Amino Acids (2000) 19: 451–468

Amino
Acids

Relationship between NO synthesis, arginine transport, and intracellular arginine levels in vascular smooth muscle cells*

N. Escobales, M. Rivera-Correa, P. I. Altieri, and **J. F. Rodriguez**

Departments of Physiology and Biochemistry, University of Puerto Rico, School of Medicine, San Juan, Puerto Rico

Accepted June 21, 1999

Summary. The present study was designed to evaluate the relevance of arginine transport in nitric oxide (NO) synthesis in vascular smooth muscle cells. For this purpose, NO synthesis and arginine transport (system $B^{0,+}$ and y^+) were evaluated in cells treated with IL-1β or angiotensin II (Ang II). In addition, the effects of 5 mM lysine and glutamine, competitive inhibitors of systems y^+ and $B^{0,+}$ respectively, were examined. L-arginine transport was estimated with ^{3}H-labelled arginine and NO was determined with the Griess reagent. These studies were done in control conditions, arginine-starved cells, and in cells incubated in media containing 10 mM arginine. Our data indicate that induction of NO biosynthesis by IL-1β depends on external arginine when cells are arginine-depleted for 24 hours. The concentration of arginine producing half maximal activation of NO synthesis in arginine-depleted cells ($[\text{arginine}]_i < 10\mu\text{M}$) was $41.1 \pm 18\mu$M. By contrast, in normal culture conditions, NO synthesis occurred independently of arginine transport. Neither 5 mM lysine or glutamine which abolished arginine transport through systems y^+ and $B^{0,+}$, respectively, reduced nitrite release in cells incubated in normal media. This suggests that the relevance of arginine uptake to NO synthesis depends on the status of intracellular arginine pools. Intracellular arginine concentrations were not affected by the stimulation of NO production using IL-1β or its inhibition using Ang II, but were markedly reduced by arginine starvation for 48 h. Aspartate levels were also reduced by arginine-depletion, but were not affected in cells incubated with 10 mM arginine. By contrast, glutamate levels were reduced in arginine-starved cells and were increased in cells incubated in arginine-supplemented medium. Ornithine levels were markedly increased by arginine supplementation. Altogether, these findings indicate that NO synthesis is normally independent of membrane transport. However in arginine-depleted cells, membrane transport is essential for NO

* This work was supported by an NIH-MBRS Grant # S06-RR8224.

synthesis. It is concluded that arginine transport is required for the long-term maintenance of intracellular arginine pools.

Keywords: Amino acids – Nitric oxide – Angiotensin II – IL-1β – Arginine – Aspartate – Ornithine – Membrane transport – Arginine-citrulline cycle – Vascular smooth muscle cells

Introduction

The importance of L-arginine metabolism to cell function has been long recognized. L-arginine, is a precursor for the synthesis of growth-stimulatory polyamines (Durante et al., 1997), and for creatine, an important high-energy phosphate source for muscle contraction (Balsom et al., 1994). L-arginine also serves as an intermediate in the urea cycle (Barbul, 1986) and in the last decade its role as a precursor of nitric oxide (NO) has been well established (Moncada et al., 1991). Since L-arginine is a semi-essential amino acid, it must gain access to the cell via transport proteins in the cell membrane (Umbarger, 1978).

We have previously shown that L-arginine transport in vascular smooth muscle cells (VSMC) takes place through Na^+-dependent and Na^+-independent transport pathways (Rivera-Correa et al., 1996). The Na^+-independent pathway shows properties similar to those of system y^+ described in other cell preparations (reviewed by White, 1985) and reported recently in VSMC (Low et al., 1993; Durante et al., 1995). The system is relatively insensitive to external pH, and is inhibited by cationic amino acids such as ornithine, lysine, and homoarginine. The Na^+-dependent arginine uptake appears to be mediated by system $B^{0,+}$ present in blastocytes and vascular endothelial cells (Van Winkle et al., 1985; Greene et al., 1993; Lind et al., 1993). The transport pathway operates as a broad scope system that transports cationic and neutral amino acids (i.e. phenylalanine, and asparagine) and mediates the transport glutamine and arginine preferentially (Taylor et al., 1996). The system is pH insensitive and does not accept lithium as a substitute for Na^+. Similar characteristics for arginine transport were reported in cultured human fibroblasts (White et al., 1982), human umbilical vein endothelial cells (Mann et al., 1989) and porcine pulmonary artery endothelial cells (Lind et al., 1993). The presence of Na^+-dependent and Na^+-independent pathways for the transport of arginine in VSMC underlines the importance of this amino acid to VSMC function.

The relevance of concentrative, Na^+-dependent arginine transport in VSMC is not clear at present. In this preparation, the Na^+-dependent, $B^{0,+}$ transport system mediates about 40% of arginine transport while the remaining 60% takes place though the Na^+-independent y^+ system. An important role of system $B^{0,+}$ in the synthesis of NO in these cells is contradicted by the finding that treatment of cell with cytokines (i.e IL-1β) co-induce NO synthesis and arginine transport through system y^+ (Durante et al., 1995; Wilemann et al., 1995). It is noteworthy that both systems (y^+ and $B^{0,+}$) express high and low affinity components for arginine transport. In the high affinity component

the K_m for the y^+ system ranges from 20 to 200μM (White et al., 1982; White and Christensen, 1982; Mann et al., 1989; Bogle et al., 1992; Lind et al., 1993; Vasta et al., 1995; Durante et al., 1995), and for system $B^{0,+}$ we have determined it to be 42.8 ± 9μM (Rivera-Correa and Escobales, unpublished observations). However, the maximal rate of transport (high affinity component) of system y^+ is 3-fold higher than that of system $B^{0,+}$. These findings indicate that although the Na^+-dependent arginine transport could be active at physiological arginine concentrations (≈100μM), system y^+ appears to be more important in terms of transport capacity and during periods of high cell activity.

A finding that could be critical in establishing the physiological relevance of system $B^{0,+}$ in VSMC is that it is inhibited by angiotensin II (Rivera-Correa et al., 1996). Angiotensin II (Ang II) is a multifunctional peptidic hormone with a potent vasoconstrictor effect and growth promoting activities in VSMC (Peach, 1977; Daemen et al., 1991). The effects of Ang II as a smooth muscle cells growth factor have been associated with the development of cardiovascular pathologies such as atherosclerosis, hypertension, and restenosis following transluminal angioplasty (Keidar, 1998; Nicholls et al., 1998). For these reasons, the effect of Ang II on system $B^{0,+}$ in VSMC was suggestive of an important event in the mechanism of action of this hormone.

