

# Interactions between L-Arginine and L-Glutamine Change Endothelial NO Production

## An Effect Independent of NO Synthase Substrate Availability

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### Abstract

The effect of extracellular L-arginine and L-glutamine on nitric oxide (NO) release was studied in cultured bovine aortic endothelial cells and in rabbit aortic rings. Increasing L-arginine (0.01 to 10 mM) did not alter NO release from cultured endothelial cells or modify endothelium-dependent relaxation to acetylcholine in isolated vessels. L-Glutamine (0.6 and 2 mM) inhibited NO release from cultured cells (in response to bradykinin) and from aortic rings (in response to acetylcholine or ADP). L-Arginine (0.1–10 mM) dose-dependently reversed the L-glutamine inhibition of receptor-stimulated NO release in both models. In contrast to its inhibitory response to receptor-mediated stimuli, glutamine alone slightly potentiated NO release in both models when the calcium ionophore, A23187, was added. Furthermore, cultured cells incubated with L-arginine (0.01–10 mM), in the presence or absence of glutamine, released similar amounts of NO in response to A23187. L-Glutamine did not affect intracellular L-arginine levels. Neither D-glutamine nor D-arginine affected NO release or endothelium-dependent vascular relaxation. L-Glutamine had no effect on the activity of endothelial NOS assessed by L-arginine to L-citrulline conversion. These findings show that in the absence of L-glutamine, manipulating intracellular L-arginine levels over a wide range does not affect NO release. L-Glutamine in concentrations circulating in vivo may tonically inhibit receptor-mediated NO release by interfering with signal transduction. One mechanism by which L-arginine may enhance NO release is via reversal of the inhibitory effect of L-glutamine, but apparently independently of enhancing NO synthase substrate. (*J. Clin. Invest.* 1995; 95:2565–2572.)

**Key words:** nitric oxide synthase • endothelial cell • arginine • glutamine

### Introduction

Over the past several years, a number of investigators have reported that the administration of L-arginine, either intra-arteri-

ally, intravenously, or by inclusion in the diet improves endothelium-dependent vascular relaxation and release of nitric oxide (NO)<sup>1</sup> (1–6). In most instances, the beneficial effect of L-arginine has been observed in disease states such as hypertension or hypercholesterolemia, although a similar effect has been demonstrated in normal subjects (5, 6). It has been assumed that the enhanced vasodilation is due to provision of intracellular substrate for the endothelial enzyme, NO synthase. There are reasons, however, to suspect that exogenous L-arginine may not enhance NO release by the constitutively expressed endothelial isoform of NO synthase. The substrate concentration at which the reaction velocity is half maximal ( $K_m$ ) is 2.9  $\mu$ M (7), and the intracellular level of L-arginine in vivo is substantially higher (0.8–2 mM). In cultured cells, intracellular L-arginine is 30–800-fold higher than the  $K_m$  for NO synthase (i.e., 0.1–0.8 mM) (8, 9). This discrepancy has been termed the “arginine paradox” (10). A possible explanation for this paradox is that in disease states, endothelial cell L-arginine may be low. A second explanation is that the “functional”  $K_m$  in intact cells is higher than that of the purified enzyme. There also may be sequestration or compartmentalization of L-arginine within the endothelial cell, so that variation of the L-arginine concentration in the vicinity of the enzyme is relatively low. A third possibility is that an inhibitor of the enzyme may be present, causing an increased requirement for L-arginine (11).

In considering variability in intracellular L-arginine, the issue is further complicated because the endothelial cell can recycle L-arginine to L-citrulline when NO production is sustained (9, 12). Hecker et al. (9) and Sessa et al. (12) have demonstrated that formation of L-arginine from L-citrulline is inhibited by concentrations of L-glutamine below those encountered in either tissue culture media or in plasma. They concluded that intracellular arginine would become rate-limiting for the formation of NO when L-glutamine was present. Moreover, they found that L-glutamine inhibited the release of endothelium-derived NO in response to ADP (9, 12). However, the levels of intracellular L-arginine in these experiments were still in excess of the  $K_m$  for NO synthase (7). This raises the possibility that L-glutamine affects endothelial release of NO by mechanisms other than depletion of intracellular L-arginine as a substrate for NO synthase.

The present experiments evaluated relationships among L-arginine, L-glutamine, and endothelial cell production of NO. We measured endothelial cell production of NO while manipulating both extracellular and intracellular concentrations of L-

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1. Abbreviations used in this paper: BAEC, bovine aortic endothelial cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KH, Krebs/Hepes; NO, nitric oxide.

arginine and L-glutamine. Parallel experiments using isolated segments of rabbit aorta examined endothelium-dependent vascular relaxation as a reflection of endothelial cell NO release. Finally, we examined the effect of L-arginine and L-glutamine on NO synthase mRNA and protein expression.

## Methods

**Cell culture and materials.** Bovine aortic endothelial cells (BAEC) were obtained as described (13, 14) and cultured in M199 supplemented with L-glutamine and amino acids (final concentrations of L-glutamine and L-arginine were 2.65 mM and 432  $\mu$ M, respectively) and 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT) at 37°C in 20% O<sub>2</sub>/5% CO<sub>2</sub>-containing humidified atmosphere. The cells were between the fifth and eight passages. All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) except when specified. Protein concentrations were determined using the Coomassie brilliant blue G-250 method, with bovine serum albumin used for standards.

