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Parallel regulation of arginine transport and nitric oxide synthesis by angiotensin II in vascular smooth muscle cells Role of protein kinase C

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Summary. Experiments were performed to characterize arginine transport in vascular smooth muscle cells (SMCs) and the effect of angiotensin II (Ang II) on this process. In addition, the role of arginine transport in the cytokine-induced nitric oxide (NO) production was assessed. Arginine transport takes place through Na^+-independent (≈60%) and Na^+-dependent pathways (≈40%). The Na^+-independent arginine uptake appears to be mediated by system y^+ because of its sensitivity to cationic amino acids such as lysine, ornithine and homoarginine. The transport system was relatively insensitive to acidification of the extracellular medium. By contrast, the Na^+-dependent pathway is consistent with system $B^{0,+}$ since it was inhibited by both cationic and neutral amino acids (i.e., glutamine, phenylalanine, and asparagine), and did not accept Li^+ as a Na^+ replacement. Treatment of SMCs with 100 nM Ang II significantly inhibited the Na^+-dependent arginine transport without affecting systems y^+, A, and L. This effect occurred in a dose-dependent manner (IC_{50} of 8.9 ± 0.9 nM) and is mediated by the AT-1 receptor subtype because it was blocked by DUP 753, a non-peptide antagonist of this receptor. The inhibition of system $B^{0,+}$ by Ang II is mediated by protein kinase C (PKC) because it was mimicked by phorbol esters (phorbol 12-myristate 13-acetate) and was inhibited by staurosporine. Ang II also inhibited the IL-1β induced nitrite accumulation by SMCs. This action was also inhibited by staurosporine and reproduced with phorbol esters, suggesting a coupling between arginine uptake and NO synthesis through a PKC-dependent mechanism. However, arginine supplementation in the medium (10 mM) failed to prevent the inhibitory action of Ang II on NO synthesis. These findings suggest that although Ang II inhibits concomitantly arginine transport and NO synthesis in SMCs, the reduction of NO synthesis is not associated with alterations in the cellular transport of arginine.

Keywords: Amino acids – Arginine transport – Nitric oxide – Smooth muscle cells – Angiotensin II – Protein kinase C

Abbreviations: Arg, arginine; Orn, ornithine; HmR, homoarginine; Lys, lysine; Gln, glutamine; Asn, asparagine; His, histidine; Phe, phenylalanine; Leu, leucine; Cys, Cysteine; Ala, alanine; Ser, serine; Thr, threonine; Glu, glutamate; mAIB, α-methyl-aminoisobutyric acid; BCH, bicyclo-aminoheptane

Introduction

Angiotensin II (Ang II) is a peptide with a wide variety of physiological actions that include the control of sodium and potassium homeostasis, positive inotropic effect in the heart, modulation of pituitary hormone release, and stimulation of thirst and glyconeogenesis. In addition, Ang II is a potent vasoconstrictor hormone and exerts growth promoting activities in vascular smooth muscle cells (SMCs) (Peach, 1977; Daemen et al., 1991).

The effects of Ang II as a SMC growth factor have been associated with pathological processes such as atherosclerosis and hypertension (Ip et al., 1990; Wong et al., 1990). Angiotensin converting enzyme (ACE) inhibitors have been shown to prevent or attenuate myointimal hyperplasia after endothelial denudation and vascular injury (Powell et al., 1989; Chobanian et al., 1990). In addition, ACE inhibitors impair the enlargement of SMC mass observed in hypertension (Harrap et al., 1990). Furthermore, DUP-753, an Ang II-receptor antagonist, effectively prevents the Ang II-stimulated proliferation and hypertrophy of these cells (Chiu et al., 1991). Therefore, Ang II seems to play a fundamental role in the genesis of vascular conditions through its effects as a SMC growth promoter.

The mechanism by which Ang II stimulates SMC growth is not well understood. Ang II increases the expression of proto-oncogenes that regulate cell growth such as *c-fos*, *c-myc* and *c-jun* (Naftilan, 1989, 1989, 1990). In addition, Ang II enhances the autocrine expression of growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β, Naftilan, 1989; Gibbons, 1990). Intracellular calcium and protein kinase C have been also implicated in the trophic actions of Ang II in these cells (Griendling, 1989).

Recent studies suggest that Ang II could also modulate SMC growth through the modulation of nitric oxide (NO) synthesis. NO has been shown to suppress SMC function, inhibiting their contraction and proliferation (Moncada et al., 1989; Garg and Hassid, 1989; Nunokawa, 1992). The enzyme that catalyzes NO synthesis is nitric oxide synthase (NOS), that cleaves the guanidino group of L-arginine to produce NO and L-citrulline (Sakuma et al., 1988; Moncada et al., 1991). The synthesis of NO in SMC is induced by cytokines or inflammatory agents found at sites with vascular damage such as interleukin 1-β (IL-1β), bacterial lipopolysaccharide (LPS), tumor necrosis factor a (TNF-α) and interferon-γ (IFN-γ). This suggests that the SMC-derived NO is a factor produced under conditions of vascular injury as a feedback mechanism to help in the control of SMC responses to the myriad of growth factors and activating agents liberated under these conditions.

