0022-3565/97/2811-0448$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 281, No. 1

Printed in U.S.A.
JPET 281:448–453, 1997

L-Glutamine Inhibits Nitric Oxide Synthesis in Bovine Venular Endothelial Cells[1]

CYNTHIA J. MEININGER and GUOYAO WU

Departments of Medical Physiology (C.J.M., G.W.) and Animal Science (G.W.) Texas A&M University, College Station, Texas

Accepted for publication December 24, 1996

ABSTRACT

This study was conducted to test the hypothesis that L-glutamine has differential effects on nitric oxide (NO) synthesis from L-arginine in bovine venular endothelial cells (EC) stimulated by A_{23187} (a Ca^{++} ionophore) and receptor-mediated vasodilators (bradykinin and substance P). EC were cultured at 37°C for 24 h in the presence of 0.4 mM L-arginine and 0.0 to 2.0 mM L-glutamine with or without 1 μM A_{23187}, 1 μM bradykinin or 10 μM substance P. The release of nitrite and nitrate by EC was used as an indicator of NO synthesis. A_{23187}, bradykinin or substance P increased NO synthesis from L-arginine by EC in the presence or absence of L-glutamine. The addition of L-glutamine (0.5 and 2 mM) markedly increased intracellular concentrations of L-glutamine, L-glutamate and L-aspartate and decreased NO synthesis by EC in a concentration-dependent manner in the presence or absence of A_{23187}, bradykinin or substance P. L-Glutamine had no effect on L-arginine uptake by EC or on intracellular L-arginine concentration. Neither L-glutamine nor its glutaminase metabolites (ammonia, L-glutamate and L-aspartate) had any effect on endothelial NO synthase activity. Taken together, these results suggest that the inhibition by L-glutamine of NO synthesis from L-arginine is unlikely to result from an effect of L-glutamine on L-arginine transport or NO synthase activity. Although the mechanism involved remains unknown, regulation of the arginine-NO pathway by L-glutamine may have pharmacologic and therapeutic implications in such conditions as inflammation and septic shock by inhibiting NO generation from L-arginine in endothelial cells.

L-Arginine is the physiological precursor of NO in EC (Ignarro *et al.*, 1987; Palmer *et al.*, 1988). NO synthase, which is present in both the plasma membrane and the cytosol of EC (Hecker *et al.*, 1994), requires BH_4, NADPH, FAD, FMN, Ca^{++}, O_2 and calmodulin, in addition to L-arginine, for its activity (Knowles and Moncada, 1994). By directly activating soluble guanylate cyclase to generate cGMP from GTP, NO is a potent mediator of various biological responses (Moncada and Higgs, 1993). Beyond its key role in host defense (Hibbs *et al.*, 1988) and neurotransmission (Bredt *et al.*, 1990) and as a signaling molecule (Bredt and Snyder, 1994), NO has recently been recognized as the endothelium-derived relaxing factor that plays an important role in regulating vascular tone and permeability (Moncada and Higgs, 1993). Thus it is important to characterize the regulation of NO synthesis in EC.

We (Wu and Meininger, 1993) and others (Hecker *et al.*, 1990b) have recently demonstrated that the citrulline produced by NO synthase can be recycled into arginine in EC, which may help to maintain a sufficient intracellular concentration of arginine for NO generation. Thus, with the production of NO from L-arginine by constitutive NO synthase in EC, there is a functional arginine-citrulline cycle in these cells, as in macrophages (Wu and Brosnan, 1992). Interestingly, the endothelial arginine-citrulline cycle has been shown to be regulated by L-glutamine (Hecker *et al.*, 1990b; Sessa *et al.*, 1990), the most abundant free amino acid in the body (Krebs, 1980). In this cycle, L-glutamine inhibits the conversion of L-citrulline into L-arginine in EC (Hecker *et al.*, 1990a; Wu and Meininger, 1993) and in the cerebral perivascular nerve tissues (Chen and Lee, 1995) in a concentration-dependent manner. L-Glutamine also markedly decreases NO synthesis from L-arginine in cultured EC (Hecker *et al.*, 1990a,b) and intact blood vessels (Swierkosz *et al.*, 1990) and inhibits cerebral neurogenic vasodilation (Lee *et al.*, 1996). It is noteworthy that Arnal *et al.* (1995) recently reported that NO synthesis in bovine aortic EC was inhibited by L-glutamine in the presence of bradykinin (a receptor-mediated vasodilator) but was increased by L-glutamine in the presence of A_{23187} (a Ca^{++} ionophore). However, the mechanism of the differential action of L-glutamine on endothelial NO synthesis has not been elucidated.