We have shown that NO synthase expression is increased in pre-confluent and just confluent cells compared with postconfluent cells (15). To ensure equivalent amounts of NO synthase were present from one experiment to the next, the cells were always split at a ratio of 1:6 and studied 4–6 d after reaching confluence.

On the day of the experiment, the medium was replaced by MEM Selectamine (Gibco Laboratories, Grand Island, NY) containing all amino acids except L-arginine, L-glutamine, and L-citrulline. Three sets of experiments were performed: cells were grown in MEM Selectamine containing various concentrations of either L-arginine, L-glutamine, or various concentrations of L-arginine and 0.6 mM L-glutamine. The cells were incubated for 3 h, subsequently washed twice with Krebs/Hepes (KH) buffer (composition [mM]: NaCl 99.0, KCl 4.69, CaCl<sub>2</sub> 1.87, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, K<sub>2</sub>HPO<sub>4</sub> 1.03, Na-Hepes 20, D-glucose 11.1) and incubated for 1 h in KH buffer containing the same concentration of L-arginine and L-glutamine.

**Measurement of endothelial cell NO release.** The production of NO was evaluated by measuring nitrite (NO<sub>2</sub><sup>-</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>), the stable degradation products of NO. BAEC were grown in 6-well dishes. The cells were washed three times with modified KH buffer and then incubated in 1 ml of the KH buffer. NO release was stimulated by adding 3  $\mu$ M calcium ionophore A23187 or bradykinin 0.1  $\mu$ M at 37°C for 1 h. 100  $\mu$ l of the buffer was injected into a reflux chamber containing vanadium III dissolved in HCl at > 85°C. These conditions reduce both nitrite and nitrate stoichiometrically to NO (16). The released NO was purged with nitrogen gas into the reaction chamber of a chemiluminescence NO analyzer. The analyzer was calibrated daily using nitrate standards, and the amount of NO released was normalized to the cell number.

**Quantification of intracellular amino acids by HPLC.** Cultured endothelial cells were washed with phosphate-buffered saline five times before ice-cold 6% perchloric acid was added. The cells and debris were collected with a rubber policeman and remaining cells were lysed by two cycles of freezing and thawing. After standing on ice for 30 min, the protein and cellular debris were removed by centrifugation (100,000 g for 5 min), and the supernatant was neutralized by Mops/NaOH (0.5 M/3 M) solution. Amino acids were quantified by HPLC after derivatization with 50  $\mu$ l of *o*-phthalaldehyde (Pierce, Rockford, IL) (17). An Ultrasphere ODS column (7.5 cm  $\times$  4.6 cm; Beckman Instruments, Inc., Fullerton, CA) was used to resolve L-arginine and L-glutamine using a linear gradient program with solvent A (0.5% tetrahydrofuran in 12.5 mM sodium acetate, pH 7.2) and solvent B (35% methanol, 15% acetonitrile in 12.5 mM sodium acetate, pH 7.2). The column was equilibrated with 20% solvent B for 15 min before the sample was injected and solvent B was then increased to 25% over 10 min and to 34% over the next 10 min. The column was isocratically washed with 34% solvent B for 8 min and then 100% solvent B for 10 min. The flow rate was held constant at 1.4 ml/min. A fluorescence detector (Shimadzu Corp., Tokyo, Japan) was used to detect the derivatized amino acids with a

sensitivity range of 0.1 pmol. L-Arginine or L-glutamine was eluted at 12.9 or 25.5 min, respectively; amino acid standards were used to quantify the amino acids before their intracellular concentrations were calculated using standard calibration curves and assuming an endothelial cell volume of 0.5 pl (9).

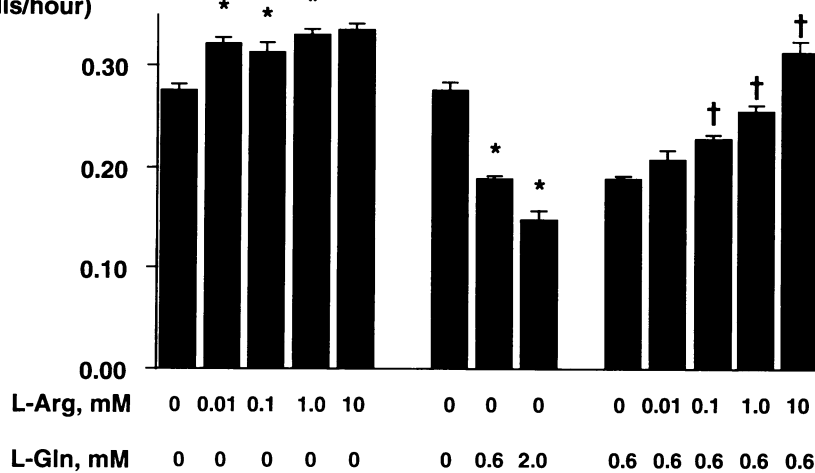
**Measurement of NO synthase activity by conversion of L-[U-<sup>14</sup>C]arginine to L-[<sup>14</sup>C]citrulline.** To study the effect of L-glutamine on the maximal activity of endothelial cell NO synthase, the conversion of L-[U-<sup>14</sup>C]arginine to L-[<sup>14</sup>C]citrulline was used as reported previously (18) with some modifications. Endothelial cells from 100-cm<sup>2</sup> dishes were washed three times with cold phosphate-buffered saline, scraped with a rubber policeman, and collected in centrifuge tubes and spun at 500 g for 5 min. The cells were then homogenized with a Dounce homogenizer in 1 ml of homogenization buffer (50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM EGTA, pH 7.5) containing the following protease inhibitors: 1  $\mu$ M pepstatin A, 2  $\mu$ M leupeptin, 1  $\mu$ M bestatin, 1 mM phenylmethylsulfonyl fluoride, and 0.1%  $\beta$ -mercaptoethanol. The homogenates were centrifuged at 100,000 g for 60 min, and the particulate fraction was washed two times with the homogenization buffer and resuspended in 100  $\mu$ l of homogenization buffer containing 20 mM 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). Each sample (100  $\mu$ l containing ~ 200  $\mu$ g of protein) was added to 37°C 50 mM Tris-HCl/0.1 mM EDTA/0.1 mM EGTA buffer, pH 7.5, containing 100 nM calmodulin, 2.5 mM CaCl<sub>2</sub>, 1 mM reduced NADPH, 3  $\mu$ M tetrahydrobiopterin, and 100  $\mu$ M L-arginine combined with L-[U-<sup>14</sup>C]-arginine (Amersham Corp., Arlington Heights, IL) (sp act, 304 mCi/mmol). The mixture also contained 1 mM L-citrulline to minimize conversion of the formed L-[<sup>14</sup>C]citrulline back to L-[<sup>14</sup>C]arginine (9). After a 15-min incubation period, the reaction was terminated by adding 1 ml of stop buffer (20 mM Hepes, pH 5.5, 2 mM EDTA, and 2 mM EGTA). The reaction mixture was applied to a 1-ml column containing Dowex AG 50WX-8 (Na<sup>+</sup> form; Bio-Rad Labs., Richmond, CA) resin that had been pre-equilibrated with the stop buffer. L-[<sup>14</sup>C]citrulline was eluted twice with 2 ml of stop buffer, and radioactivity was determined by liquid scintillation counting (Scintiverse II; Fisher Scientific Co., Pittsburgh, PA).