Preliminary evidence obtained by us (Rivera-Correa et al., 1995) indicates that Ang II inhibits arginine transport in SMC. This suggests that under conditions in which Ang II levels are elevated such as atherosclerosis, restenosis and hypertension, the intracellular arginine concentration should decrease. Reduced intracellular arginine levels in SMCs could lead to reduced NO synthesis because the cytokine-induced NO production is dependent on the presence of extracellular arginine (Hibbs et al., 1987). This effect would be independent of the suppression of the inducible NO synthase mRNA by Ang II reported by Nakayama and coworkers (1994). Reduced NO production by SMCs could secondarily lead to activation of SMCs resulting in increased proliferation, contraction and migration. Smooth muscle cell activation has been related to vasospasm and vessel wall remodeling (Rubanyi, 1993). To determine the relation between arginine transport to NO synthesis in SMCs, we characterized L-arginine transport in this cell preparation and evaluated the effect of Ang II on this process. In addition, the effect of this hormone on the cytokine-induced NO synthesis was assessed.

Materials and methods

Culture media, serum, trypsin-EDTA (ethylenediaminetetraacetic acid) were obtained from GIBCO BRL (Grand Island, NY), and Interleukin 1β, Genzyme Diagnostics (Cambridge, MA). Thapsigargin, BAPTA [1,2-*bis*-(o-Aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid tetra-(acetomethyl)-ester], FURA 2/AM {1-[2-(5-carboxy-oxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-N,N,N′,N′-tetraacetic acid, pentaacetoxymethyl ester} were from Calbiochem (La Jolla, CA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). DUP 753 was a gift from Merck, Sharp, and Dohme Co. (Rahway, NJ).

Tissue culture

Rat aortic smooth muscle cells were isolated from thoracic aortas of adult Long-Evans female rats by enzymatic digestion as described previously (Travo et al., 1986). The cells then were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf serum and 1% antibiotics (100 U/mL; streptomycin 100μg/mL and amphotericin-B 25μg/mL) and maintained in a humidified atmosphere of 5% CO_2-95% air at 37°C. SMC identity was verified by observation of their characteristic multilayered hill and valley morphology under phase-contrast microscopy and by smooth muscle specific myosin immunoreactivity. When confluent, 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), 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 at approximately 6–8 $\times 10^4$ cells/ml 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 L-^{3}H-[arginine] (Arg), ^{3}H-[α-methyl aminoisobutyric acid] (mAIB), and ^{14}C-[leucine] into SMCs. 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). SMCs were incubated for 1 hr at 37°C in Na^+

medium and amino acid uptake was then determined by incubating cells in media with 0.1 mM L-arginine (0.5 μCi/μmol). The uptake was terminated by aspirating the radioactive medium and washing the cells 4 times with ice-cold Na^+-free medium at pH 7.4. 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 assay. Bovine serum albumin was used as standard. Unless otherwise indicated, results were expressed as nmol/mg protein × hr. To estimate the fraction of amino acid uptake that is Na^+-dependent, uptake in choline medium was subtracted from that observed in Na^+-media. Uptake in choline media was used as an estimate of the Na^+-independent amino acid uptake.

Intracellular calcium measurements

To determine the effect of Ang II on intracellular calcium mobilization, the calcium-sensitive fluorescent dye FURA 2/AM was used. For these experiments, confluent cells (T75 cm^2 flasks) were trypsinized and incubated 20 min at 37°C with 2 μM FURA 2/AM. The cells were then washed twice and resuspended with serum-free DMEM plus 0.1% bovine serum albumin and kept on ice ready for use. Prior to intracellular calcium measurements, the cells were washed with Na^+ medium, resuspended and placed in a cuvette for fluorescence determination. The samples were excited alternately at 340 and 380 nM (bandwidth = 10 nm) light and fluorescent emission intensity was monitored at 505 nm. To calibrate the samples, digitonin (75 μM) and 4 mM EGTA (ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′,-tetraacetic acid) were used as described by Griendling (1986).

Measurement of nitrite (NO_2^-)

To determine NO levels, the presence of nitrite (NO_2^-) in the culture media was measured using the method described by Green et al. (1982). This method takes advantage of the fact that NO is rapidly converted within the cells into nitrites (95%) and nitrates (5%) which remain in solution and can be determined spectrophotometrically (Ignarro et al., 1994). Briefly, equal volumes of cell-conditioned incubation media and Griess reagent (1% sulfanilamide and 0.1% naphtylethylenediamine in 5% phosphoric acid) were mixed. After 10 min incubation at room temperature, the sample's absorbance were read at 550 nm. 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 mmol/mg protein × 24 hours.

Intracellular L-arginine content determination

L-arginine content was determined using a Hewlett Packard HPLC 1050 coupled to a fluorescence detector. Cell samples were prepared as described by White et al. (1982). Arginine measurements were done using the Waters AccQ-Tag chemistry package with a 3.9 mm × 300 mm column (Cohen and De Antonis, 1994).

Statistical analysis

Results are expressed as mean ± SEM of the indicated number of experiments done with triplicate samples. Statistical comparisons between different groups were done using analysis of variance (ANOVA). Differences between treatments were considered significant as determined by Scheffés's F test using the Abacus Concepts StatView II program (Abacus Concepts, Berkeley, CA) on a Macintosh SE computer (Apple Computer, Cupertino, CA). Values were considered statistically significant at $P \leq 0.05$. The molar concentration of Ang II producing 50% inhibition (IC_{50}) of arginine transport or activation of intracellular calcium mobilization (EC_{50}) was calculated using the program Tablecurve (Jandel, San Rafael, CA) in a IBM-XT and reported as the mean ± SEM.

Results

Time course of L-[^{3}H]-arginine uptake

The time course of arginine uptake by SMC is shown in Fig. 1. The uptake was determined in the presence and absence of sodium at different time intervals. Under all conditions examined, the uptake was found to be linear for at least two minutes. Arginine uptake in these cells is composed of a Na^+-independent component which accounts for about 60% of the total uptake and a Na^+-dependent component which represents about 40% of arginine uptake. The non-saturable (diffusional) component, demonstrated by addition of a 5mM excess of unlabeled arginine to the uptake medium in the absence of sodium, was negligible.