A role for system $B^{0,+}$ in the mechanism of action of Ang II was supported by the observation that the effect of Ang II is specific for system $B^{0,+}$ (Rivera-Correa et al., 1996). Systems A, L, or y^+, were not affected by Ang II. The insensitivity of system y^+ to Ang II is in contrast with reports by Low and Grigor (1995) and Gill et al. (1996) indicating stimulation of system y^+ by Ang II in cultured smooth muscle cells. The action of Ang II on system $B^{0,+}$ is mediated by the receptor subtype-1 (AT1 receptor) because it was blocked by DUP-753, a non-peptidic blocker of this receptor. It thus appears that the action of Ang II occurs through similar pathways as those described for its Ca^{2+}-mobilizing action. Indeed, both of these processes, the inhibition of system $B^{0,+}$ and the release of intracellular calcium, display similar K_d for Ang II (≈5μM), and are sensitive to pertusis toxin, an inhibitor of G_i, G_o, and G_t proteins (Neer, 1986). Protein Kinase C (PKC) plays a critical role in the modulation of Na^+-dependent arginine transport by Ang II because phorbol esters (i.e. phorbolmyristate acetate) mimicked the effect of Ang II, and PKC inhibitors such as staurosporine, prevented the inhibitory actions of Ang II on arginine transport. These actions of Ang II are therefore in line with the well characterized transmembrane signaling events associated with Ang II action in smooth muscle cells (see Griendling et al., 1997).

The effects of Ang II on Na^+-dependent arginine transport in VSMC lead us to consider the possibility that Ang II could inhibit NO synthesis by reducing Na^+-dependent arginine transport. This notion was supported by the fact that Ang II also inhibits the cytokine-induced NO synthesis via a PKC dependent mechanism (Nakayama et al., 1994; Geng et al., 1994). However, supplementation of media with 10 mM arginine did not prevent the inhibitory effect of Ang II on NO synthesis in this preparation, suggesting that the inhibition of NO production was not secondary to low cellular arginine levels.

Indeed, Nakayama et al. (1994) and Geng et al. (1994), have reported that Ang II inhibits the expression of the inducible NOS mRNA. Thus, it appears that Ang II inhibits Na^+-dependent arginine transport and NO synthesis through a PKC-mediated pathway that operates in parallel rather than sequentially. Notwithstanding with these findings, arginine transport appears to be critical for NO synthesis because incubation of smooth muscle cells in media free of L-arginine for 24 hours makes them insensitive to IL-β1 stimulation. Therefore, it appears that the relevance of arginine transport, in particular of system $B^{0,+}$, to NO synthesis is dependent on the status of intracellular arginine pools. However, data is lacking to support this notion.

In this study, we evaluated the role of arginine transport in NO synthesis in IL-β1 stimulated cells. To carry out this goal, we studied the effect of amino acids such as glutamine and lysine that compete with arginine for systems $B^{0,+}$ and y^+, respectively. In addition, we determined the effects of Ang II and IL-β1 on the intracellular levels of arginine, aspartate, glutamate, and ornithine in smooth muscle cells. These experiments were performed with cells incubated in control conditions, in virtually arginine-free medium, and 10 mM arginine supplemented medium. Finally, we determined the dependence of NO synthesis on the external arginine concentration in IL-β1-stimulated cells. The data obtained indicate that under normal conditions, NO synthesis is relatively independent of external arginine concentration and arginine transport by systems y^+ and $B^{0,+}$. We found normal arginine levels in cells treated with IL-1β and Ang II. We postulate that the independence of NO synthesis from external arginine results from the activity of the arginine-citrulline cycle and the high affinity of the synthase for arginine. By contrast, in arginine depleted cells membrane transport through systems y^+ and $B^{0,+}$ appear to play a critical role in sustaining NO synthesis. These data indicate that the dependence of NO synthesis on membrane transport depends on the status of intracellular arginine pools, and that arginine transport is required for the long-term maintenance of intracellular arginine pools.

Materials and methods

Reagents

Culture media, serum, trypsin-EDTA (ethylenediaminetetraacetic acid) and trypan-blue stain was obtained from GIBCO BRL (Grand Island, NY). Interleukin-1β was from Genzyme Diagnostics (Cambridge, MA). Amino acid standards, AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, and all other derivatization reagents were from the AccQ·Tag Chemistry Package (Waters Corp., Milford, MA). Acetonitrile and ethanol were obtained from Fisher Scientific (Pittsburgh, PA) and the protein dye reagent was from BioRad (Hercules, CA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]L-arginine was purchased from New England Nuclear (Boston, MA).

Tissue culture

Rat aortic smooth muscle cells were isolated from thoracic aortas of adult Long-Evans female rats by enzymatic digestion according to the method of Travo et al. (1986). The

cells, which grow attached to the culture surface, were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) calf serum and 1% (v/v) antibiotics (100 units/ml; streptomycin 100 μg/ml and amphotericin-B 25 μg/ml) and kept in a humidified atmosphere of 5% CO_2-95% air at 37°C. VSMC identity was verified by observation of their characteristic multilayered hill and valley morphology under phase-contrast microscopy and by smooth muscle specific α-actin immunoreactivity. When confluent, usually at 7 days, the cells were harvested in a mix of trypsin-EDTA (0.05%, 2 mM) diluted with Hanks balanced salt solution (HBSS; 1:3 v/v) and passaged weekly (1:4 ratio). The cells were then plated in T 75 cm^2 culture flasks and fed every other day. For the experiments, the cells were seeded in 35-mm culture dishes, 12-Multiwell plates or T 75 cm^2 culture flasks at approximately 12–14 $\times$ 10^3 cells/cm^2 and used when confluent. Cultures were used between the 5th and the 13th passages.

Transport assays

Amino acid uptake was performed by measuring the entry of [^{3}H]L-arginine (Arg), [^{3}H]-α-methyl aminoisobutyric acid (mAIB), and [^{14}C]-leucine into VSMC. Uptake measurements were done in Na^+-containing medium (mM): 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 glucose, 10 HEPES (N-2-Hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid]), pH = 7.4 or Na^+-free media (choline substitution). VSMC were incubated for 1 h at 37°C in Na^+ medium and amino acid uptake was then determined by incubating cells in media with 0.1 mM L-arginine with tracer amounts of radioactive arginine (0.5 μCi/μmol). Aspirating the radioactive medium and washing the cells 4 times with ice-cold Na^+-free medium at pH 7.4 terminated the uptake. Cell-associated radioactivity was extracted with 0.1 N KOH and aliquots were counted using a β-counter. To standardize data, the protein content of the cells was measured using the BioRad Protein assay. Bovine serum albumin was used as a standard. Unless otherwise indicated, results were expressed as nmol amino acid /mg protein $\times$ h. To estimate the fraction of amino acid uptake that is Na^+-dependent, the uptake in Na^+-free (choline) medium was subtracted from that observed in sodium containing media. The uptake in choline media was used as an estimate of the Na^+-independent amino acid uptake.

Measurement of nitrite (NO_2^-)

To determine NO levels, the presence of nitrite (NO_2^-) in the culture media was measured using the Griess reaction (Green et al., 1982). Under physiological conditions, NO is oxidized to nitrites and nitrates that can be determined spectrophotometrically. The procedure involved the mixing of equal volumes of cell-conditioned incubation media and Griess reagent (1-% sulfanilamide and 0.1% naphtylethylenediamine in 5% phosphoric acid). After 10-min incubation at room temperature, the absorbance of the sample was read at 550 nm using a Beckman spectrophotometer. NO_2^- concentration of the samples was determined by comparison with values obtained from a standard curve using $NaNO_2$ dissolved in culture medium. Results are expressed as μmol/mg protein $\times$ 24 h.