Separate experiments were performed to examine the effect of L-glutamine on the K<sub>m</sub> of the endothelial cell NO synthase. Because these experiments required several samples of the enzyme, we used a baculovirus-expressed endothelial cell NO synthase. 10  $\mu$ g of the enzyme was used in the above assay in the presence or absence of 2 mM L-glutamine. To assess specific activity at varying concentrations of L-arginine, experiments were performed in the presence of 1, 5, 10, 30, 50, and 100  $\mu$ M unlabeled L-arginine.

**Northern blotting.** Endothelial cells were grown in 60-cm<sup>2</sup> dishes and incubated in MEM Selectamine with or without L-glutamine or L-arginine for 24 h. The cells were then washed with 10 ml PBS twice and then lysed in guanidinium isothiocyanate. Total RNA was isolated using phenol extraction according to the method described by Chomczynski and Sacchi (19). The RNA (20  $\mu$ g) was size-fractionated on a 1.0% agarose/3% formaldehyde gel and subsequently transferred to a nitrocellulose membrane. Northern hybridizations were performed overnight using a [<sup>32</sup>P]dCTP (Amersham Corp.), random primed labeled, 2.1-kb cDNA fragment of bovine endothelial NO-synthase obtained by SstI (Gibco Laboratories) digestion of the full-length bovine NO-synthase cDNA (20). The hybridization solution contained 50% formamide, 100  $\mu$ g/ml sheared salmon sperm DNA in 5  $\times$  SSC, 5  $\times$  Denhardt's solution, 10% dextran sulfate, and 1% SDS at 42°C. The membranes were then washed twice with 2  $\times$  SSC and 1% SDS for 30 min at 55°C, and subsequently once with 0.2  $\times$  SSC and 0.1% SDS for 30 min at 55°C. In all studies, the nitrocellulose membranes were stripped and subsequently hybridized with cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control.

**Isolated vascular ring experiments.** For these experiments, New Zealand White rabbits (wt 3–5 kg) were killed by injection of pentobarbital into an ear vein and the thoracic aorta removed. Eight 5-mm ring segments of rabbit thoracic aorta were suspended in individual organ chambers filled with Krebs buffer (25 ml) of the following composition

**Bradykinin-stimulated  
NO<sub>x</sub> release  
(nmol/10<sup>6</sup> cells/hour)**



*Figure 1.* Effect of extracellular levels of L-arginine (L-Arg) and L-glutamine (L-Gln) on NO release in response to bradykinin. BAEC were incubated for 3 h in Selectamine medium and then 1 h in KH buffer, both of which contained the indicated L-arginine and L-glutamine concentrations. BAEC were stimulated by bradykinin 0.1 μM, and the nitrogen oxides (NO<sub>x</sub>) released in the medium were quantified by chemiluminescence. Data were analyzed using one-way ANOVA: \* *P* < 0.05 versus L-arginine 0 mM and L-glutamine 0 mM; † *P* < 0.05 versus L-arginine 0 mM and L-glutamine 0.6 mM. Two-way ANOVA taking into account 0 and 0.6 mM L-glutamine: effect of L-arginine: F-test 44, *P* < 0.0001; effect of L-glutamine: F-test 260, *P* < 0.0001; interaction: F-test 10, *P* < 0.0001.