Amino acid inhibition of L-[^{3}H]-arginine uptake by SMCs

To characterize the Na^+-dependent and Na^+-independent transport systems in SMCs, the transport of 100μm arginine was measured at external pH (pHo) = 6.0 and at pHo = 7.4, in the presence of the L-amino acids shown in Fig. 2, each at a concentration of 5mM. The selected amino acids were chosen because they have been reported to be markers of amino acid transport systems in other cell preparations (reviewed by Saier, 1988; Kilberg, 1993). As depicted in Fig. 2A, the Na^+-independent arginine transport was inhibited (20%) by acid pHo ($P \geq 0.05$), but was abolished by unlabeled arginine and substrates of system y^+ (homoarginine, ornithine and lysine). In addition, histidine, which at pH 7.4 bears a positive net charge, significatively inhibited

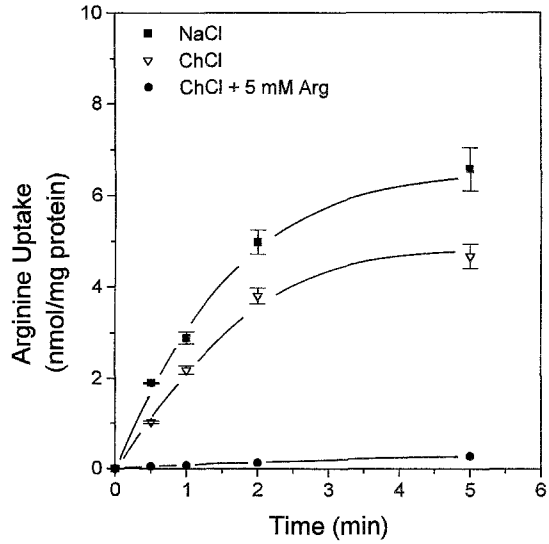

Fig. 1. L-Arginine uptake by smooth muscle cells (SMCs) as a function of time. Uptake was initiated by adding 100μM arginine to cells incubated in Na^+-containing or Na^+-free medium. Uptake was terminated at the indicated time. The diffusible component was determined in the absence of sodium and in the presence of a 5mM excess of unlabeled arginine. Points represent mean ± SEM for six determinations

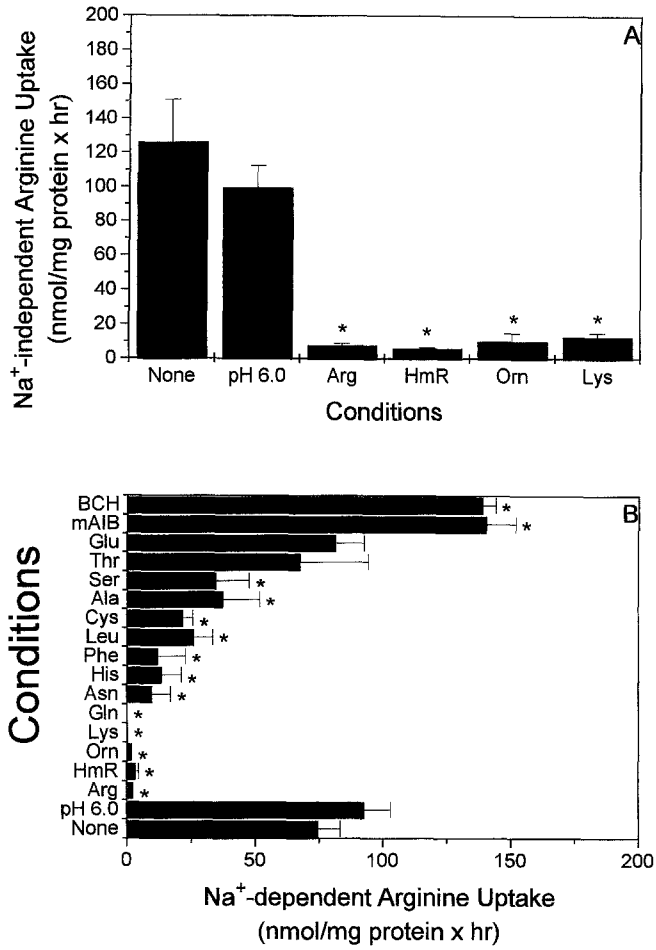

Fig. 2. *Cis*-inhibition of arginine uptake by SMCs. **A** Na^+-independent uptake. Transport of 100μM arginine was measured for 2 min at 37°C in Na^+-free media, in the presence or absence of the indicated L-amino acids (5 mM) or acid pH_0 (6.0). Significant differences ($P \leq 0.05$) were found for None vs.: Arg, Orn, HmR and, Lys. **B** Na^+-dependent uptake. Uptake determinations of 100μM arginine were done in the presence or absence of sodium and in presence or absence of the indicated L-amino acids at 5 mM concentration. The Na^+-dependent component was determined by subtracting the uptake in choline medium from the uptake in sodium medium. With the exception of Glu, Thr and pH 6.0, all differences from control values (None) were found to be statistically significant with $P \leq 0.05$. Data represent mean ± SEM for 3 experiments

arginine transport by 84% ($P \leq 0.05$). These data indicate that the Na^+-independent arginine transport must occur through a transport system similar to system y^+ as described in other cell preparations. The presence of system $b^{0,+}$ in these cells was suggested by a small inhibition (10%) of the Na^+-independent arginine transport observed with neutral amino acids such as alanine, serine, threonine, and glutamine (data not shown).