Received for publication July 10, 1996.
[1] This research was supported by Grants-in-Aid from the American Heart Association (No. 95013030 and 95009150) and by an interdisciplinary grant from Texas A&M University.

ABBREVIATIONS: BH_4, (6R)-5,6,7,8-tetrahydro-L-biopterin; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; EC, endothelial cells; ECGF, endothelial cell growth factor; FBS, fetal bovine serum; KHB, Krebs-Henseleit bicarbonate; NO, nitric oxide; PBS, phosphate-buffered saline.

On the basis of our work on the inhibition by L-glutamine of L-citrulline recycling into L-arginine in bovine venular EC (Wu and Meininger, 1993), the present study was conducted to test the hypothesis that L-glutamine has differential effects on NO synthesis from L-arginine in bovine venular EC stimulated by A_{23187}, bradykinin and substance P (a receptor-mediated vasodilator) (Ziche *et al.*, 1994). Our results demonstrate that L-glutamine inhibited NO synthesis from L-arginine in bovine venular EC in a concentration-dependent manner in the presence or absence of A_{23187}, bradykinin and substance P.

Materials and Methods

Chemicals. The following drugs and chemicals were used in this study: L-arginine, L-glutamine, HEPES, BSA (essentially fatty acid-free), calmodulin, NADPH, FAD, FMN, EDTA, dithiothreitol, L-valine, and L-amino acid standards (Sigma Chemical Co., St. Louis, MO), BH_4 (Research Biochemicals Inc., Natick, MA), L-[U-^{14}C]arginine (50 mCi/mmol) (American Radiolabeled Chemicals, Inc., St. Louis, MO), calcium- and magnesium-free DPBS and DMEM (Gibco, Grand Island, NY), FBS (Summit, Greeley, CO), ECGF (Biomedical Technologies, Stoughton, MA), collagenase Type II (Cooper Biochemicals, Malvern, PA), polystyrene beads for cell isolation (3M, St. Paul, MN) and Biosilon^R microcarrier beads (Nunc, Naperville, IL).

Isolation and culture of bovine venular EC. Bovine venular EC were isolated from coronary venules 15 μm in diameter using a bead perfusion system previously described (Meininger and Granger, 1990; Wu and Meininger, 1993). Briefly, the coronary circulation was perfused *via* the aortic ostia with Ca^{++}- and Mg^{++}-free DPBS at 37°C to clear the vasculature of blood. A suspension of 15 μm polystyrene beads at a concentration of 8000 beads/ml in DPBS containing 0.02% EDTA was perfused at 4°C *via* small veins connecting the coronary sinus with the venous side of the microcirculation. Antegrade perfusion of DPBS (37°C) from the aortic ostia washed the bead-cell complexes from the microcirculation, allowing them to be collected at the coronary sinus. The complexes were collected by centrifugation, washed and plated in gelatin-coated (1.5% in DPBS) dishes. EC were cultured in DMEM supplemented with ECGF (100 μg/ml), 2 mM L-glutamine, 0.4 mM L-arginine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (Fungizone) (PSF). After the cells neared confluence, the ECGF was replaced with 20% FBS. Cell lines were passaged by trypsinization in DPBS containing 0.25% trypsin and 0.02% EDTA and used at passages 9–15.

NO synthesis by bovine venular EC. The release of nitrite and nitrate (two major stable end products of NO oxidation) by cultured venular EC was measured as an indicator of NO synthesis, as previously described (Wu and Meininger, 1995). Briefly, EC (1.5×10^6 cells) were rinsed with DPBS and then cultured at 37°C for 24 h in 0.7 ml of DMEM containing 0, 0.5 or 2 mM L-glutamine in the presence of 0, 1 μM A_{23187}, 1 μM bradykinin or 10 μM substance P. The culture medium contained 0.4 mM L-arginine, 20 mM D-glucose, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (Fungizone). Medium contained no phenol red or serum. In all experiments, culture medium without cells was used as a blank. At the end of the 24-h culture period, the conditioned media were used for the determination of nitrite and nitrate by the Griess reagent, with $NaNO_2$ and $NaNO_3$ as standards. Cell numbers were determined using a hemacytometer. Cell viability was greater than 95% as assessed by 0.2% Trypan Blue exclusion.