(mM): NaCl 118.3, KCl 4.69, CaCl<sub>2</sub> 1.87, MgSO<sub>4</sub> 1.20, K<sub>2</sub>HPO<sub>4</sub> 1.03, NaHCO<sub>3</sub> 25.0, and glucose 11.1, pH 7.40. The solution was aerated continuously with 95% O<sub>2</sub>/5% CO<sub>2</sub> and maintained at 37°C. Care was taken not to injure the endothelium during preparation of the rings. Tension was recorded with a linear force transducer. Over a period of 1 h, the resting tension was gradually increased and the ring segment was frequently exposed to 80 mM KCl until the optimal tension for generating force during isometric contraction was reached. The vessels were left at this resting tension throughout the remainder of the study. To prevent the synthesis of prostaglandins, we performed all experiments in the presence of 10 mM indomethacin. The vessels were then precontracted with L-phenylephrine (0.5 mM). After a stable contraction plateau was reached, the rings were exposed cumulatively to either acetylcholine (1 nM to 3 mM), ADP (3 μM to 0.3 mM), or the calcium ionophore A23187 (3 nM to 1 μM) in the absence or presence of L-glutamine (0.6 and 2.0 mM) and/or L-arginine (0.1 to 10 mM).

*Statistical analysis.* The data are expressed as mean ± standard error. For studies of NO release from cultured cells, comparisons between different groups were made by one-way ANOVA, and a Fishers PLSD post-hoc test was used when differences were indicated. Two-way ANOVA was also used to examine potential interactions between L-arginine and L-glutamine. Studies of isolated vascular segments were analyzed by one-way ANOVA with Student-Newman-Keuls post-hoc test when differences were indicated. *P* values < 0.05 were considered significant.

## Results

### *Effect of extracellular L-arginine and L-glutamine on endothelial cell release of NO*

*Responses to bradykinin.* In the absence of either L-arginine or L-glutamine, the release of NO averaged 0.275 ± 0.007 nmol/10<sup>6</sup> cells/h (Fig. 1). This value increased to 0.321 ± 0.006 when as little as 0.01 mM L-arginine was added. As L-arginine was further increased by 10-, 100-, or 1,000-fold, NO release was unaffected. L-Glutamine, in concentrations of 0.6 and 2 mM, decreased the release of NO by 32 and 47%, respectively (Fig. 1). The plasma concentration of L-glutamine averages ~ 0.6 mM. We therefore examined the effect of L-arginine on NO release when this concentration of L-glutamine was present. In

the presence of 0.6 mM L-glutamine, addition of L-arginine caused a concentration-dependent increase in NO release. In the presence of 10 mM L-arginine, the effect of 0.6 mM L-glutamine was completely reversed. Thus, in contrast to the lack of effect of L-arginine on NO release in the absence of L-glutamine, increasing L-arginine concentrations from 0.01 to 10 mM rather markedly increased the release of NO in the presence of L-glutamine.

*Responses to the calcium ionophore A23187.* In the absence of either L-arginine or L-glutamine, the release of NO in response to A23187 averaged 0.91 ± 0.02 nmol NO/10<sup>6</sup> cells/h (Fig. 2). This was increased by 52% upon adding as little as 0.01 mM L-arginine. Higher concentrations of L-arginine did not increase the release of NO beyond the value measured with 0.01 mM. In contrast to the inhibitory effect of L-glutamine on the release of NO in cells stimulated by bradykinin, adding L-glutamine alone slightly increased (12%) NO release when the stimulus was A23187. Concentrations of L-arginine above 0.01 mM did not increase the release of NO in response to A23187 when L-glutamine was present (Fig. 2).

### *Effect of extracellular level of L-arginine on its intracellular concentration*

The effect of varying concentrations of L-arginine on intracellular L-arginine and L-glutamine after 1 h of stimulation with bradykinin is shown in Table I. In both the presence and absence of L-glutamine, increasing extracellular L-arginine produced a dose-dependent increase in intracellular L-arginine. The addition of 0.6 mM L-glutamine increased the intracellular L-glutamine by 10-fold but had no effect on the intracellular L-arginine concentrations.

Fig. 3 A shows the relationship between intracellular L-arginine and NO release in the presence or absence of L-glutamine. When intracellular levels of L-arginine are equal to or less than ~ 3 mM, it is clear that the presence of L-glutamine markedly inhibits NO release in response to bradykinin. Thus, for any given intracellular L-arginine concentration below 3