Intracellular amino acid content determination

For these experiments, cells were grown in T 75 cm^2 culture flasks. After the experimental period, the culture medium was removed by aspiration and the cells were washed three times with PBS to remove traces of culture medium. To extract the amino acid content of the cells, 3.0 ml of 75% ethanol were added to each culture flask and incubated for at least 12 hours at 4°C (White et al., 1982). The ethanol was nitrogen-evaporated and the amino acids resuspended in 0.5 ml eluent containing 140 mM Na-Acetate, 5 mM triethylamine,

and 3 μM EDTA titrated to pH = 5.8 with 50% phosphoric acid. The samples were then filtered though a 0.45 μm nylon filter, and duplicate dilutions (1:12.5, sample:water) of each sample were prepared for HPLC analysis and stored at −20°C until used. α-Aminobutyric acid (20 picomol) was used as internal standard.

The samples were derivatized using the Waters AccQ-Tag method (Cohen and De Antonis, 1994) with AQC as derivatizing agent. AQC reacts rapidly with primary and secondary amino acids to yield highly stable ureas that fluoresce strongly. The method involved mixing of 10 μl of sample with 70 μl of derivatization buffer (0.2 M sodium borate, 5 mM disodium EDTA, pH = 8.8) and 20 μl of the derivatization agent (AQC 3 mg/ml in acetonitrile). The mixture was then heated for 10 min at 55°C, and the amino acid content of the samples was determined using a Hewlett Packard HPLC 1050, coupled to a fluorescence detector (Ex I = 250 nm, Em I = 395 nm). Separations were done using a 3.9 mm × 150 mm AccQ·Tag C_{18} reversed-phase column (Waters Corp).

Amino acid concentration of the samples was determined by comparison with values obtained from a standard curve prepared for each amino acid. Intracellular amino acid concentrations are expressed in μM.

Determination of the intracellular water space

The intracellular water space of VSMC was determined using the protocol described by Kletzien et al. (1975). This procedure allows for the determination of intracellular water space in cultured cells while the cells are attached to the culture dish, thus preventing disruption of cell morphology. In this protocol, the uptake of [^{14}C]3-O-Methyl-D-Glucose (3-MG), a non-metabolizable hexose, is measured in the presence of phloretin, an inhibitor of sugar transport. For these determinations, confluent dishes were washed three times with 150 mM Na^+ medium to remove the culture medium. The cells were then incubated in 150 mM Na^+ medium containing different concentrations of 3-MG (0, 1.0, 2.5, 5.0 and 10 mM; [^{14}C]-3-MG 60 mCi/mmol) for 1 hour at 37°C. The radioactive media was then aspirated and the cells washed 5 times with ice-cold 110 mM $MgCl_2$ and 10 mM HEPES-TRIS buffered pH = 7.4 containing 1 mM phloretin (in 1% ethanol). Cells were digested for 15 minutes with 0.1 N NaOH to extract the cell-associated radioactivity and aliquots were counted using a scintillation β-counter. The protein content of the cells was measured using the BioRad Protein assay. The slope of the 3-MG uptake (nmol/mg protein) versus 3-MG concentration (nmol/μliter) plot represents the intracellular water, expressed as μl cell water/mg protein. The value of intracellular water space obtained for VSMC was 4.2 ± 1 μl cell water/mg protein.

Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM) of the indicated number of experiments, done with triplicate samples. Statistical comparisons between different groups were done using analysis of variance (ANOVA) followed by Scheffé's F test or by unpaired t-test analysis using the SAS Institute StatView 5.0 program (Cary, NC). Values were considered statistically significant at $p \leq 0.05$.

Results

Effect of lysine and glutamine on arginine transport

The effect of 5 mM lysine and glutamine on arginine transport was determined to evaluate their suitability as inhibitors of this process. Figure 1 shows [^{3}H]L-arginine transport through Na^+-dependent (system $B^{0,+}$), and Na^+-independent pathways (system y^+), in the presence and absence of 5 mM arginine, lysine, and glutamine. The transport media contained 100 μM

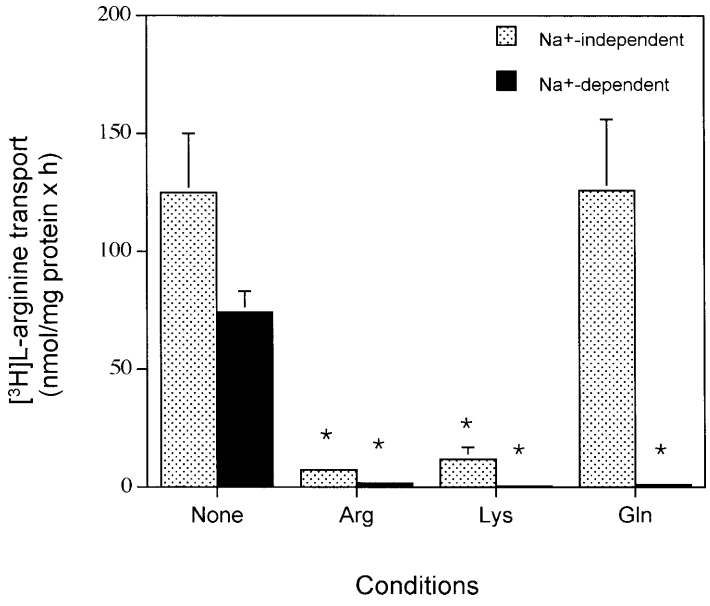

Fig. 1. Effect of lysine and glutamine on arginine transport in VSMC. The bars represent the means ± SEM for three experiments. Transport of [^{3}H]L-arginine (100μM) was measured for 2 minutes in Na^+-free and Na^+-containing media, in the presence or absence of 5 mM arginine, lysine, and glutamine. (*): Statistically different ($p < 0.05$) from control values

labelled arginine. As depicted in the figure, [^{3}H]-arginine transport through systems $B^{0,+}$ and y^+, was specific because it was abolished by an excess of unlabelled arginine in the media. Arginine transport by these pathways was also inhibited by the presence of 5 mM lysine in the incubation media. By contrast, glutamine did not affect system y^+ but abolished transport by system $B^{0,+}$. These findings indicate that these amino acids competitively inhibit arginine transport by systems $B^{0,+}$ and y^+. Glutamine is a selective inhibitor of arginine transport through system $B^{0,+}$, whilst lysine inhibits both systems $B^{0,+}$ and y^+.