The concentrations of L-glutamine used in the present study (0, 0.5 and 2 mM) were based on the concentrations used in other studies with endothelial cells (*e.g.*, Arnal *et al.*, 1995; Sessa *et al.*, 1990) and on plasma concentrations of L-glutamine in animals and humans (0.4–1 mM) (Wu and Marliss, 1992; Wu *et al.*, 1991; Wu *et al.*, 1996). The 0 mM L-glutamine was included as a control in the present study to determine an effect of glutamine on NO synthesis in endothelial cells as in other studies (*e.g.*, Arnal *et al.*, 1995; Sessa *et al.*, 1990; Lee *et al.*, 1996).

Determination of amino acids in bovine venular EC. Venular EC were cultured at 37°C for 24 h, as described above. At the end of the culture, EC cells were harvested from the culture dish by trypsinization in DPBS containing 0.25% trypsin and 0.02% EDTA. Cells were washed twice in PBS by centrifugation (10,000 $\times g$, 1 min) and separated from the medium through a layer of silicone oil (Wu and Meininger, 1993) by centrifugation (10,000 $\times g$, 1 min). EC were lysed in an underlying 0.2 ml of 1.5 M $HClO_4$, and the acidified extracts were neutralized with 0.1 ml of 2 M K_2CO_3 and centrifuged at 10,000 $\times g$ for 1 min. The supernatant was used for analysis of amino acids by a sensitive fluorescent HPLC method involving precolumn derivatization with o-phthaldialdehyde, as previously described (Wu and Meininger, 1993).

NO synthase activity in bovine venular EC. NO synthase activity was measured in venular EC extracts as previously described (Wu and Meininger, 1995). Briefly, EC (2×10^7 cells/ml) were washed three times with PBS containing protease inhibitors (5 μg/ml phenylmethylsulfonylfluoride, 5 μg/ml aprotinin, 5 μg/ml chymostatin, 5 μg/ml pepstatin A). Cells were lysed by 3 cycles of freezing in liquid nitrogen and thawing at 37°C in a water bath. The whole-cell extracts were used for the NO synthase assay. The assay medium (0.2 ml) contained 0.3 mM BH_4, 3 mM dithiothreitol, 1 μg/ml calmodulin, 1 mM $CaCl_2$, 1 mM NADPH, 0.2 mM FAD, 0.1 mM FMN, 0.1 mM L-[U-^{14}C]arginine (1500 dpm/nmol), cell extracts (0.2 mg protein), and 10 mM L-valine. The addition to the assay mixture of L-glutamine (5 and 10 mM), ammonia (0.5 and 2 mM), L-glutamate (10 and 40 mM) and L-aspartate (5 and 10 mM) is indicated in Table 3. The enzyme reaction was initiated by addition of cell extracts and was terminated after a 30-min incubation at 23°C by addition of 50 μl of 1.5 M $HClO_4$. The acidified medium was neutralized with 20 μl of 2 M K_2CO_3 and then mixed with 1 ml of 10 mM HEPES (pH 5.5). L-[^{14}C]citrulline was separated from L-[^{14}C]arginine *via* Dowex 50W-X8 resin (Na^+ form), and the radioactivity of L-[^{14}C]citrulline was measured by a Packard 1900 liquid scintillation counter. Blanks without cell extracts were run and were subtracted from the sample values. It was established in preliminary experiments that the enzyme assay was linear with time and the amount of cell protein used. Protein in cell extracts was determined by a modified Lowry procedure using BSA as a standard (Markwell *et al.*, 1978).

NO synthesis from L-arginine occurs inside endothelial cells, and thus intracellular concentrations of L-glutamine and its metabolites, but not their plasma concentrations, should be used to determine the effect of L-glutamine and its metabolites on NO synthase activity. The concentrations of L-glutamine and its metabolites used in this study were based on the intracellular concentrations of L-glutamine and its metabolites in bovine venular EC, which were calculated on the basis of our HPLC analysis of amino acids (nmol/10^6 cells) (Table 2) and endothelial cell volume of 0.462 $\pm$ 0.043 μl/10^6 cells (n = 6) measured with ^3H_2O as previously described (Wu and Flynn 1995). For example, in the absence of vasodilators, intracellular concentrations of L-glutamine were calculated to be 0.28, 5.2 and 7.7 mM, respectively, in the presence of 0, 0.5 and 2 mM L-glutamine. Similarly, in the absence of vasodilators, intracellular concentrations of L-glutamate (the major product of L-glutamine metabolism in endothelial cells) were calculated to be 19.8, 32.0 and 46.7 mM, respectively. Therefore, 0, 5 and 10 mM L-glutamine and 10 and 40 mM L-glutamate were used in the NO synthase assay.