**A23187-stimulated  
NO<sub>x</sub> release  
(nmol/10<sup>6</sup> cells/hour)**

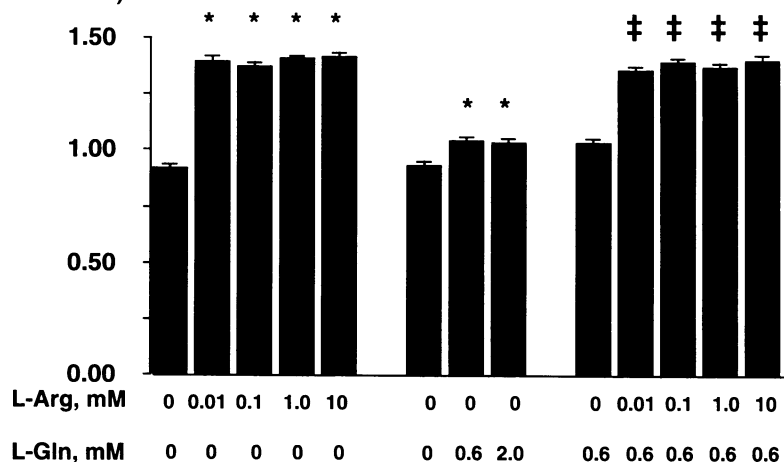


Figure 2. Effect of extracellular levels of L-arginine (L-Arg) and L-glutamine (L-Gln) on NO<sub>x</sub> release in response to calcium ionophore A23187. BAEC were incubated for 3 h in Selectamine medium and then 1 h in KH buffer, both of which contained the indicated L-arginine and L-glutamine concentrations. BAEC were stimulated by 3 μM A23187, and the NO<sub>x</sub> released in the medium were quantified by chemiluminescence. Data were analyzed using one-way ANOVA: \* *P* < 0.05 versus L-arginine 0 mM and L-glutamine 0 mM; † *P* < 0.05 versus L-arginine 0 mM and L-glutamine 0.6 mM. Further analysis using two-way ANOVA taking into account 0 and 0.6 mM L-glutamine indicated: effect of L-arginine: F-test 163, *P* < 0.0001; effect of L-glutamine: F-test 1,3, *P* = NS; interaction: F-test 1.8, *P* = NS.

mM, NO release was dramatically altered by addition of L-glutamine. Increasing the intracellular concentration of L-arginine to above 10 mM reversed the inhibitory effect of L-glutamine, but had no effect on the release of NO evoked by bradykinin in the absence of extracellular L-glutamine. Such an effect of L-glutamine was not observed when the calcium ionophore A23187 was used as the stimulus for NO release (Fig. 3 B).

*L-Arginine, L-glutamine, and endothelium-dependent vascular relaxations*

In isolated segments of rabbit aorta, addition of L-glutamine inhibited relaxation in response to acetylcholine (Fig. 4 A). The addition of increasing concentrations of L-arginine progressively reversed the inhibitory effects of 0.6 mM L-glutamine (Fig. 4 B). 10 mM L-arginine completely reversed the inhibition of acetylcholine-induced relaxation caused by 0.6 mM L-glutamine. In a similar fashion, relaxations to ADP were inhibited by ~ 25% when 0.6 mM L-glutamine was present (Fig. 5). This effect of L-glutamine was abolished by adding 10 mM L-arginine.

In contrast to the inhibition of relaxation in response to acetylcholine or ADP, L-glutamine slightly but significantly enhanced relaxation in response to the calcium ionophore A23187 (EC<sub>50</sub> shifted from -7.10±0.02 to -7.32±0.07, *P* < 0.05). The relaxation in response to A23187 was completely abolished by

the addition of the NO synthase antagonist, L-N-nitro arginine (L-NNA) (Fig. 6).

*D-Glutamine, D-arginine, and NO release and endothelium-dependent vascular relaxation*

Neither D-arginine nor D-glutamine changed NO release in response to bradykinin (Fig. 7 A). Furthermore, D-glutamine did not alter the endothelium-dependent vascular relaxation to acetylcholine (Fig. 7 B).

When the arginine antagonist, 1 mM N<sup>w</sup>-monomethyl-L-arginine (L-NMMA), was added to KH buffer containing 0.1 mM L-arginine and 0.6 mM L-glutamine, the inhibition of A23187- and bradykinin-stimulated NO release averaged 75±4 and 79±5% (*n* = 3 each). When 1 mM L-NMMA was added in both the medium (3-h preincubation) and subsequently in the KH buffer (1-h incubation), the inhibition of A23187- and bradykinin-stimulated NO release averaged 89±2 and 87±3% (*n* = 3 each). These findings confirm that the nitrogen oxides detected by this technique were likely due to conversion of L-arginine to NO by NO synthase.

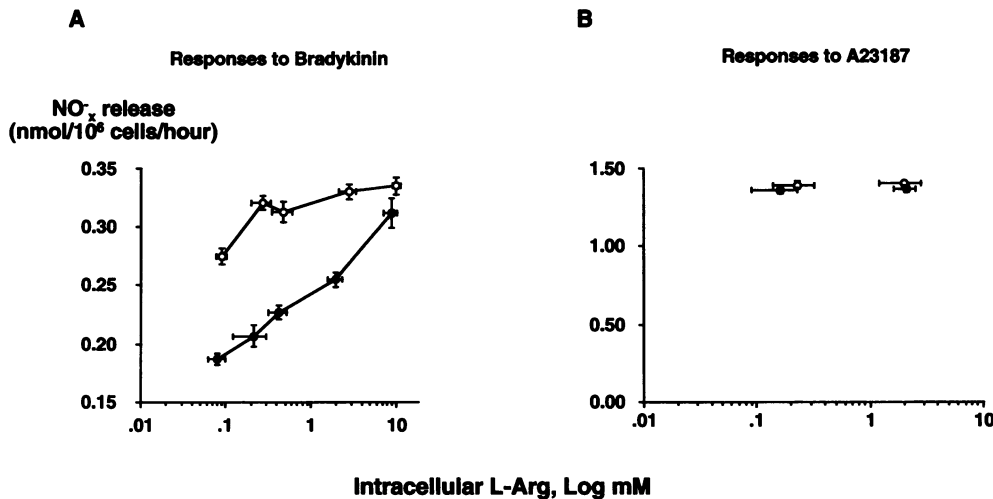
*Effects of L-glutamine and L-arginine on NO synthase activity and mRNA*

Endothelial cell NO synthase activity was assessed via the conversion of L-[<sup>14</sup>C]arginine to L-[<sup>14</sup>C]citrulline using membrane

Table 1. Effects of Extracellular L-Arginine with and without L-Glutamine on Intracellular L-Arginine and L-Glutamine Concentrations