*Induction of arginine transport and nitrite synthesis by IL-1*β

To establish the temporal relationship between arginine transport and NO synthesis induced with IL-1β, we determined these processes as a function of time in cells treated with the cytokine. Figure 2a shows that after treatment of cells with IL-1β, arginine transport increased in a time-dependent manner by 56% ($p < 0.01$) at 8 h. At 24 h, however, the stimulation was reduced to 28% ($p < 0.02$). Figure 2b illustrates the production of nitrates in the same batch of cells. As depicted in the figure, nitrate production also increased in a time-dependent manner. Significant increases were observed at 4 (6-fold, $p < 0.001$), 8 (11-fold, $p < 0.001$), and 24 h (20-fold, $p < 0.001$). These data agree with previous reports indicating the co-induction of arginine transport and NO synthesis in VSMC. However, as found by Durante et al. (1995)

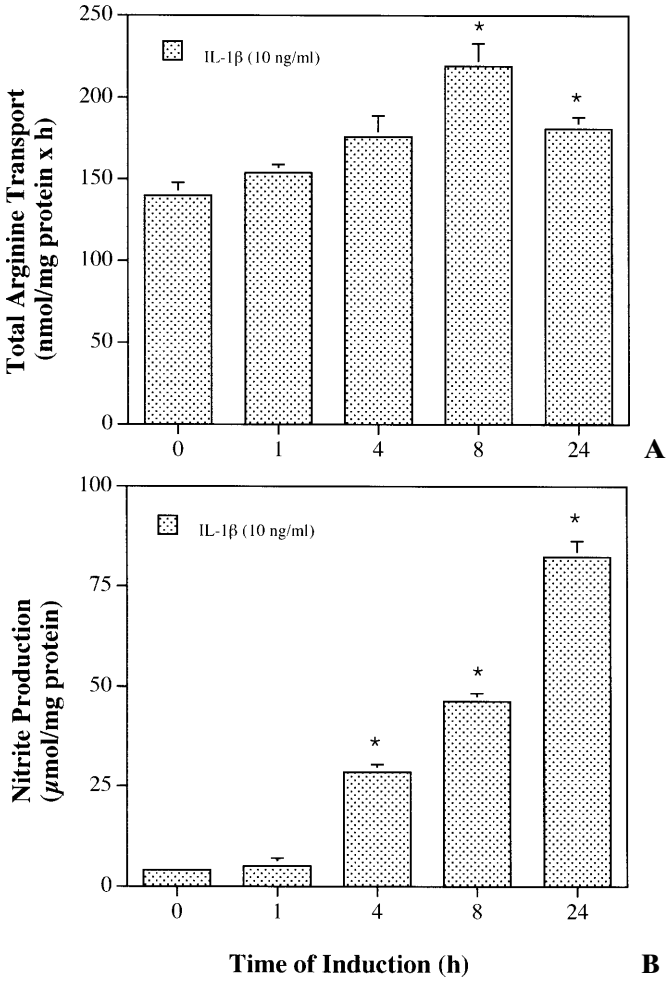

Fig. 2. Co-induction of arginine transport and NO synthesis by IL-1β. The data are representative of three studies performed in triplicates. In this procedure, the cells were grown for 4 days under normal conditions and thereafter, the medium was replaced with DMEM 0.5% calf serum containing IL-1β for variable periods of time up to 24 hours. At the indicated time intervals the conditioned medium was collected and nitrite accumulation determined as described in Materials and methods. Total arginine transport (in 145 mM NaCl media) in the cytokine-treated cell preparation was concomitantly determined. (*) Statistically significant difference when compared to zero time, $p < 0.05$

the activation of transport appears to be small when compared to nitrite production.

*Relationship between intracellular arginine levels and nitrite production in VSMC treated with Ang II and IL-1*β

We have previously shown that Ang II inhibits concomitantly arginine transport and NO synthesis in VSMC by a protein kinase C-dependent mechanism

Table 1. Effect of IL-1β and Ang II on intracellular arginine levels in vascular smooth muscle cells

Conditions	Intracellular arginine	Nitrite production
	μM	
None	88 ± 17	2 ± 1
IL-1β	107 ± 27	170 ± 24*
Ang II	92 ± 28	1 ± 1
IL-1β + Ang II	102 ± 25	80 ± 16*$^{+}$
Arg-free	9 ± 9*	3 ± 2
Arg-free + IL-1β	10 ± 8*	8 ± 5
Arg	5,694 ± 365*	2 ± 1
Arg + IL-1β	5,536 ± 432*	119 ± 24*
Arg + Ang II	5,819 ± 448*	3 ± 3
Arg + IL-1β + Ang II	5,093 ± 344*	70 ± 15*$^{+}$

Relationship between intracellular arginine concentration and nitrite production in VSMC. The cells were grown for 4 days, afterwards the media was replaced with DMEM (0.5% Calf Serum) for 48 hours. The media of the arginine-free experimental group was replaced on day 5 with Arg-free DMEM (0.5% calf serum). On day 6, the media was removed and substituted with DMEM 0.5% calf serum (with or without arginine) in the absence or presence of IL-1β (10 ng/ml), Ang II (100 nM) or Arginine (10 mM). After a 24 hours incubation, aliquots were collected and nitrite concentration quantitated. The remaining medium was aspirated, and the cells washed with cold PBS (4°C). The amino acid content of the cells was extracted in 75% ethanol. The ethanol was evaporated and the amino acids resuspended in buffer and processed for HPLC determination as described in Materials and methods. Bars represent mean ± SEM for 7 experiments. (*) Statistically different from control (None) values, $p < 0.05$. ($^{+}$) Statistically different from its respective control (IL-1β or Arg + IL-1β) value, $p < 0.05$.

(Rivera-Correa et al., 1996). We provided data suggesting that the reduction of arginine transport by Ang II is not the cause of NO synthesis inhibition because supplementation of media with 10 mM arginine did not prevent the inhibition of NO synthesis induced by this hormone. To be certain that the effect of Ang II was unrelated to alterations in intracellular arginine levels, nitrite accumulation and intracellular arginine content were measured in the same cell population in control conditions and in cells treated with 100 nM Ang II or IL-1β (10 ng/ml) for 24 hours (Table 1). For these experiments, the cells were divided in three groups: cells incubated in normal media ($[\text{arginine}]_0 = 400\mu\text{M}$), cells incubated in arginine-free media, and cell incubated in arginine-supplemented media (10 mM). Arginine-deprived cells were obtained after a 24 hour incubation period in arginine-free media. The cells were then treated in a similar media with IL-1β for an additional 24 hours.

The effect of arginine-starvation and supplementation, and the effect of IL-1β and Ang II on the intracellular arginine concentration are shown in Table 1. Treatment of cells with IL-1β or Ang II, had no effect on the intracellular arginine concentration. These results indicate that in normal smooth muscle cells, alterations of NO synthesis and of arginine transport do not cause significant changes in the intracellular arginine concentration. Incubation in arginine-free media for 48 hours reduced the intracellular arginine levels 9-fold ($p < 0.04$, $n = 12$). By contrast, addition of 10 mM arginine to the extracellular medium markedly increased the intracellular arginine level by ~100-fold ($p < 0.05$).

Table 1 also illustrates the effect of arginine-starvation and supplementation, and the effect of IL-1β and Ang II on nitrite production using the Griess reagent (Green et al., 1982). IL-1β stimulated nitrite production by 91-fold ($p < 0.05$). Ang II and arginine removal inhibited the IL-1β-stimulated nitrite accumulation by 53% and 93%, respectively ($p < 0.05$). Arginine supplementation, however, caused 30% inhibition ($p < 0.05$) of the IL-1β-stimulated nitrite accumulation but did not affect the basal nitrite production by the cells. Notice that even in the presence of 10 mM arginine, Ang II caused a 60% inhibition of nitrite accumulation ($p < 0.05$). These experiments demonstrate that extracellular arginine is required for nitric oxide synthesis and that the reduction in this parameter caused by Ang II is not a consequence of reduced arginine availability.