Figure 2B illustrates similar studies for the Na^+- dependent arginine transport. Acidification of the external medium to pH = 6.0 slightly increased arginine uptake by 24% ($P \geq 0.05$). In contrast, cationic amino acids (arginine, lysine, ornithine and homoarginine) abolished this fraction of arginine trans-

port ($P \leq 0.05$). The neutral amino acids glutamine, asparagine, histidine, phenylalanine and leucine also inhibited transport activity. Similar results were observed with alanine, serine, cysteine and threonine, although these amino acids were less effective inhibitors of arginine transport. BCH and mAIB, the synthetic markers for systems L and A respectively, stimulated arginine uptake. Lithium, a cation that could substitute Na^+ as a co-solute in system N, was ineffective for the Na^+-dependent arginine uptake (data not shown). These results strongly suggest that the Na^+-dependent arginine uptake is mediated by system $B^{0,+}$. The latter is a Na^+-dependent transport system that accepts neutral and cationic amino acids (Van Winkle et al., 1985).

Effect of angiotensin II on L-[^{3}H]-arginine uptake

As depicted in Fig. 3, incubation of cells with 100nM Ang II (1 hour) decreased the Na^+-dependent arginine uptake by 94% ($P \leq 0.05$) while the Na^+-independent transport was unaffected by this hormone. The inhibition of arginine transport by Ang II was mediated by the AT-1 receptor since DUP 753, a non-peptidic antagonist specific for this receptor reversed this effect. The inhibition of Na^+-dependent arginine transport was a sustained event since cells that were treated for four days with Ang II also showed reduced rates of arginine uptake (from 54.1 ± 2.0 to 2.2 ± 0.2nmol/mg protein × hr, $n = 3$, $P \leq 0.05$).

To investigate whether the effect of Ang II on the Na^+-dependent arginine transport was specific for system $B^{0,+}$, the effect of Ang II was also evaluated

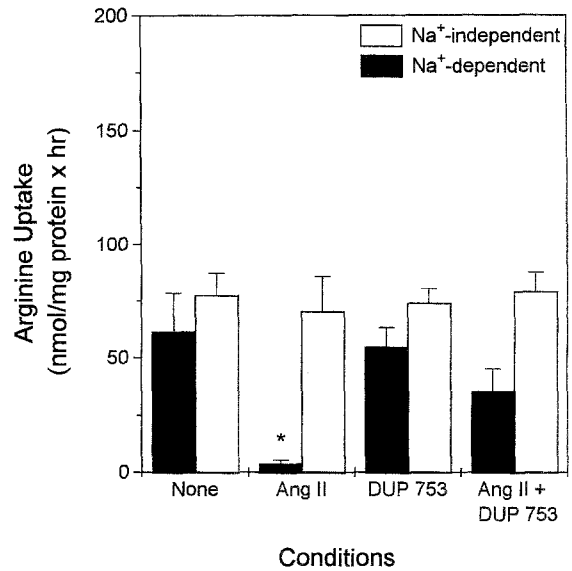

Fig. 3. Effect of angiotensin II and DUP 753 on arginine uptake in SMCs. Cells were incubated with 100nM Ang II in the presence and absence of DUP 753 (250μM) during the 1 hour incubation period. These agents were also present during the [^{3}H]-arginine uptake period. Data represent mean ± SEM for 3 experiments. Significant differences ($P \leq 0.05$) in the Na^+-dependent fraction were found for None vs. Ang II, and for Ang II vs. the combination of DUP 753 + Ang II

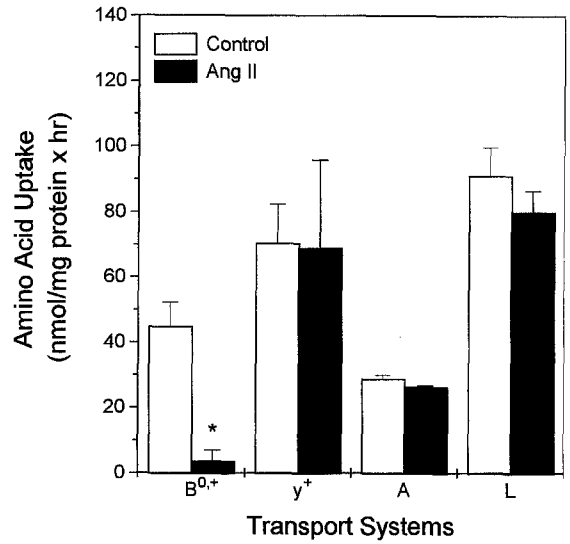

Fig. 4. Effect of Ang II on amino acid transport systems. SMCs were incubated with 100nM Ang II during the 1 hour incubation period. System y^+ and $B^{0,+}$ activities were determined as described in the Materials and methods. System A activity and system L activities were estimated by the Na^+-dependent uptake of [^{3}H]-α-methyl-aminoisobutyric acid uptake, and the Na^+-independent [^{14}C]-leucine transport, respectively. Bars represent mean ± SEM for 3 experiments. Significant differences ($P \leq 0.05$) were only found for the $B^{0,+}$

on system y^+, A and L. System y^+ was estimated from the Na^+-independent arginine uptake whereas system A and L activities were determined as the Na^+-dependent mAIB uptake and the Na^+-independent leucine transport, respectively. System A and L were chosen because they are affected by cell transformation, starvation and are subjected to hormonal regulation (Moreno et al., 1985; Collarini and Oxender, 1987; Saier, 1988). Figure 4 shows that Ang II affected only the Na^+-dependent arginine uptake, suggesting that the inhibition of transport by this hormone is specific for system $B^{0,+}$.