	Extracellular L-arginine (mM) without L-glutamine					Extracellular L-arginine (mM) with 0.6 mM L-glutamine				
	0	0.01	0.1	1.0	10	0	0.01	0.1	1.0	10
L-Arginine	0.09±0.01	0.27±0.07*	0.48±0.13*	2.8±0.64*	10.3±1.1*	0.08±0.022	0.21±0.09*	0.42±0.1*	2.0±0.39*	8.99±1.6*
L-Glutamine	0.13±0.02	0.13±0.02	0.16±0.02	0.14±0.01	0.18±0.02	1.7±0.2 <sup>‡</sup>	1.2±0.2 <sup>‡</sup>	1.6±0.2 <sup>‡</sup>	1.9±0.3 <sup>‡</sup>	2.2±0.3 <sup>‡</sup>

Values in the table are intracellular L-arginine and L-glutamine concentrations in mM. Data are mean±SEM. \* *P* < 0.05 versus lower concentrations of extracellular L-arginine. <sup>‡</sup> *P* < 0.05 versus without L-glutamine.



**Figure 3.** (A) Correlation between intracellular concentration of L-arginine and NO<sub>x</sub> release from BAEC in presence (closed circles) or absence (open circles) of 0.6 mM L-glutamine in response to 0.1 μM bradykinin. (B) Correlation between intracellular concentration of L-arginine and NO<sub>x</sub> release from BAEC in presence (closed circles) or absence (open circles) of 0.6 mM L-glutamine in response to 3 μM A23187.

fractions (centrifugation at 100,000 g for 1 h) and a baculovirus-expressed endothelial cell NO synthase. The  $K_m$  of the enzyme derived from endothelial cells, determined by varying the amount of unlabeled L-arginine in the assay, was  $5.7 \pm 1.2 \mu\text{M}$  ( $n = 2$ ), a value similar to that reported previously (7). Similarly, the  $K_m$  of baculovirus-expressed enzyme was  $5 \mu\text{M}$  ( $n = 2$ ).

The addition of L-glutamine did not alter NO synthase activity in vitro, as assessed by the conversion of L-[<sup>14</sup>C]arginine to L-[<sup>14</sup>C]citrulline. Enzyme activity was  $301 \pm 4$  pmol/mg protein/min in the absence of L-glutamine and  $307 \pm 9$  pmol/mg protein/min in the presence of 2 mM L-glutamine (each  $n = 3$ ). Likewise, the  $K_m$  of the baculovirus-expressed enzyme was not altered by L-glutamine. Furthermore, incubating endothelial cells with L-glutamine (2 mM) and/or L-arginine (1 mM) for 4 h did not influence NO synthase activity subsequently measured in vitro. NO synthase activity was  $307 \pm 12$ ,  $314 \pm 13$ ,  $301 \pm 7$ , and  $308 \pm 18$  pmol/mg protein/min in control cells and after exposure to either L-glutamine, L-arginine, or both, respectively ( $n = 4$  in each group).

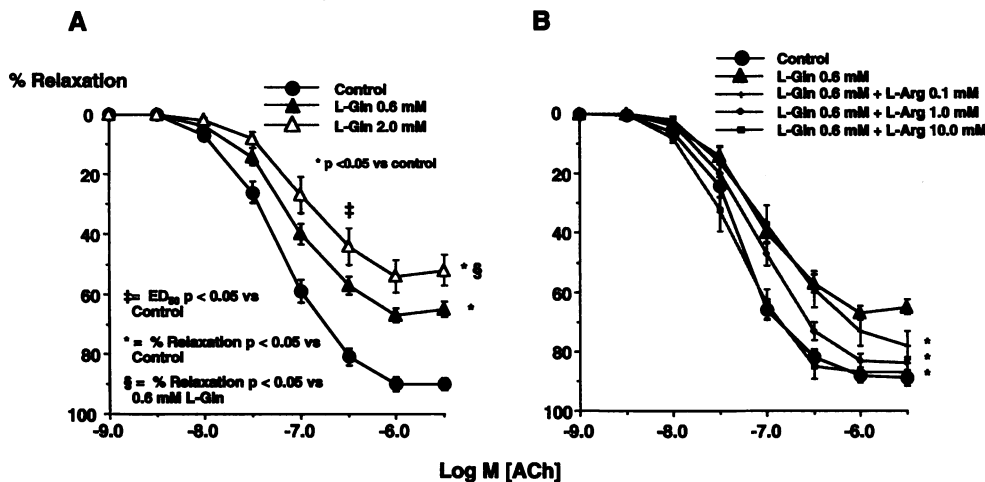
L-Arginine might also change the expression of NO synthase. This possibility was tested by incubating BAEC with or without extracellular L-glutamine or L-arginine for 24 h before quantifying NO synthase mRNA. Fig. 8 shows a representative

Northern blot analysis of NO-synthase mRNA levels of cells exposed to L-arginine 1 mM (A), L-glutamine 2 mM (G), both (AG), or none (C) of these amino acids. The amounts of NO-synthase and GAPDH transcripts were similar under these conditions.

## Discussion

The present experiments demonstrate that L-glutamine, in concentrations present in plasma (and standard tissue culture media), has different effects on the release of NO from BAEC depending on the stimulus used. When bradykinin was used, L-glutamine markedly reduced NO release. In contrast, when the calcium ionophore A23187 was used, L-glutamine slightly but significantly enhanced NO release. This discrepancy between receptor-mediated and receptor-independent stimuli was confirmed in isolated segments of rabbit aorta. In intact vascular segments, L-glutamine markedly inhibited endothelium-dependent vascular relaxations to acetylcholine and ADP, while slightly stimulating relaxation to the calcium ionophore A23187.