Role of extracellular arginine concentration on nitrite production

Since removal of arginine from the culture medium abolished nitrite accumulation, the dependency of nitrite formation on extracellular arginine was evaluated. For these experiments, the IL-1β-stimulated nitrite production was measured in the presence of increasing L-arginine concentrations (0–1 mM). As shown in Fig. 3, nitrite accumulation is a saturable function of the external arginine concentration. Saturation was observed at arginine values above 250μM. The calculated K_m for nitrite production under these conditions was $41.1 \pm 18\mu$M, and V_{max} was $65.7 \pm 5\mu$mol/mg protein $\times$ 24 h. These values are similar to those previously reported by Granger et al. (1990) and Bogle et al. (1992).

Effect of inhibition of L-arginine transport on NO synthesis

We have previously shown that 5 mM glutamine selectively abolishes arginine uptake through the Na^+-dependent $B^{0,+}$ pathway whereas 5 mM lysine inhibits both the Na^+-dependent and the Na^+ independent y^+ pathway (Fig. 1). To determine if NO synthesis is dependent on L-arginine uptake through systems $B^{0,+}$ or system y^+, the IL-1β-stimulated nitrite production was evaluated in the presence of 5 mM glutamine and lysine. VSMC were also treated with Ang II to compare the effect of this hormone with the actions of glutamine and lysine. These experiments were conducted in normal cells ($[arginine]_0 = 400\mu$M) and in arginine depleted cells, to elucidate any effect that alterations

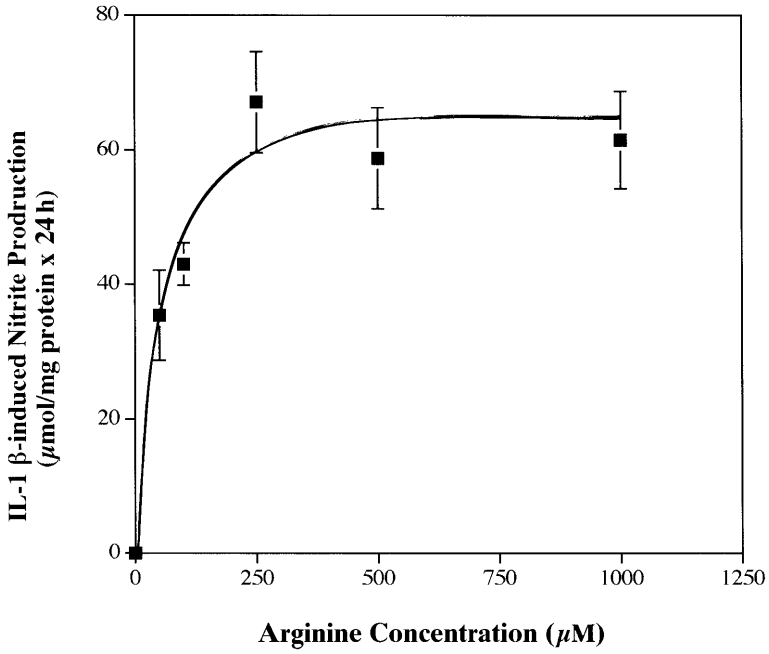

Fig. 3. Concentration dependence curve for the IL-1β-induced nitrite production as a function of the extracellular arginine concentration. The method used was similar to that described in the legend of Fig. 2, with the exception that following the initial 4 days of cell growth, the cells were incubated in arginine-free DMEM (0.5% calf serum) for 24 hours. The cells were then incubated at the indicated arginine concentrations in the absence (none) and presence of IL-1β (10 ng/ml) for 24 hours. The basal nitrite determined (none) was subtracted from that measured in the IL-1β-treated cells. The data was analyzed using a Woolf-Augustinsson-Hofstee plot (Segel, 1976). $K_{0.5} = 41.1 \pm 18\mu M$ and Vmax = 65.7 $\pm$ 5.0μmol/mg protein $\times$ 24 h. $Y = -41.05X + 65.71$. $R^2 = 0.952$, $p < 0.05$. Points represent mean $\pm$ SEM for 3 experiments

in the arginine pools may have cationic amino acid transport (White et al., 1982).

As illustrated in Fig. 4, basal nitrite production was not affected under any condition evaluated. In normal cells ($[arginine]_0 = 400\mu M$), 5 mM lysine reduced the IL-1β-induced nitrite accumulation by 28% but the effect did not reach statistical significance ($p > 0.05$). Glutamine did not affect nitrite accumulation. In arginine-starved cells, however, lysine inhibited the IL-1β-stimulated nitrite production by 65% ($p < 0.05$), while glutamine increased this parameter by 30% ($p < 0.05$). Ang II inhibited the IL-1β-induced nitrite production about 70% ($p < 0.05$) in both arginine containing medium and arginine-free conditions. Neither lysine nor glutamine affected the inhibition caused by this hormone. In the presence of 5 mM lysine, Ang II abolished nitrite production by IL-1β-treated cells. Altogether, these data indicate that basal nitrite accumulation is independent of arginine transport. It also appears that arginine uptake through systems y^+ and $B^{0,+}$ are important elements in NO synthesis under conditions of arginine starvation, and that the inhibition