Uptake of L-arginine by bovine venular EC. The uptake of L-arginine by venular EC was measured at 37°C using L-[U-^{14}C]arginine, as previously described (Wu and Meininger, 1995). Briefly, the oxygenated (95% O_2/5% CO_2) KHB medium (final volume of 0.2 ml) contained 5 mM glucose and 0.1, 0.4 or 1.0 mM L-[U-^{14}C]arginine (0.05 μCi/ml), with or without L-glutamine (0.5 and 2 mM). L-Arginine transport was initiated by addition of 2×10^6 cells and was terminated in 5 min by addition of 0.2 ml of ice-cold 10 mM L-

arginine containing 0.05 μCi D-[2-^{3}H]mannitol as an extracellular marker. The solution was immediately transferred to a 1.6-ml microcentrifuge tube, which contained 0.7 ml of a mixture of bromododecane and dodecane (20:1, v/v) overlaid on 0.2 ml of 1.5 M $HClO_4$. Cells were separated from the medium through the oil layer into the acid layer by centrifugation in a microcentrifuge (12,000 $\times$ *g*, 1 min). After washing the oil layer three times with KHB buffer containing no radioisotopes, the oil layer was removed, and the acid layer was used for the measurement of ^{3}H and ^{14}C radioactivity *via* a dual-channel counting program in a Packard 1900 liquid scintillation counter. A very small amount of ^{3}H radioactivity was present in the acid layer, which was used to correct for contamination by the incubation medium. The specific radioactivity of L-[^{14}C]arginine in the incubation medium was used to calculate L-arginine uptake. It was demonstrated in preliminary experiments that L-[^{14}C]arginine uptake by bovine venular EC was linear over a 5-min incubation period.

Effect of L-glutamine on NO synthesis in bovine aortic EC cultured in the presence or absence of A_{23187}. Bovine aortic EC were prepared as previously described (Wu and Meininger 1993). Cells were cultured in DMEM supplemented with ECGF (100 μg/ml), 2 mM L-glutamine, 0.4 mM L-arginine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (Fungizone) (PSF). After the cells neared confluence, the ECGF was replaced with 20% FBS. Cell lines were passaged by trypsinization in DPBS containing 0.25% trypsin and 0.02% EDTA and used at passages 9–15. For the measurement of NO synthesis, bovine aortic EC were cultured at 37°C for 24 h in 0.7 ml of DMEM containing 0.4 mM L-arginine or 0 or 2 mM L-glutamine in the presence of 0 or 1 μM A_{23187}, as described above. At the end of the 24-h culture period, the conditioned media were used for the determination of nitrite and nitrate by the Griess reagent, with $NaNO_2$ and $NaNO_3$ as standards.

Statistical analyses. Results were statistically analyzed by two-way or one-way ANOVA, with the Student-Newman-Keuls multiple range test to identify significance among means (Steel and Torrie, 1980), as indicated in the tables. Probability values of less than .05 were taken to indicate statistical significance.

Results

NO synthesis in bovine venular EC. Table 1 summarizes the effect of 0.5 and 2 mM L-glutamine on NO synthesis from L-arginine in venular EC. Under the culture conditions used, nitrate was the predominant end product of NO, compared with nitrite. A_{23187} (1 μM), 1 μM bradykinin and 10 μM substance P increased ($P < .05$) NO synthesis by 25% to 40%. L-Glutamine (0.5 and 2 mM) inhibited ($P < .05$) NO synthesis by 22% to 44%, in a concentration-dependent manner, regardless of the presence or absence of A_{23187}, bradykinin or substance P. Increasing medium L-glutamine from 2 to 4 mM decreased ($P < .05$) NO synthesis in bovine venular EC by 23% (data not shown).

Intracellular concentrations of amino acids in bovine venular EC. The intracellular concentrations of L-arginine, L-glutamine and L-glutamine metabolites (L-aspartate, L-glutamate and L-alanine) are shown in table 2. In the absence of L-glutamine from the culture medium, EC were remarkably depleted of this amino acid. Increasing extracellular L-glutamine concentrations from 0 to 2 mM increased ($P < .05$) intracellular concentration of L-glutamine, L-aspartate and L-glutamate in EC in a concentration-dependent manner. L-Glutamine had no effect ($P > .05$) on intracellular concentrations of L-arginine, L-alanine and other amino acids (data not shown). In the presence of 0.5 and 2 mM L-glutamine, 1 μM A_{23187}, 1 μM bradykinin and 10 μM substance P slightly increased ($P < .05$) intracellular concentrations of L-glutamate by 15% to 22%.