Figure 5A shows arginine transport as a function of Ang II concentration in a Na^+-containing medium. As depicted in the figure, Ang II inhibited 40% of total arginine uptake in a concentration dependent manner. The half maximal effect (IC_{50}) was at 8.9 ± 1.0nM. Notice that at the highest Ang II concentration used, 60% of arginine uptake is still observed. This represents the Na^+-independent transport of arginine taking place in a Na^+-containing medium. To determine if the IC_{50} for the Na^+-dependent arginine transport was similar to that observed for other processes activated by Ang II, the mobilization of intracellular calcium by Ang II was determined (Fig. 5B). The concentration that induced 50% activation (EC_{50}) of calcium mobilization was 3.8 ± 1.0nM, respectively. As expected, DUP 753 (250μM) blocked the intracellular calcium mobilization by 100nM Ang II. These results indicate that both actions of Ang II are mediated by the AT-1 receptor and support a common mechanism of action for Ang II in these cellular processes.

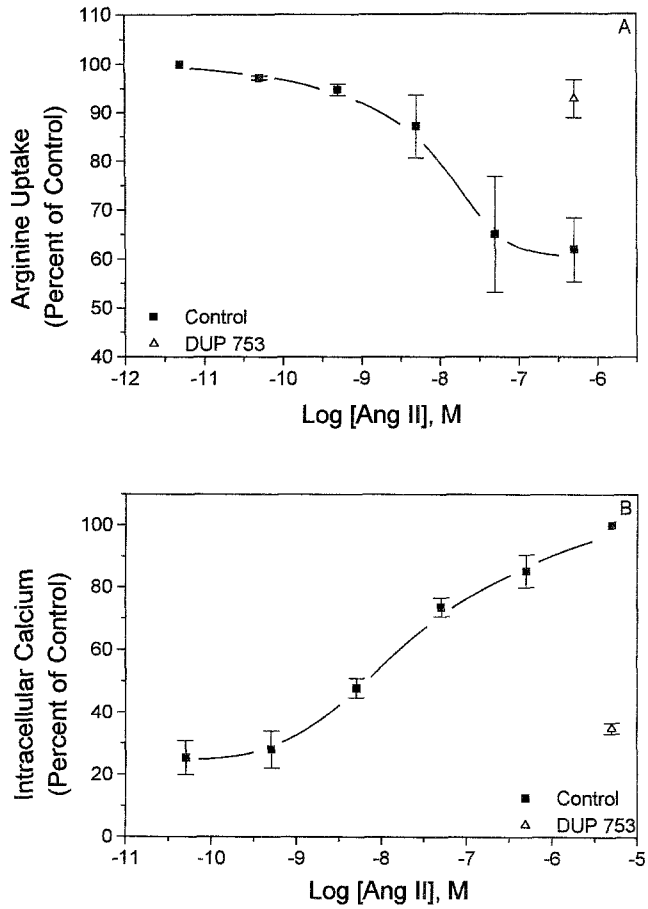

Fig. 5. Concentration dependence curves for the inhibition of arginine uptake and the stimulation of intracellular calcium mobilization by Ang II in SMCs. **A** Arginine uptake as a function of Ang II concentration. The cells were incubated with different concentrations of Ang II or 1 μM Ang II plus 250 μM DUP 753 for 2 min during uptake measurements. The remaining procedure was determined as previously described. Calculated IC_{50} was 8.9 ± 1.0 nM. **B** Intracellular calcium mobilization as a function of Ang II concentration. FURA 2/AM loaded cells were placed in a fluorimeter cuvette at 37°C to determine the effect Ang II addition. In the DUP 753 (250 μM) determination, the cells were pre incubated in the cuvette for 1 min prior to the addition of Ang II (1 μM). Values represent mean ± SEM for 4 experiments. EC_{50} = 3.8 ± 1.4 nM

Regulation of the Na^+-dependent [^{3}H]-arginine uptake by Ang II

Ang II is known to activate G proteins, increase intracellular calcium mobilization, stimulate phosphoinositide turnover and activate protein kinase C (PKC) (Smith, 1986; Griendling et al., 1989). To elucidate which of these elements of the signal transduction pathway of Ang II are involved in the regulation the Na^+-dependent arginine uptake, drugs known to interfere with these regulatory pathways were used. As shown in Fig. 6, PMA, a PKC-activating phorbol, inhibited the Na^+-dependent arginine uptake by 71% ($P \leq 0.05$). No further inhibition of transport was observed when PMA and Ang II were added together, indicating that PMA and Ang II share a

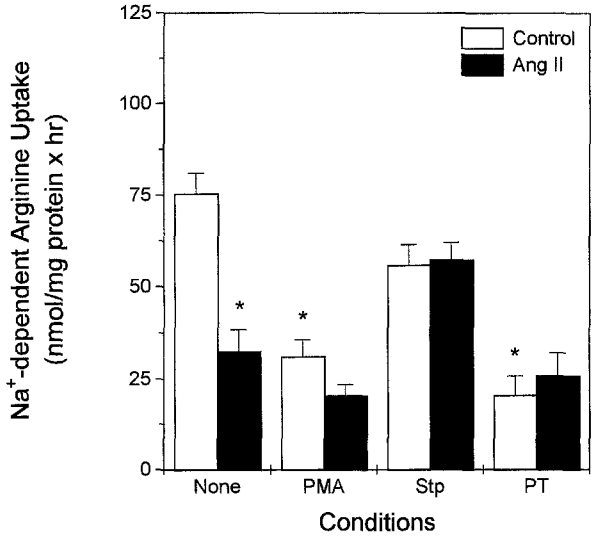

Fig. 6. Effect of phorbol esters (*PMA*), staurosporine (*Stp*) and pertussis toxin (*PT*) on the Na^+-dependent arginine uptake. The cells were pre incubated for 30 min with 100 nM PMA, 100 nM Stp or 400 ng/ml PT before the addition of 100 nM Ang II. The cells were then washed with Na^+-containing medium and incubated in this medium for an additional hour with the agents under study. Bars represent mean ± SEM for 3 experiments. Significant differences ($P \leq 0.05$) were found for None versus Ang II, PMA, and PT