Our findings are in agreement with those of Sessa et al. (12), who showed that the release of endothelium-derived re-



**Figure 4.** Effect of extracellular levels of L-arginine (L-Arg) and L-glutamine (L-Gln) on endothelium-dependent relaxation in response to acetylcholine (ACh). (A) Rabbit aortic rings were incubated for 1 h in Krebs buffer containing the indicated L-glutamine concentrations. The vessels were contracted with phenylephrine, and acetylcholine was then added in a cumulative fashion. (B) Rabbit aortic rings were incubated for 1 h in Krebs buffer containing 0.6 mM L-glutamine and the indicated L-arginine concentrations. Endothelium-dependent relaxations in response to acetylcholine were then examined.

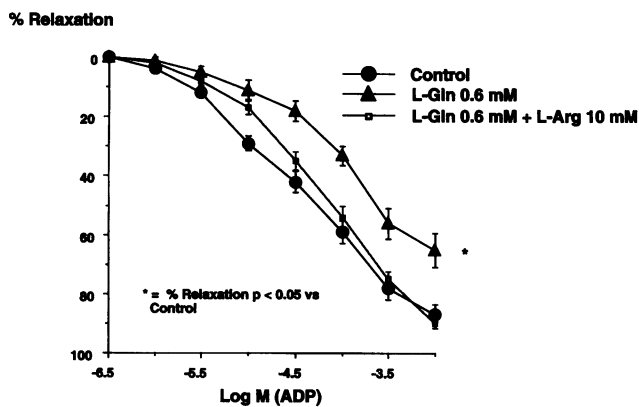


Figure 5. Effect of extracellular levels of L-arginine (*L-Arg*) and L-glutamine (*L-Gln*) on endothelium-dependent relaxation in response to ADP. Vessels were studied after contraction with phenylephrine and ADP added in a cumulative fashion.

laxing factor (EDRF) in response to ADP could be inhibited by L-glutamine. In contrast to this report, however, we would conclude that L-glutamine did not deplete intracellular L-arginine, because the release of NO evoked by A23187 was unchanged. Furthermore, for any given level of intracellular L-arginine, the production of NO in response to bradykinin was markedly depressed by the presence of L-glutamine (Fig. 3 A), and, in striking contrast, L-glutamine had a stimulatory effect when A23187 was used to evoke NO release.

In parallel with our findings in cultured cells, L-glutamine produced a concentration-dependent inhibition of endothelium-dependent vascular relaxation in response to the receptor-mediated agonists, acetylcholine or ADP, while slightly augmenting relaxation in response to A23187. We did not use identical agonists in the cultured cell and isolated vessel experiments because of receptor differences which exist in the two preparations. Nevertheless, these data support the concept derived from our studies of endothelial cells that L-glutamine selectively inhibits receptor-mediated NO release.

The effect of L-arginine on endothelium-dependent vasodilation has largely been observed in in vivo experiments (1, 2, 4–6). In vitro, L-arginine either had no effect or only modest effects on endothelium-dependent vasodilation (3, 21). Based on the present experiments, a potential explanation for this discrepancy is that in the in vitro experiments, L-glutamine was not present. Importantly, removal of L-glutamine from the media of cultured endothelial cells results in a 10–25-fold decline in intracellular glutamine over a brief period (12, 22). We found that intracellular L-glutamine declined 10-fold 4 h after removal of L-glutamine. Thus, levels of extracellular L-glutamine present in vivo are important in sustaining intracellular levels of L-glutamine. We considered the possibility that L-arginine given in vivo might overcome the inhibition caused by L-glutamine. This was found to be the case. By varying extracellular L-arginine from 0.1 to 10 mM in the absence of L-glutamine, we found no increased production of NO in response to bradykinin (Fig. 1). However, when L-glutamine was added, L-arginine produced a concentration-dependent increase in NO production and improvement in endothelium-dependent vascular relaxation. The inhibitory effect of L-glutamine was completely reversed by 10 mM L-arginine. While this concentration may

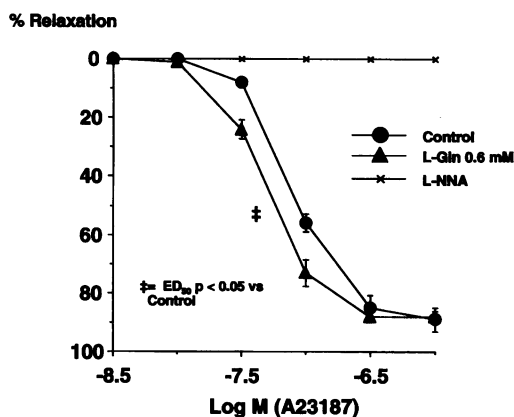


Figure 6. Effect of extracellular levels of L-arginine (*L-Arg*) and L-glutamine (*L-Gln*) on endothelium-dependent relaxation in response to calcium ionophore A23187. Rabbit aortic rings were incubated for 1 h in Krebs buffer containing 0.6 mM L-glutamine or 0 mM (*Control*). The endothelium-dependent relaxation in response to acetylcholine was then studied. The relaxation in response to A23187 was completely abolished by the addition of the NO synthase antagonist, L-N-nitro arginine (*L-NNA*).

seem high, similar levels have been seen in in vivo experiments in both animals and humans (1–6).