NO synthase activity in and arginine uptake by bovine venular EC. L-Glutamine and its metabolites (L-glutamate, NH_4^+ and L-aspartate) had no effect on NO synthase activity (table 3). The uptake of L-arginine increased ($P < .05$) with increasing extracellular concentrations (0.1 to 1.0 mM) of L-arginine (table 4). L-Glutamine (0.5 and 2 mM) had no effect ($P > .05$) on L-arginine uptake in the presence of 0.1, 0.4 and 1 mM L-arginine.

Effect of L-glutamine on NO synthesis by bovine aortic EC. L-Glutamine (2 mM) decreased ($P < .05$) NO synthesis from L-arginine by bovine aortic EC in the presence or absence of 1 μM A_{23187} (table 5). The rates of NO synthesis in bovine aortic and venular EC were similar.

TABLE 1

Production of nitrite and nitrate in bovine venular endothelial cells

Bovine venular EC were cultured at 37°C for 24 h in the presence of phenol red- and serum-free DMEM containing 0.4 mM L-arginine. L-Glutamine, bradykinin, A_{23187} and substance P were added to the culture medium as indicated. The medium was used for analysis of nitrite and nitrate. Data are mean ± S.E.M., $n = 6$, and are analyzed by two-way ANOVA.

Addition to culture medium		Nitrite	Nitrate	Nitrite + Nitrate
L-Gln (mM)	Vasodilator			
			pmol/10^6 cells/h	
0	None	93 ± 11^a	318 ± 30^a	411 ± 44^a
0.5	None	73 ± 10^b	245 ± 27^b	318 ± 34^b
2	None	56 ± 6^c	174 ± 14^c	231 ± 18^c
0	1 μM A_{23187}	125 ± 11^{a,*}	419 ± 34^{a,*}	543 ± 43^{a,*}
0.5	1 μM A_{23187}	101 ± 8^{b,*}	305 ± 32^{b,*}	407 ± 38^{b,*}
2	1 μM A_{23187}	83 ± 8^{c,*}	234 ± 21^{c,*}	317 ± 26^{c,*}
0	1 μM Bradykinin	132 ± 10^{a,*}	436 ± 36^{a,*}	567 ± 29^{a,*}
0.5	1 μM Bradykinin	105 ± 8^{b,*}	313 ± 28^{b,*}	419 ± 32^{b,*}
2	1 μM Bradykinin	85 ± 8^{c,*}	240 ± 22^{c,*}	324 ± 25^{c,*}
0	10 μM Substance P	120 ± 9^{a,*}	408 ± 33^{a,*}	529 ± 38^{a,*}
0.5	10 μM Substance P	96 ± 9^{b,*}	301 ± 33^{b,*}	396 ± 35^{b,*}
2	10 μM Substance P	79 ± 7^{c,*}	229 ± 19^{c,*}	308 ± 23^{c,*}

* $P < .05$; different from the corresponding group without the addition of vasodilators (None group).
$^{a-c}$ $P < .05$: means that have different superscripts within each vasodilator treatment are different.

TABLE 2

Intracellular concentrations of amino acids in bovine venular endothelial cells

Bovine venular EC were cultured at 37°C for 24 h in the presence of phenol red- and serum-free DMEM containing 0.4 mM L-arginine. L-Glutamine, bradykinin, A_{23187} and substance P were added to the culture medium as indicated. The medium was used for analysis of amino acids. Data are mean ± S.E.M., $n = 6$, and are analyzed by two-way ANOVA.