common intracellular signaling mechanism. Furthermore, staurosporine, an inhibitor of protein phosphorylation, prevented the inhibition by Ang II on arginine uptake. These data are in agreement with the notion that the Na^+-dependent arginine transport is regulated by Ang II through a PKC-dependent mechanism.

Pertussis toxin, an inhibitor of certain G-proteins, inhibited the Na^+-dependent arginine transport by 55% ($P \leq 0.05$), indicating that a G-protein is involved in the basal regulation of transport. No effect of Ang II was observed in the presence of this agent. In addition, agents that interfere with the intracellular calcium mobilization (i.e., BAPTA and thapsigargin), and protein synthesis (i.e., cycloheximide) did not affect the Na^+-dependent arginine transport (data not shown).

Effect of Ang II on nitric oxide synthesis

To determine if under the conditions in which Ang II inhibits arginine uptake NO synthesis is similarly affected, NO synthesis was evaluated using the Griess reaction. This reaction is used to estimate nitrite production, a stable NO breakdown product. For these experiments, the cells were incubated with the different agents used for 24 hours after which aliquots from the medium were collected and nitrite levels were determined.

As illustrated in Fig. 7A, IL-1β (5 ng/ml) markedly stimulated the accumulation of nitrites in the culture medium 24-fold ($P \leq 0.05$). The IL-1β induced

nitrite accumulation was abolished by 1 mM aminoguanidine (AG), a selective inhibitor for the inducible NOS (Misko et al., 1993). AG, however, did not affect the basal nitrite production. This indicates that the stimulated nitrite production under these experimental conditions is the result of NOS activity. Furthermore, removal of arginine from the medium also abolished the IL-1β-stimulated nitrite accumulation, supporting previous reports (Hibbs et al., 1987) on the requirement of extracellular arginine for NO synthesis.

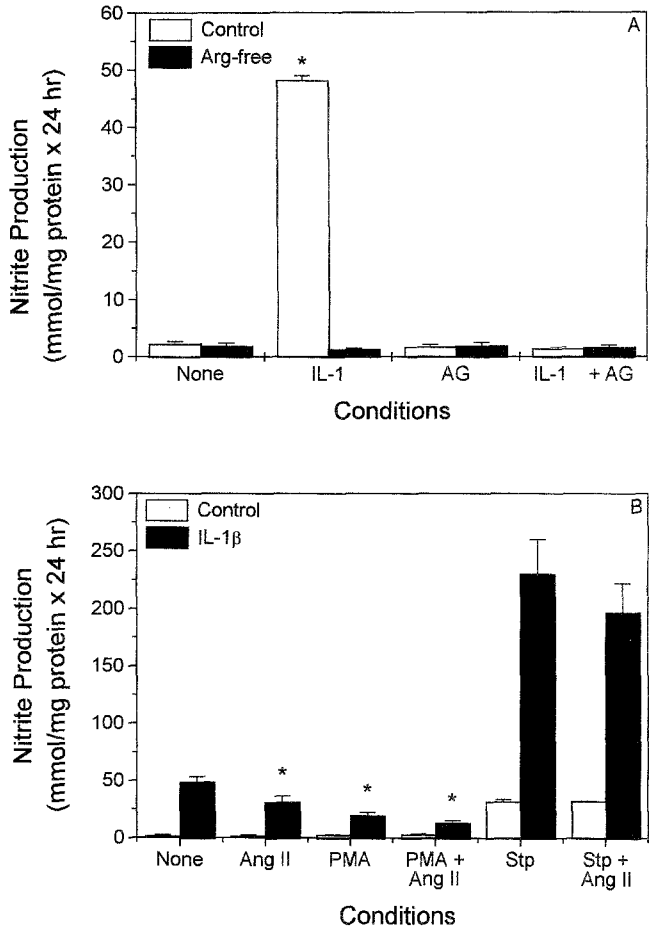

Fig. 7. Characterization and regulation of nitrite production in SMC cultures. **A** The cells were washed twice with HBSS and were then transferred to the CO_2-air incubator in complete or arginine-free medium in the presence or absence of 5 ng/ml Interleukin-1β (*IL-1*β), 1 mM aminoguanidine or both. After 24 hours, aliquots from the medium were collected and nitrite concentration quantitated as described in materials and methods. Bars represent means ± SEM for 4 experiments. Significant differences ($P \leq 0.05$) were found only for None vs. IL-1β. **B** Effect of 100 nM Ang II, 100 nM PMA and 10 nM Stp on the IL-1β-stimulated nitrite accumulation by SMC cultures. Experiments were performed as described above. Data are representative of mean ± SEM for 4 experiments. Significant differences ($P \leq 0.05$) were found for None vs. IL-1β, IL-1β vs. IL-1β + Ang II, IL-1β vs. PMA and IL-1β vs. PMA + Ang II. In addition, statistically significant differences were observed between None vs. Stp, None vs. Stp + Ang II, IL-1β vs. Stp + IL-1β and IL-1β vs. Stp + IL-1β + Ang II. Comparison between PMA versus PMA + Ang II did not reach statistical significance