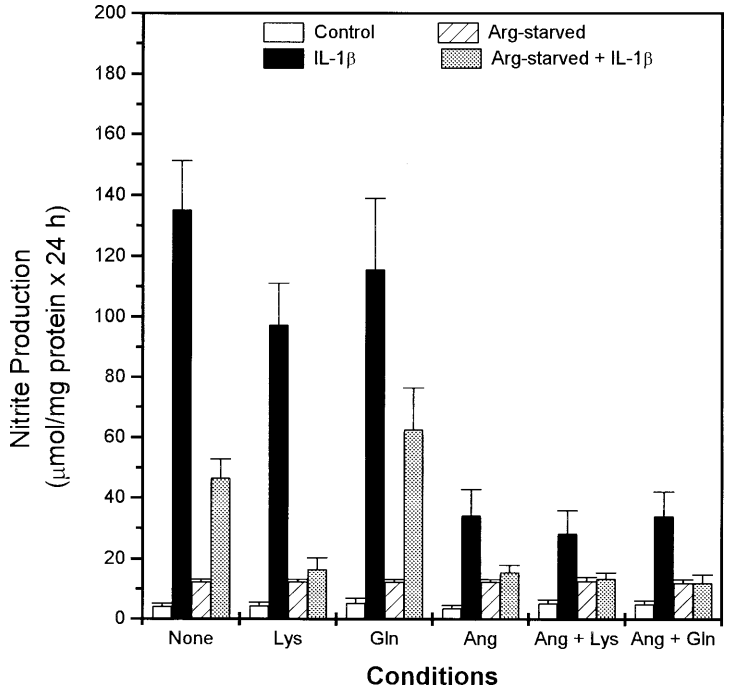

Fig. 4. Effect of lysine, glutamine and Ang II on the IL-1β-induced nitrite production. Cells were grown for 4 days under normal conditions, afterwards, the cell media was replaced with DMEM (0.5% calf serum) for 48 hours. On day 5, the media of the arginine-free group was replaced with arginine-free DMEM (0.5% calf serum) for 24 hours. On day 6, 5 mM lysine or glutamine, 100 nM Ang II (100 nM) and IL-1β (10 ng/ml) were added to the culture medium in the absence and presence of arginine for another 24 hours. Aliquots of the conditioned medium were then collected and assayed for nitrite accumulation as described in Materials and methods. Bars represent mean ± SEM for 4 experiments. Basal nitrite production was unaffected by Ang II, lysine or glutamine in control cells. However, nitrite accumulation in arginine-starved cells was significantly higher ($p \leq 0.05$) than controls in all experimental conditions. In all conditions evaluated, 1L-1β significantly ($p < 0.05$) stimulated nitrite production, an effect of that was inhibited by arginine depletion ($p < 0.05$). Ang II reduced $p < 0.05$ the IL-1β nitrite production in control cells as well as in arginine-free cells. Statistical differences ($p < 0.05$) were found between IL-1β vs IL-1β + Gln and IL-1β vs IL-1β + Lys in arginine-free medium. Comparison between IL-1β vs IL-1β + Lys and IL-1β vs IL-1β + Gln in normal medium (400μM external arginine) did not reach statistical significance

of NO synthesis by Ang II is not due to reductions in intracellular arginine levels secondary to inhibition of arginine transport.

*Effect of Ang II, IL-1*β*, arginine depletion, and arginine supplementation on the intracellular levels of aspartate, glutamate, and ornithine*

To determine a role for the arginine-citrulline cycle (ACC) in the generation of NO in VSMC we determined the levels of aspartate, glutamate and ornithine in normal cells, arginine-starved cells, and arginine supplemented cells

Table 2. Effect of IL-1β and Ang II on the intracellular levels of aspartate, glutamate, and ornithine in vascular smooth muscle cells

Conditions	Aspartate	Glutamate	Ornithine
		μM	
None	2,890 ± 709	6,704 ± 717	74 ± 39
IL-1β	2,022 ± 343	4,996 ± 489	59 ± 30
Ang II	2,115 ± 555	6,022 ± 256	26 ± 18
IL-1β + Ang II	2,122 ± 502	5,083 ± 126	81 ± 11
Arg-free	959 ± 78*	3,879 ± 667*	101 ± 64
Arg-free + IL-1β	1,263 ± 138*	3,601 ± 370*	98 ± 74
Arg	2,573 ± 450	7,931 ± 769	1,516 ± 206*
Arg + IL-1β	2,608 ± 502	7,511 ± 749*	1,590 ± 189*
Arg + Ang II	2,893 ± 491	8,019 ± 434*	1,699 + 137*
Arg + IL-1β + Ang II	2,979 ± 553	7,541 ± 405*	1,491 ± 132*

Effect of arginine depletion and arginine supplementation on the intracellular levels of aspartate, glutamate and ornithine in VSMC. The conditions used were similar to those indicated in the legends of Table 1. Bars represent mean ± SEM for 7 experiments. (*) Statistically different from control (None) values, $p < 0.05$.

(10 mM $[arginine]_0$) in the presence and absence of Ang II (100 nM) and IL-1β (10 ng/ml). The arginine-citrulline cycle, an aborted pathway of the urea cycle, combines aspartate with the citrulline produced by iNOS to produce new arginine (Hecker et al., 1990; Hattory et al., 1994). The activity of this cycle in VSMC could make NO synthesis to proceed independently of arginine transport. Glutamate was chosen because it is related to arginine metabolism through the citric acid cycle (Stryer, 1988) and ornithine is produced from arginine in VMSC by arginase activity (Durante et al., 1997).

The results of this experiment are shown in Table 2. Aspartate and glutamate levels were reduced by arginine starvation (66% and 42%, respectively, $p < 0.05$). However, incubation of cells in media containing high levels of arginine (10 mM) increased glutamate levels by an average of 37 ± 7% (None vs Arg: 18%, $p = 0.11$; Ang II vs Arg + Ang II: 50%, $p = 0.031$; IL-1β vs Arg + IL-1β: 33%, $p = 0.0018$; Ang II + IL-1β vs Arg + Ang II + IL-1β: 48%, $p = 0.008$), but did not affect the intracellular aspartate concentration. These experiments are consistent with the activation of ACC because aspartate levels were only affected (decreased) by arginine starvation. Ornithine levels were markedly increased (>18-fold, $p < 0.05$) by incubation of cells in arginine supplemented medium, indicating the presence of arginase activity in these cells.

Discussion

The relevance of arginine transport for the synthesis of NO synthesis in VSMC is still a matter of controversy. Durante and colleagues (1995) have reported coinduction of these processes by IL-1β and tumor necrosis factor-α

in bovine aortic smooth muscle cells. They suggested that the activation of NO synthesis and arginine transport could represent a mechanism to provide increased levels of substrate to the cells during activation of NO synthesis. In the same study, however, INF-γ and db-cAMP stimulated NO synthesis without affecting arginine transport. Wilemann et al. (1995) also reported coinduction of NO synthesis and arginine transport by lipopolysaccharide in cultured smooth muscle cells from rat aorta. The results of the present study on the activation of these processes by IL-1β in rat aortic smooth muscle cells are in agreement with these reports. However, as reported by Durante et al. (1995) and Wilemann et al. (1995), the induction of arginine transport observed in the present study was small (56%) compared to the marked stimulation of nitrite synthesis (20-fold). These findings are consistent with the notion that although a correlation of arginine transport and NO synthesis exists in cytokine-treated VSMC, the uptake of arginine may not be critical for NO synthesis. This notion is at variance with studies in preparations of macrophages (Bogle et al., 1992; Shibazaki et al., 1996) and in the isolated perfused liver (Pastor et al., 1995) indicating the requirement of arginine transport for the inducible NO production.

The results of the present study indicate that under normal conditions, NO synthesis in VSMC is independent of arginine transport. Thus when arginine transport ($[\text{arginine}]_o = 400\mu\text{M}$) was completely blocked with 5 mM lysine or glutamine, nitrite production by IL-1β-treated cells was not significantly affected. Furthermore, the intracellular arginine levels did not vary when NO synthesis was stimulated using IL-1β or inhibited by Ang II. Only in arginine depleted cells, lysine inhibited the nitrite production by cytokine-treated cells. This finding indicates that arginine transport through systems y^+ and $B^{0,+}$ is essential for NO synthesis in arginine depleted cells. Therefore, the relevance of arginine transport to NO synthesis appears to be a function of the status of intracellular arginine pools; being dependent on transport only when arginine pools are depleted.