Addition to Culture Medium		L-Asp	L-Glu	L-Gln	L-Arg	L-Ala
L-Gln (mM)	Vasodilator					
				nmol/10⁶ cells		
0	None	2.35 ± 0.26[c]	8.71 ± 0.76[c]	0.13 ± 0.02[c]	0.51 ± 0.05	2.21 ± 0.19
0.5	None	3.41 ± 0.37[b]	14.8 ± 1.20[b]	2.40 ± 0.32[b]	0.47 ± 0.04	2.09 ± 0.16
2	None	4.70 ± 0.41[a]	21.6 ± 0.98[a]	3.56 ± 0.40[a]	0.48 ± 0.05	2.16 ± 0.17
0	1 μM A_{23187}	2.26 ± 0.24[c]	8.86 ± 0.95[c]	0.14 ± 0.02[c]	0.48 ± 0.05	2.28 ± 0.21
0.5	1 μM A_{23187}	3.68 ± 0.43[b]	18.1 ± 1.25[b,*]	2.50 ± 0.31[b]	0.51 ± 0.06	2.21 ± 0.19
2	1 μM A_{23187}	5.11 ± 0.57[a]	25.2 ± 1.26[a,*]	3.48 ± 0.45[a]	0.49 ± 0.05	2.28 ± 0.24
0	1 μM Bradykinin	2.19 ± 0.28[c]	9.14 ± 0.82[c]	0.14 ± 0.02[c]	0.49 ± 0.04	2.16 ± 0.23
0.5	1 μM Bradykinin	3.72 ± 0.41[b]	17.2 ± 1.33[b,*]	2.28 ± 0.34[b]	0.49 ± 0.05	2.25 ± 0.20
2	1 μM Bradykinin	5.03 ± 0.55[a]	24.9 ± 1.13[a,*]	3.42 ± 0.43[a]	0.47 ± 0.04	2.31 ± 0.19
0	10 μM Substance P	2.30 ± 0.25[c]	9.07 ± 0.86[c]	0.14 ± 0.02[c]	0.52 ± 0.04	2.09 ± 0.24
0.5	10 μM Substance P	3.55 ± 0.40[b]	17.6 ± 1.30[b,*]	2.37 ± 0.29[b]	0.50 ± 0.04	2.16 ± 0.22
2	10 μM Substance P	5.08 ± 0.54[a]	24.7 ± 1.44[a,*]	3.51 ± 0.39[a]	0.46 ± 0.06	2.07 ± 0.25

* $P < .05$: different from the corresponding group without the addition of vasodilators (None group).
[a-c] $P < .05$: means that have different superscripts within each vasodilator treatment are different.

TABLE 3

NO synthase activity in bovine venular endothelial cells

The enzyme activities were measured as described in the text. Data are mean ± S.E.M., $n = 5$, and are analyzed by one-way ANOVA. The addition of L-glutamate, L-glutamine, NH_4Cl or L-aspartate had no effect ($P > .05$) on NO synthase activity.

Addition to Assay Mixture		NO synthase activity (pmol/mg protein/min)
L-Glutamine	0 mM	154 ± 18
	5 mM	139 ± 24
	10 mM	146 ± 20
L-Glutamate	10 mM	143 ± 21
	40 mM	150 ± 23
NH_4Cl	0.5 mM	141 ± 23
	2 mM	137 ± 25
L-Aspartate	5 mM	128 ± 26
	10 mM	140 ± 29

TABLE 4

Uptake of L-arginine by bovine venular endothelial cells

Cultured bovine venular endothelial cells were washed three times with 10 ml of KHB buffer and then incubated in 0.2 ml of KHB buffer (pH 7.4) at 37°C for 5 min. The incubation medium contained 0.1, 0.4 and 1.0 mM L-[U-^{14}C]arginine (2×10^6 dpm/0.2 ml). Data are mean ± S.E.M., $n = 5$, and are analyzed by two-way ANOVA.

Medium L-Glutamine (mM)	L-Arginine Concentrations in Medium (mM)		
	0.1	0.4	1
	nmol/5 min/10⁶ cells		
0	0.91 ± 0.08[c]	3.48 ± 0.42[b]	7.46 ± 0.81[a]
0.5	0.87 ± 0.09[c]	3.26 ± 0.44[b]	7.73 ± 0.92[a]
2	0.93 ± 0.11[c]	3.57 ± 0.48[b]	7.85 ± 0.96[a]

[a-c] $P < .05$: means that have different superscripts within a row are different.

