altered. NO release was still detectable, although levels of cGMP were reduced in reporter LLC-PK_1 cells. In contrast, cells exposed to a Ca^{2+}-free medium for 20 min failed to generate detectable NO in response to bradykinin, whereas L-arginine transport was enhanced to the same extent as in cells superfused with Ca^{2+}-containing medium. The small decrease in agonist-stimulated PGI_2 release, in the absence of Ca^{2+}, may reflect partial depletion of internal Ca^{2+} stores during prolonged perfusion with Ca^{2+}-free solution.

With regard to the possible influence of NO on system y^+, we previously reported that inhibition of NO release by L-NNA marginally attenuated bradykinin-induced stimulation of L-arginine transport (Bogle *et al.* 1991), suggesting that agonist modulation of system y^+ may in part be regulated by NO generation. However, in the current study, sodium nitroprusside (SNP), which spontaneously liberates NO and elevates cGMP (Schini *et al.* 1990), did not affect L-arginine transport, indicating that neither acute generation of NO nor cGMP directly influences system y^+ activity.

Thus, although bradykinin-stimulated NO release from endothelial cells does not appear to be coupled directly to the transient increase in L-arginine transport, we cannot exclude the possibility that the rate of L-arginine utilization by the NO pathway partly determines the rate of L-arginine entry. Both system y^+ and eNOS are located in the plasma membrane. Therefore, it is plausible that preferential delivery of extracellular L-arginine to eNOS may occur. Indeed, if L-arginine is compartmentalized in endothelial cells, then the rate of emptying of the arginine pool may determine the rate of filling from the extracellular space. In this context, it is relevant to note that impaired release of NO from superfused endothelial cells following L-arginine deprivation is rapidly restored on application of extracellular L-arginine (Palmer *et al.* 1988; Buga, Gold, Fukuto & Ignarro, 1991).

Figure 6 illustrates our present view of regulation of L-arginine transport in vascular endothelial cells. Translocation of L-arginine is mediated by the Na^+-independent system y^+, which is sensitive to *cis*-inhibition by cationic L-arginine analogues, including the eNOS inhibitors L-NMMA and L-NIO. Stimulation of endothelial cells with agonists elevates intracellular Ca^{2+} resulting in: (i) activation of particulate eNOS and conversion of

L-arginine into NO and L-citrulline; (ii) activation of soluble guanylate cyclase and elevation of cGMP by NO; (iii) opening of Ca^{2+}-activated K^+ channels resulting in hyperpolarization of the cell membrane and an increased driving force for L-arginine entry into endothelial cells via system y^+; and (iv) a subsequent intracellular alkalinization which sustains NO release in response to bradykinin (Fleming, Hecker & Busse, 1994). Deprivation of L-arginine is associated with an enhanced transport activity of system y^+, and it is possible that a pool of L-arginine to which eNOS has access is depleted. Whether this pool is specific to eNOS or is shared by other metabolic pathways remains to be investigated.

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Acknowledgements

We gratefully acknowledge support from the British Heart Foundation (BHF Grant 91075). R.G.B. was supported by a SERC-CASE PhD collaborative award with Dr Salvador Moncada FRS, Wellcome Research Laboratories, Beckenham, UK. We thank Dr Moncada for his helpful discussion of this work.

Author's present address

R. G. Bogle: Clinical Pharmacology Unit, Department of Pharmacology and Clinical Pharmacology, St. George's Hospital Medical School, London SW17 0RE, UK.

Received 17 March 1995; accepted 12 September 1995.