Durante et al., (1995) found that 10 mM lysine abolished the IL-1β-induced nitrite production by VSMC, a finding which is consistent with our results in arginine-starved cells but which is at variance with the findings in normal arginine-containing cells. However, in that study the extracellular arginine concentration was set at 100μM for 24 hours in serum-free media for the IL-1β-treatment, while in the present study the external arginine concentration was 400μM (normal media) or zero in 0.5% Calf serum-media. Under these conditions normal and arginine-deprived cells had intracellular arginine levels of $88 \pm 17\mu$M and $9 \pm 9\mu$M, respectively (Table 1). Therefore, it is possible that the cells used by Durante and coworkers (1995) had low arginine levels close to those found by us in arginine-starved cells where 5 mM lysine did inhibit nitrite production. It is also possible that lysine at concentrations above those required for complete inhibition of arginine transport (5 mM, Fig. 1), exerts effects on the inducible NO synthesis that are independent of its action on the transport system. Indeed, Bogle et al. (1992) reported that at concentrations above 5 mM lysine have direct inhibitory effects on the isolated NO synthase activity. These obser-

vations underline the importance of assessing the intracellular arginine pools when evaluating the role of arginine transport in NO production and complicate the interpretation of lysine studies at concentrations above 5 mM.

The results of this study also indicate that glutamine, an amino acid that has been shown to selectively abolished arginine transport though system $B^{0,+}$, did not affect nitrite release in normal or in arginine-starved cells. This finding indicates that even cell when transport through system $B^{0,+}$ is blocked by glutamine, sufficient arginine for NO synthesis can be provided by system y^+. In this context, the relevance of Na^+-dependent arginine transport for NO synthesis is still questionable. However, since lysine competitively inhibits arginine uptake through both transport systems, a role for system $B^{0,+}$ in NO synthesis cannot be ruled out.

In arginine-depleted cells, the cytokine-induced nitrite synthesis was a saturable function of the concentration of arginine in the medium. The concentration of arginine yielding half-maximal activity was $41.1 \pm 18\,\mu$M. These results agree with similar experiments published by others investigators (Granger et al., 1990, Bogle et al., 1992) indicating app. K_m in the range 30–75 μM. These values represent an average of the affinity constants of the arginine transport processes and that of the intracellular iNOS. In studies involving purified iNOS, it has been estimated that the K_m ranges from 2 to 35 μM (Föstermann et al., 1994). Laubach et al. (1996) have recently reported a K_m of 1.8 μM for the purified, human iNOS. These values are clearly lower than those estimated in this study with intact cells while varying arginine concentrations in the external medium. Since physiological plasma arginine concentrations are in the range from 100 to 200 μM (Bogle et al., 1992), the arginine concentrations available to iNOS should be enough to sustain near maximal NO production. This would make NO synthesis practically independent of arginine transport from the extracellular milieu. The data obtained in this study support this notion because normal arginine levels in VSMC are about 90 μM (Table 1) at $[\text{arginine}]_o = 400\,\mu$M. These results suggest that arginine transport could play a role in the long-term maintenance of arginine pools but not for the provision of substrate for immediate NO synthesis. It is plausible to postulate that the synthesis of NO normally proceeds with arginine derived from a pool that does not mix rapidly with arginine entering the cell across the plasma membrane.

Our results on intracellular arginine levels in cells incubated at normal arginine concentrations (400 μM), suggest that arginine pools are maintained relatively constant even when the production of NO is markedly stimulated by IL-1β or is inhibited by Ang II. If one assumes a constant rate of arginine transport of 225 nmol/mg protein $\times$ hour (maximal rate at 8 hours), about 54 μmol of arginine/mg protein should have entered the cells in 24 hours. This value which represents a constant maximal rate of transport, is lower than the IL-1β-induced production of nitrite (or NO) in the same period of time (82 μmol/mg protein), suggesting that in addition to arginine transport, another source is providing arginine for NO synthesis. Although it is possible that arginine could have been derived from protein breakdown, the marked reduc-

tion in aspartate levels in arginine-depleted cells suggest that the arginine-citrulline cycle (ACC) could be an important contributor for the regeneration of arginine from citrulline (and aspartate) in stimulated conditions. Indeed, a key enzyme of this cycle, arginossuccinate synthase (AS), is induced by cytokines in VSMC and basal activity ($\approx$10% maximal activity) can be detected in control cells by the formation of arginosuccinate from aspartate (Hattory and coworkers, 1994). However in arginine-starved cells, NO production was negligible in the presence of aspartate (Fig. 4 and Table 2) suggesting that citrulline levels must have been limiting for AS activity. Indeed, Hattory and coworkers (1994) reported that citrulline can restore the capacity of arginine-depleted smooth muscle cells to synthesize NO. For this reason, the reduction in aspartate levels observed in arginine-depleted cells must represent, at least in part, the consumption of the amino acid for arginine synthesis while citrulline was available at concentrations above those required for AS activity ($K_m >$ 200μM, Meijer et al., 1990). Glutamate levels were also reduced by arginine starvation, but in contrast to the alterations observed with aspartate, it increased by 37 $\pm$ 7%, $p < 0.05$ in arginine-supplemented cells. Thus, glutamate levels followed cellular arginine levels, whereas aspartate levels were only affected by arginine-depletion. Additional experiments are necessary to establish the role of ACC for arginine and NO production by VSMC. The latter, however, is limited by the absence of suitable inhibitors of this cycle.

In summary, in VSMC NO synthesis is relatively independent of the transport of arginine from the external media. We found normal arginine levels in cells treated with IL-1β and Ang II. This independency appears to be the result of the high affinity of iNOS for arginine and possibly, the activity of the arginine-citrulline cycle. By contrast, in arginine depleted cells membrane transport, through system y^+ and possibly $B^{0,+}$ appear to play a critical role in sustaining NO synthesis. The data indicate that the dependence of NO synthesis on membrane transport depends on the status of intracellular arginine pools. It appears that arginine transport is required for the long-term maintenance of intracellular arginine pools but not for the immediate NO synthesis.

References

Balsom PD, Soderlund K, Ekblom B (1994) Creatine in humans with special reference to creatine supplementation. Sports Med 18: 268–280

Barbul A (1986) Arginine: biochemistry, physiology, and therapeutic implications. J Parenter Enteral Nutr 10: 227–238

Bogle RG, Baydoun A, Pearson JD, Moncada S, Mann GE (1992) L-Arginine transport is increased in macrophages generating nitric oxide. Biochem J 284: 15–18

Cohen SA, De Antonis K (1994) Applications of amino acid derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate: analysis of feed grains, intravenous solutions and glycoproteins. J Chomatography 661: 25–34

Daemen MJAP, Lombardi D, Bosman FT, Schwartz S (1991) Angiotensin II induces smooth muscle cell proliferation in the normal and in the injured rat arterial wall. Circ Res 68: 450–456

Durante W, Liao L, Schafer A (1995) Differential regulation of L-arginine transport and inducible NOS in cultured vascular smooth muscle cells. Am J Physiol 268: H1158–H1164