Discussion

This is the first report of NO synthesis from L-arginine in microvascular venular EC. The results of the present study demonstrate that A_{23187}, bradykinin and substance P increased NO synthesis from L-arginine in bovine venular EC (table 1), as previously reported for bovine and porcine aortic EC (Arnal *et al.*, 1995; Bugle *et al.*, 1991; Gooch and Frangos, 1996; Ziche *et al.*, 1994). A_{23187} is a Ca^{++} ionophore and increases the flux of Ca^{++} into EC, whereas bradykinin and substance P cause vasodilation *via* receptor-mediated mechanisms (Gooch and Frangos, 1996; Ziche *et al.*, 1994). A new, important finding from this study is that L-glutamine inhibited NO synthesis from L-arginine by bovine EC in a concentration-dependent manner in the presence or absence of A_{23187}, bradykinin or substance P (table 1). Our results are consistent with the previous findings that 1) L-glutamine decreased NO synthesis in bovine aortic EC stimulated by ADP (Hecker *et al.*, 1990a,b) or bradykinin (Arnal *et al.*, 1995) and in intact rabbit aorta (Swierkosz *et al.*, 1990) and 2) L-glutamine inhibited porcine cerebral neurogenic vasodilation (Lee *et al.*, 1996). However, our results are in contrast to the recent report that L-glutamine increased NO synthesis in bovine aortic EC stimulated by A_{23187} (Arnal *et al.*, 1995). The reason for this discrepancy is not known at present, but it is unlikely to be due to the use of EC prepared from different vessels (venular *vs.* aortic), because we also found that L-glutamine (2 mM) decreased NO synthesis from L-arginine in bovine aortic EC cultured in the presence or absence of 1 μM A_{23187} (table 5).

The mechanism whereby L-glutamine inhibited NO synthesis from L-arginine in EC remains unknown. L-Glutamine is extensively metabolized to ammonia, L-glutamate and L-aspartate in bovine venular EC (our unpublished data). This is consistent with high activities of glutaminase and aspartate aminotransferase in EC (Leighton *et al.*, 1987) and with our result that the addition of L-glutamine to the culture medium markedly increased intracellular concentrations of L-glutamate and L-aspartate in a concentration-dependent manner (table 2). Although the addition of L-glutamine to the culture medium markedly increased intracellular concentrations of L-glutamine (table 2), neither L-glutamine nor its glutaminase metabolites (ammonia, L-glutamate and L-aspartate) had any effect on NO synthase activity in bovine venular EC (table 3). To the best of our knowledge, this study demonstrates for the first time that endothelial glutamine metabolites (L-glutamate, L-aspartate and ammonia) had no direct effect on NO synthase activity in venular EC. Our data suggest that neither L-glutamine nor its glutaminase products directly regulates endothelial NO synthase activity. Pre-

TABLE 5

Production of nitrite and nitrate in bovine aortic endothelial cells

Bovine venular EC were cultured at 37°C for 24 h in the presence of phenol red- and serum-free DMEM containing 0.4 mM L-arginine. L-Glutamine and A_{23187} were added to the culture medium as indicated. The medium was used for analysis of nitrite and nitrate. Data are mean ± S.E.M., $n = 6$, and are analyzed by two-way ANOVA.

Addition to Culture Medium		Nitrite	Nitrate	Nitrite + Nitrate
L-Gln (mM)	Vasodilator			
			pmol/10⁶ cells/h	
0	None	86 ± 7[a]	287 ± 25[a]	374 ± 31[a]
2	None	43 ± 5[b]	166 ± 12[b]	209 ± 14[b]
0	1 μM A_{23187}	118 ± 9[a,*]	402 ± 38[a,*]	520 ± 36[a,*]
2	1 μM A_{23187}	80 ± 7[b,*]	225 ± 24[b,*]	304 ± 28[b,*]

* $P < .05$: different from the corresponding group without A_{23187} (None group).
[a–b] $P < .05$: means that have different superscripts within each vasodilator treatment are different.

vious studies have shown that neither ammonia nor L-glutamate inhibited NO synthesis from L-arginine in cultured EC (Hecker *et al.*, 1990) or cerebral neurogenic vasodilation (Lee *et al.*, 1996). These results, however, do not necessarily suggest that the metabolism of L-glutamine is not required for an inhibition of NO synthesis, because the effect of L-glutamine metabolites produced by glutamine transaminase, amidotransferase and other glutamine-utilizing enzymes (Sayeski and Kudlow, 1996; Wu *et al.*, 1991) was not determined in the present study. Other possible mechanisms for inhibition of NO synthesis by L-glutamine may involve 1) synthesis of cofactors of NO synthase, 2) Ca^{++} uptake by EC and 3) change in intracellular pH due to ammoniagenesis from L-glutamine.