The mechanism by which L-glutamine inhibits endothelium-dependent vascular relaxation in response to receptor activation is complicated because it is now clear that NO synthase is not simply regulated by levels of intracellular calcium but also by posttranslational processing, phosphorylation (23, 24), and possibly other processes (25). These could be independently modulated by L-glutamine in different fashions depending on the stimulus used (receptor- versus non-receptor-mediated). Regardless, our experiments measuring NO synthase activity in vitro exclude a direct inhibition of enzyme activity by L-glutamine. It is unlikely that L-glutamine affects intracellular calcium signaling. In preliminary studies, we have found that 2.0 mM L-glutamine has no effect on bradykinin-stimulated increases in intracellular calcium as assessed by the calcium-sensitive indicator Fluo-3 imaged with confocal microscopy.

Similarly, the mechanism whereby L-arginine reverses the effect of L-glutamine remains unclear. In other cell types as in the endothelium, these two amino acids do not have similar transport mechanisms (26). In our studies, addition of L-arginine did not alter the intracellular levels of L-glutamine, suggesting that large concentrations of one amino acid does not significantly alter the uptake of the other. Moreover, L-glutamine did not change the  $K_m$  of NO synthase measured in vitro.

An important observation is that the inhibitory effects of L-glutamine occurred in concentrations encountered in normal mammalian plasma and tissue culture conditions. This leads to the speculation that endogenous L-glutamine tonically inhibits the release of endothelium-derived NO in vivo. It is important to note that even modest increases in L-glutamine (from 0.6 to 2 mM) produced significantly greater inhibitions of NO release and endothelium-dependent vascular relaxation. Whether disease processes associated with abnormal endothelium-dependent vasodilation are associated with alterations of L-glutamine metabolism or storage is unknown. It is interesting to speculate that moderate changes in plasma levels of L-glutamine associ-

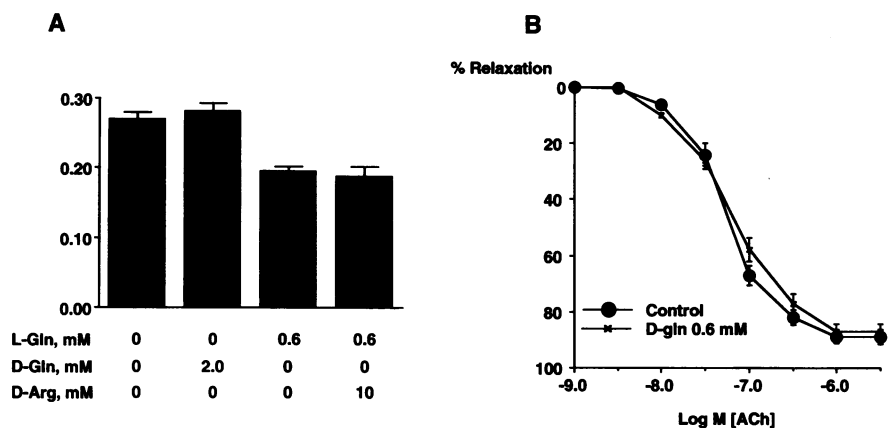


Figure 7. Effects of D-arginine and of D-glutamine. (A) Nitrogen oxides release from BAEC in response to bradykinin. (B) Endothelium-dependent relaxations of rabbit aorta in response to acetylcholine (ACh).

ated with changes in diet might influence endothelial production of NO.

In the absence of L-glutamine, large changes in extracellular L-arginine (from 0.1 to 10 mM) and intracellular levels of L-arginine (from 0.48 to 10.3 mM) did not affect NO production. It is unlikely that these extremes of intracellular L-arginine would occur in vivo even in pathophysiological conditions. Thus, it is doubtful that the administration of L-arginine would raise NO production by the endothelial isoform of NO synthase simply by raising enzyme substrate. On the other hand, removal of L-arginine from the culture media markedly reduced intracellular levels and NO release. On first inspection, this would seem to indicate that the functional  $K_m$  of the intracellular enzyme might be higher than that observed in broken cell preparations. Such a conclusion should be taken with caution however, because this degree of depletion of L-arginine may affect cellular function in a nonspecific fashion and could alter activation of NO synthase in a manner independent of NO synthase substrate availability.

These findings may have implications for future studies of isolated vessels and cultured cells. In general, such studies are done without adding L-glutamine. It would seem prudent to add L-glutamine to such preparations if one wishes to mimic in vivo conditions. It is of interest that in vivo responses to endothelium-dependent vasodilators are often quite blunted compared with what is observed in vitro (27). The presence of endogenous L-glutamine may in part explain these discrepancies.

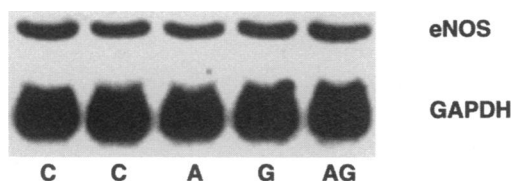


Figure 8. Effect of 24 h of incubation in presence of L-arginine (1 mM, A) and of L-glutamine (2 mM, G), none (C), or both (AG) on expression of NO-synthase mRNA in BAEC. Equivalent quantities and quantities (20  $\mu$ g/lane) of RNAs were verified by ethidium bromide staining of 18S and 28S ribosomal RNA. Representative Northern blot probed with bovine endothelial NO-synthase cDNA and GAPDH cDNA. Quantification of bovine endothelial NO-synthase mRNA by densitometry of three separate Northern analyses did not reveal any statistically significant differences between these various conditions.

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