Figure 7B shows the effect of Ang II on the IL-1β induced nitrite accumulation, and the action of PMA (100nM) and staurosporine (10nM) on this process. Ang II significantly inhibited (46%, $P \leq 0.05$) the cytokine stimulated nitrite accumulation without affecting the basal production. The cytokine-induced nitrate accumulation was also inhibited 60% by PMA ($P \leq 0.05$), and this effect was not additive with that of Ang II, supporting a common mechanism for the action of these agents. By contrast, staurosporine markedly stimulated the induced nitrate production by 114-fold ($P \leq 0.05$) and the basal nitrite production by 19-fold ($P \leq 0.05$). These experiments support an important role for PKC in the inhibitory action of Ang II.

Effect of arginine supplementation on nitrite production

To ascertain if the inhibition of Ang II on the IL-1β-stimulated nitrite production was due to reduced cellular arginine levels, nitrite accumulation was determined in cells incubated in the presence of 10mM arginine for 24 hours. This treatment increases the intracellular arginine levels 3-fold (from 99 ± 2 to 307 ± 7nmol/mg protein, n = 3). Figure 8 shows that 10mM arginine did not affect basal nitrite production nor prevented the inhibition of the cytokine-induced nitrite accumulation by Ang II. Similar results were obtained at lower (0.5mM) arginine concentrations (data not shown). These results suggest that the effect of Ang II on NO synthesis is not secondary to the inhibition of arginine transport.

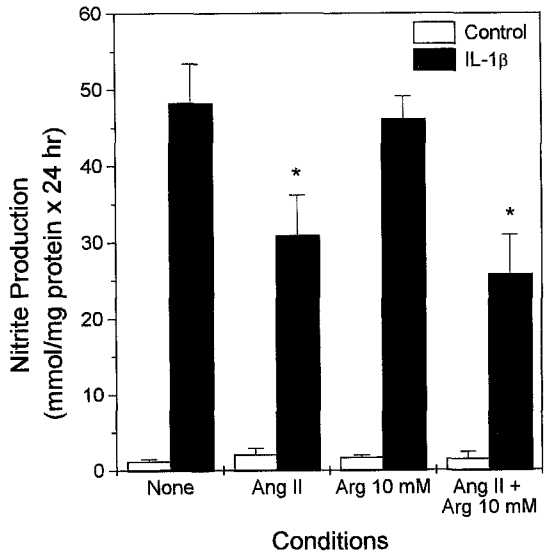

Fig. 8. Effect of arginine supplementation on nitrite synthesis in the presence or absence of Ang II. SMCs were incubated in the presence or absence of 10mM arginine for 24 hours after which nitrite accumulation was determined. Bars represent mean ± SEM for 4 experiments. Significant differences ($P \leq 0.05$) were found for None vs. IL-1β, IL-1β vs. IL-1β + Ang II. In addition, comparison of nitrite production in the presence of Arg 10mM and IL-1β with Arg 10mM + IL-1β + Ang II was also statistically significant ($P \leq 0.05$)

Discussion

The main objective of the present study was to characterize arginine transport in vascular SMC and to study its regulation by Ang II. Furthermore, we considered it relevant to assess the role of arginine transport in the inducible production of NO by this cell preparation. Our results indicate that arginine uptake takes place through Na^+-dependent and Na^+-independent transport pathways. 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 SMCs (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. By contrast, 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). This transport pathway operates as a broad scope system that transports cationic and neutral amino acids (i.e. glutamine, phenylalanine, and asparagine). The transport system is pH insensitive and does not accept lithium as a substitute for Na^+. The presence of system $B^{0,+}$ in SMCs is at variance with earlier studies (Low et al., 1993; Low and Grigor, 1995; Durante et al., 1995) that negate the existence of a Na^+-dependent arginine transport in these cells. The reason for this discrepancy is not known. However, the present investigation was carried out with cells cultured and maintained in the presence of 10% serum as opposed to the use of quiescent cells in the previous studies. This suggests that the Na^+-dependent arginine transport could be dependent on growth factors or agents present in serum. This idea is supported by reports of arginine transport through the $B^{0,+}$ pathway in endothelial cells maintained in the presence of serum (Greene et al., 1993; Cendan et al., 1995). Modulation of amino acid transport systems by hormones, growth factors and serum (Boerner and Saier, 1982; Escobales et al., 1994) is well documented. The presence of Na^+-dependent and Na^+-independent pathways for the transport of arginine in vascular smooth muscle cells underlines the importance of this amino acid to vascular SMC function. In this context, concentrative, Na^+-dependent arginine transport could be of relevance to maintain high levels of this amino acid under conditions of high cellular activity.

A major finding of this work is that the Na^+-dependent arginine transport is modulated by Ang II. The regulation of arginine transport (system $B^{0,+}$) by Ang II is not shared by systems A, L, and y^+, indicating that this action is specific for system $B^{0,+}$. Our finding regarding the lack of effect of Ang II on system y^+ is at variance with that reported by Low and Grigor (1995) which indicated stimulation of this transport system by Ang II. Although the precise reason for this discrepancy is unknown, it is possible that incubation of cells in serum-free medium for 48 hrs and/or depletion of amino acid pools before the determinations of arginine transport cause a different hormonal response.