The effect of L-glutamine on intracellular concentrations of amino acids with regard to NO synthesis in EC deserves comment. L-Glutamine had no effect on L-arginine uptake (table 4) or on intracellular concentrations of L-arginine in EC (table 2). Because of the intracellular compartmentalization of L-arginine metabolism in mammalian cells (Cynober *et al.*, 1995), this result should not be interpreted to mean that L-arginine concentration at the site of NO synthesis was unaltered. When endothelial cells were lysed for amino acid analysis, only the total intracellular amino acid concentrations were determined in the present study, as in other studies (*e.g.*, Sessa *et al.*, 1990; Hecker *et al.*, 1990b). At present, there are no techniques that allow for the determination of amino acid concentrations in different organelles or compartments of the cell. The concentration of L-arginine in a particular compartment or at the site of NO synthesis may be very much different from the total intracellular L-arginine concentration in the endothelial cell. As suggested in other studies, there may be intracellular sequestration or compartmentalization of arginine in endothelial cells (Arnal *et al.*, 1995). This may help to explain the paradox that although the K_m value of purified endothelial NO synthase for L-arginine is 2.9 μM (Pollock *et al.*, 1991), which is substantially higher than intracellular L-arginine concentrations in cultured endothelial cells (0.1–1 mM) (table 2) (Hecker *et al.*, 1990b), NO synthesis by endothelial cells increased with increasing extracellular L-arginine concentrations from 0 to 10 mM in the presence of 0.6 mM L-glutamine (Arnal *et al.*, 1995). Whether L-glutamine decreased L-arginine concentration in a particular compartment of endothelial cells is not known. L-Glutamine inhibits the synthesis of L-arginine from L-citrulline (a coproduct of NO synthase) in endothelial cells (Sessa *et al.*, 1990; Wu and Meininger, 1993), as reported for perivascular nerves of cerebral artery (Chen and Lee, 1995). It is possible that NO synthase and the enzymes for the synthesis of arginine from citrulline (argininosuccinate synthase and lyase) are colocalized in a particulate compartment within the endothelial cell, as reported for the brain (Vincent, 1994). In light of the recent evidence for an inhibition of argininosuccinate synthase activity by L-glutamine in EC (Su and Block, 1995), it is conceivable that L-glutamine decreases the recycling of L-citrulline into L-arginine and thus reduces the local L-arginine concentration at the site of NO synthesis in EC.

The regulation of NO synthesis by L-glutamine may have pharmacologic and therapeutic implications. For example, the hypotension in sepsis, trauma, infection and inflammation has been reported to result from increased NO synthesis in vascular EC in both laboratory animals and humans (Nava *et al.*, 1991; Petros *et al.*, 1991; Thiemermann and Vane, 1990). Interestingly, plasma L-glutamine concentrations are markedly decreased under such clinical conditions (Parry-Billings *et al.*, 1990) because of increased utilization of L-glutamine by activated lymphocytes and macrophages (Newsholme *et al.*, 1987). The findings from this and other studies have shown that L-glutamine markedly inhibits the activity of the arginine-citrulline cycle in endothelial cells, with the net result of decreased NO synthesis (Hecker *et al.*, 1990a; Sessa *et al.*, 1990; Wu and Meininger, 1993) (table 1). It can be surmised that a decrease in plasma concentration of L-glutamine may lead to increased NO generation from L-arginine *in vivo* by relieving an inhibiting effect of L-glutamine on the synthesis of NO from L-arginine in EC. This may partially account for increased NO synthesis in EC and subsequent hypotension in septic shock. An increase in extracellular L-glutamine concentration may attenuate the increase in endothelial NO synthesis induced by substance P and other mediators of inflammation (Ralevic *et al.*, 1995), thereby increasing blood pressure, as recently reported for the rabbit pulmonary artery (Xu and Pearl, 1994). Because L-glutamine is the most abundant free amino acid in the body (*e.g.*, in plasma and skeletal muscle) (Krebs, 1980) and yet is the most susceptible to depletion under such clinical conditions as sepsis, trauma, infection and inflammation (Parry-Billings *et al.*, 1990), L-glutamine may play an important role in regulating NO synthesis and thus the function of the cardiovascular system.

In summary, L-glutamine decreased the synthesis of NO from L-arginine in bovine venular EC *via* an unknown mechanism that is unlikely to involve an inhibition of L-arginine

uptake by EC or a direct effect of glutamine or its glutaminase metabolites on NO synthase activity. L-Glutamine may play an important role in regulating endothelial NO synthesis, which may have pharmacologic and therapeutic implications.

Acknowledgments

The authors thank Tony E. Haynes and Wene Yan for their excellent technical assistance.

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Send reprint requests to: Cynthia J. Meininger, Department of Medical Physiology, Texas A&M University, College Station, TX 77843-1114.