Durante W, Liao L, Peyton KJ, Schafer A (1997) Lysophosphatidylcholine regulates cationic amino acid transport and metabolism in vascular smooth muscle cells. Role in polyamine biosynthesis. J Biol Chem 272: 30154–30159

Förstermann U, Closs EI, Pollock JS, Nakame M, Schwarz P, Gath I, Kleinert H (1994) Nitric oxide synthase isozymes: characterization, purification, molecular cloning and functions. Hypertension 23: 1121–1131

Geng Y, Wu Q, Hansson GK (1994) Protein kinase C activation inhibits cytokine-induced nitric oxide synthesis in vascular smooth muscle cells. Biochim Biophys Acta 1223: 125–132

Gill D, Low B, Grigor MR (1996) Interleukin-1β and tumor necrosis factor-α stimulate the CAT-2 gene of the L-arginine transporter in cultured vascular smooth muscle cells. J Biol Chem 271: 11280–11283

Granger DL, Hibbs J, Perfect JR, Durack DT (1990) Metabolic fate of L-arginine in relation to microbiostatic capability in murine macrophages. J Clin Invest 85: 264–273

Green LC, Wagner DA, Glogowsski J, Skipper PI, Wishnok JS, Tannenbaum S (1982) Analysis of nitrate, nitrite and [^{15}N] nitrate in biological fluids. Anal Biochem 126: 131–138

Greene B, Pacitti A, Souba WW (1993) Characterization of L-arginine transport by pulmonary artery endothelial cells. Am J Physiol 264: L351–L356

Griendling K, Ushio-Fukai M, Lasseue J, Alexander RW (1997) Angiotensin II signaling in vascular smooth muscle: new concepts. Hypertension 29: 366–373

Hattori Y, Campbell E, Gross S (1994) Argininosuccinate synthase mRNA and activity are induced by immunostimulants in vascular smooth muscle. J Biol Chem 269: 9405–9408

Hecker M, Sessa W, Harris WC, Anggard E, Vane JR (1990) The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: Cultured endothelial cells recycle L-citrulline to L-arginine. Proc Natl Acad Sci USA 87: 8612–8616

Keidar S (1998) Angiotensin, LDL peroxidation and atherosclerosis. Life Sci 63/1: 1–11

Kletzien RF, Pariza M, Becker J, Potter VR (1975) A method using 3-O-methyl-D-glucose and phloretin for the determination of intracellular water space of cells in monolayer culture. Anal Biochem 68: 537–544

Laubach VE, Garvey EP, Sherman PA (1996) High-level expression of human inducible nitric oxide synthase in Chinese Hamster ovary cells and characterization of the purified enzyme. Biochem Biophys Res Commun 218: 802–807

Lind DS, Copeland III E, Souba WW (1993) Endotoxin stimulates arginine transport in pulmonary artery endothelial cells. Surgery 114: 199–205

Low BC, Grigor M (1995) Angiotensin II stimulates system y^+ and cationic amino acid transporter gene expression in cultured vascular smooth muscle cells. J Biol Chem 270: 27577–27583

Low BC, Ross I, Grigor M (1993) Characterization of system L and y^+ amino acid transport activity in cultured vascular cells. J Cell Physiol 156: 626–634

Mann GE, Pearson JD, Sheriff CJ, Toothill VJ (1989) Expression of amino acid transport in human umbilical vein endothelial cells. J Physiol (London) 410: 325–339

Meijer AJ, Lamers WH, Chamuleau FM (1990) Nitrogen metabolism and ornithine cycle function. Physiol Rev 70: 701–730

Moncada S, Higgs E, Palmer R (1991) Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol Rev 43: 109–142

Nakayama I, Kawahara T, Tsuda M, Okuda M, Yokoyama M (1994) Angiotensin II inhibits cytokine-stimulated inducible nitric synthase expression in vascular smooth muscle cells. J Biol Chem 269: 11628–11633

Neer EJ (1986) Guanine nucleotide-binding proteins involved in transmembrane signaling. Symp Fundam Cancer Res 39: 123–136

Nicholls MG, Richards AM, Agarwal M (1998) The importance of the renin-angiotensin system in cardiovascular disease. J Hum Hypertens 12/5: 295–299

Pastor CM, Morris SM Jr, Billiar TR (1995) Sources of arginine for induced nitric oxide synthesis in the isolated perfused liver. Am J Physiol 269/32: G861–G866

Peach MJ (1977) Renin-angiotensin system: biochemistry and mechanisms of action. Physiol Rev 57: 313–370

Rivera-Correa MM, Altieri PI, Escobales N (1996) Parallel regulation of arginine transport and nitric oxide synthesis by angiotensin II in vascular smooth muscle cells. Role of protein kinase C. Amino Acids 11: 153–170

Segel IH (1976) Biochemical calculations, 2nd edn. John Wiley & Sons, New York, pp 208–323

Shibazaki TM, Fujiwara M, Sato H, Fujiwara K, Abe K, Bannai S (1996) Relevance of arginine transport activity to the nitric oxide synthesis in mouse peritoneal macrophages stimulated with bacterial lipopolisaccharide. Biochim Biophys Acta 1311/3: 150–154

Stryer L (1988) Biochemistry, 3rd edn. W.H. Freeman & Company, New York, pp 500–505

Taylor P, Kaur S, Mackenzie B, Peter B (1996) Amino acid-dependent modulation of amino acid transport in *Xenopus laevis* oocytes. J Exp Biol 199: 923–931

Travo P, Barret G, Burnstock G (1980) Differences in proliferation of vascular smooth muscle cells taken from male and female rats. Blood Vessels 17: 110–116

Umbarger HE (1978) Amino acid biosynthesis and its regulation. Annu Rev Biochem 47: 533–606

Van Winkle LJ, Christensen H, Campione A (1985) Na^+-dependent transport of basic, zwitterionic, and bicyclic amino acids by a broad-scope system in mouse blastocysts. J Biol Chem 260: 12118–12123

Vasta V, Meacci E, Farnararo M, Bruni P (1995) Identification of a specific transport system for L-arginine in human platelets. Biochem Biophys Res Commun 206/3: 878–884

White MF (1985) The transport of cationic amino acids across the plasma membrane of mammalian cells. Biochim Biophys Acta 822: 355–374

White MF, Christensen HN (1982) Cationic amino acid transport into cultured animal cells. 2. Transport system barely perceptible in ordinary hepatocytes, but active in hepatoma cell lines. J Biol Chem 257: 4450–4457

White MF, Gazzola G, Christensen HN (1982) Cationic amino acid transport into cultured animal cells. 1. Influx into cultured human fibroblasts. J Biol Chem 257: 4443–4449

Wileman SM, Mann GE, Baydoun AR (1995) Induction of L-arginine transport and nitric oxide synthase in vascular smooth muscle cells: synergistic actions of proinflammatory cytokines and bacterial lipopolysaccharide. Br J Pharmacol 116/8: 3243–3250

Authors' address: Dr. Nelson Escobales, University of Puerto Rico, School of Medicine, Department of Physiology, GPO Box 36-5067, San Juan, Puerto Rico 00936-5067, E-mail: nescobal@prtc.net

Received February 7, 1999