The action of Ang II is mediated by the AT-1 receptor subtype because DUP-753, an AT-1 receptor blocker, prevented the inhibition of arginine

transport. This blocker also inhibited the stimulation of intracellular calcium mobilization by Ang II. Furthermore, the effects of Ang II on arginine transport and Ca_i^{2+} mobilization occurred with apparent Kd values that were very similar (9 and 4 nM, respectively), suggesting a common receptor type for the action of Ang II. 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 and Alexander, 1990). In these cells, Ang II binds to the AT-1 receptor to activate the phosphoinositide-specific phospholipase C via pertussis toxin-insensitive and pertussis toxin-sensitive G proteins (Anand-Srivastava, 1983; Timmermans et al., 1992). Activation of phospholipase C, in turn, hydrolyses membrane phospholipids into inositol trisphosphate ($InsP_3$) and *sn*-1,2-diacylglycerol (DAG). $InsP_3$ stimulates the release of intracellularly stored calcium and DAG stimulates PKC. PKC is recognized to play a fundamental role in Ang II action. For example, intracellular calcium homeostasis (Smith, 1986; Griendling et al., 1989), SMC contraction (Walsh et al., 1994), and SMC proliferation (Rozengurt, 1986), have been reportedly regulated by PKC.

Intracellular Ca^{2+} mobilization is not involved in this aspect of the action of Ang II because agents that affect this cellular process (i.e., BAPTA and thapsigargin) were ineffective on the inhibition of arginine transport. By contrast, agents that affect PKC activity markedly affect arginine uptake and the inhibitory effect of Ang II. Thus, activation of this enzyme with PMA mimicked Ang II action and staurosporine, a potent PKC inhibitor, prevented the inhibition of system $B^{0,+}$ by Ang II. Collectively, these findings support the notion that the regulation of Na^+-dependent arginine transport by Ang II in SMCs is dependent on PKC activity. This action appears to be mediated by a G_i protein because the inhibition of arginine transport by Ang II was blocked by pertussis toxin.

Evidence accrued in the last decade indicates that NO is derived from the terminal guanidino group of L-arginine. Our observation that Ang II inhibits the Na^+-dependent arginine transport in SMCs raised the possibility that this hormone could inhibit NO synthesis through the reduction of intracellular arginine levels. To establish the role that arginine transport could have on NO synthesis, we determined the effect of Ang II on the IL-1β induced NO synthesis. Our results indicate that Ang II significantly reduced NO synthesis by cytokine-stimulated cells. This action of Ang II was mimicked by PMA and was abolished by staurosporine, which agrees with arginine transport experiments. Furthermore, the inhibitory actions of Ang II and PMA were not additive supporting a common mechanism for the inhibition of NO synthesis and arginine uptake. These findings are consistent with a mechanism by which NO synthesis is coupled to arginine uptake via a PKC-dependent pathway.

The results of this study agree with earlier reports indicating that Ang II inhibits the cytokine-induced NO synthesis via a PKC dependent mechanism (Nakayama et al., 1994). Inhibition of the cytokine-induced NO synthesis in SMCs by PKC was also reported by Geng et al. (1994). The modulation of NO synthesis reported in these studies was ascribed to the suppression of the

inducible NOS mRNA transcription since it was sensitive to actinomycin-D. The lack of effect of an excess of arginine in the medium (10mM) on the inhibitory effect of Ang II on IL-1β induced nitrite accumulation supports the existence of such a mechanism in these cells. It is clear, however, that NO synthesis is totally dependent on the entry of arginine into the SMC, because entry is negligible in virtually arginine-free medium. Therefore, although NO production is dependent on the presence of arginine, its regulation by Ang II appears to be independent of arginine transport. This notion agrees with preliminary studies indicating that Ang II does not affect intracellular arginine levels in our preparation (data not shown). From these studies we conclude that the observation of concomitant inhibition of arginine transport and NO synthase activity should represent a parallel depression of these processes by PKC rather than a causal effect of arginine transport on NO biosynthesis.

Durante and coworkers (1993) reported that arginine transport through the y^+ transport system and NO synthesis are upregulated by IL-1β and TNF-α in quiescent SMCs. Yet, although nitrate production was stimulated by INF-γ and dibutyryl cyclic AMP in these cells, arginine uptake was not affected. These findings, together with those of the present study concerning the Na^+-dependent $B^{0,+}$ system, suggest that arginine transport in SMCs could be differentially regulated (y^+ versus the $B^{0,+}$ system) depending on the condition of cells (i.e. quiescent versus serum-activated). It would be of interest to assess the role of PKC in such regulation.

What purpose does a parallel regulation of Na^+-dependent arginine transport and nitric oxide synthase in SMCs serve? The answer to this question remains to be elucidated. However, SMC incubated in media containing 10mM arginine for 24 hours exhibited reduced viability as determined by trypan-blue exclusion (21%, $n = 12$, $P \leq 0.01$). In addition, high arginine levels are known to induce potassium exit from cells (Dickerman and Walker, 1964) which could cause alterations of enzymatic activity and membrane potential. Therefore, a parallel regulation of arginine transport and NO synthase by Ang II could be a protective mechanism employed by SMCs to prevent the possible damaging effects of accumulated arginine under conditions of low metabolic requirements. Experiments are thus needed to address these important points.

In summary, the results of this study indicate that the Na^+-dependent arginine transport in vascular smooth muscle cells is mediated by system $B^{0,+}$ and is subjected to regulation by Ang II through a mechanism dependent on PKC. A parallel regulation for NO synthesis by this hormone is also present in these cells. However, no direct relationship between these processes could be determined because under conditions in which intracellular arginine levels are elevated 3-fold (i.e. $[arginine]_0 = 10mM$), nitrite accumulation was still depressed by Ang II.

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