L-Glutamine in vitro regulates rat aortic glutamate content and modulates nitric oxide formation and contractility responses

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Schachter D. L-Glutamine in vitro regulates rat aortic glutamate content and modulates nitric oxide formation and contractility responses. Am J Physiol Cell Physiol 293: C142–C151, 2007. First published February 28, 2007; doi:10.1152/ajpcell.00589.2006.—These studies test the hypothesis that L-glutamine at its physiological plasma concentration, ~0.5 mM, can increase tissue content and net synthesis of glutamate in rat aortic segments in vitro, thereby mediating relaxation of the underlying smooth muscle in the elastic reservoir region of the thoracic aorta. Aortic segments were incubated in an isotonic medium with and without 21 amino acids at their normal plasma concentrations. Of these amino acids only L-glutamine and L-leucine at their plasma concentrations increased glutamate synthesis and content. Tissue glutamate content resulting from increasing concentrations of each precursor reached an upper level of ~1.3–1.6 μmol/g wet wt. Regulation of the tissue glutamate content involves an interaction of the synthetic pathways in which L-glutamine inhibits the endothelial leucine-to-glutamate pathway. L-Glutamine increases nitric oxide (NO) formation, and NO inhibits the controlling enzyme of the endothelial leucine-to-glutamate pathway, the branched-chain α-ketoacid dehydrogenase complex. Treatment of precontracted aortic rings with 0.5 mM L-glutamine elicits smooth muscle relaxation, a response that requires endothelial nitric oxide synthase activity and an intact endothelium. The results demonstrate that in vivo L-glutamine at its normal concentration in plasma can regulate rat aortic glutamate content and modulate NO formation and contractility responses of the thoracic aortic wall.

L-leucine; α-ketoisocaproate; thoracic aorta; cGMP; endothelium; smooth muscle

THE STUDIES IN THIS REPORT test the specific hypothesis that L-glutamine at its physiological concentration in rat plasma can regulate the glutamate content of the thoracic aortic wall in the elastic reservoir (“windkessel”) region and, thereby, the contractility responses of the vessel wall. The hypothesis stems from prior studies of rat thoracic aortic segments in vitro (1, 34–36) that characterize a regional pattern of differentiation of the endothelium for three signal pathways regulating the cGMP content and contractility of the underlying smooth muscle. Endothelial formation of nitric oxide (NO) from L-arginine (1) and of glutamate from the carbon atoms of L-leucine via the leucine-to-glutamate (Leu→Glu) signaling pathway (35, 36) increases cGMP to mediate relaxation; formation of prostaglandin H2 via the cyclooxygenase arm of the eicosanoid pathway decreases cGMP (34) and elicits contraction (23). All three endothelial pathways reach maximal activity in the segment just distal to the aortic arch and decrease peripherally (36). Observations of this endothelial differentiation pattern in the thoracic aorta led to the general hypothesis that the foregoing pathways interact for dynamic regulation of the compliance and contractility responses of the aortic wall. The compliance is a functionally significant determinant of such hemodynamic parameters as the arterial systolic and pulse pressures, work of the left ventricle, and pulse-wave velocity (17). The studies described here were undertaken to characterize the plasma amino acids used as precursors of aortic glutamate and to explore possible interactions of the synthetic pathways for regulating aortic glutamate content and contractility responses.

Attempts to study directly the effects of L-glutamine on aortic segments in vitro have been hampered by its poor penetration of the tissue (35, 36). Thus, while 10–20 mM L-glutamate acting on rat aortic rings (36) and 100 mM L-glutamate acting on rabbit aortic rings (27) elicited relaxation responses, the physiological relevance of responses to these pharmacological concentrations is unclear. Glutamate permeates many tissues poorly (30, 37, 43), and prior investigators have utilized L-glutamine, which penetrates cells more readily, as an effective precursor of cellular glutamate owing to the widespread tissue distribution of glutaminase. The importance of L-glutamine as a precursor and metabolite is well documented and reviewed (10, 37, 43). L-Glutamine is utilized for protein biosynthesis, cell growth and tissue repair, acid-base balance and renal excretion of NH4+, hepatic and renal gluconeogenesis (41), synthesis of purines, pyrimidines, nucleic acids, and amino sugars, recycling of neurotransmitter glutamate in the brain (11), and maintenance of immunocompetent lymphocytes and macrophages (29). Synthesized mainly in skeletal muscle, lung, adipose tissue, and liver, L-glutamine is reported to be the most abundant amino acid in the plasma. The experiments described here support the working hypothesis by demonstrating that physiological concentrations of L-glutamine in vitro can increase the content of aortic glutamate, enhance the formation of NO by nitric oxide synthase (NOS), and elicit relaxation responses of aortic rings.

L-Leucine at its physiological concentration in rat plasma is also an effective precursor of the carbon atoms of aortic glutamate via the endothelial Leu→Glu pathway (35, 36). In this pathway uptake of L-leucine into the endothelium is followed by deamination to α-ketoisocaproate (α-KIC) and oxidative decarboxylation of α-KIC to isovaleryl coenzyme A and CO2. This last step, mediated by the mitochondrial branched-chain α-ketoacid dehydrogenase complex (BCDC), is a controlling reaction in the Leu→Glu sequence. Further mitochondrial reactions metabolize isovaleryl CoA to acetyl...
CoA, which enters the tricarboxylic acid cycle to yield α-ketoglutarate, which can be converted to glutamate by amination catalyzed by transaminases or glutamate dehydrogenase. The results presented here demonstrate that the pathways for aortic glutamate formation from t-glutamine and l-leucine interact: l-glutamine-stimulated NO inhibits the BCDC and thereby the Leu→Glu pathway.

MATERIALS AND METHODS

**Animals.** Sprague-Dawley strain male rats weighing 150–300 g were obtained from Charles River and maintained on a nutritionally complete pellet diet (Purina rat chow 5000) and water ad libitum. Rats usually weighed 250–450 g when used for studies and were not fasted except as indicated below. All animal procedures were submitted and approved by our Institutional Animal Care and Use Committee under Protocol AAAA4794.

**Preparation and incubation of aortic slices.** As previously described (1, 34–36), rats were anesthetized by carbon dioxide inhalation and exsanguinated. The thoracic aorta was excised from the aortic valve ring to the level of the left renal artery, chilled immediately in 145 mM NaCl-5 mM KCl, and maintained at 2°C until the onset of incubation (~10–20 min later). Adherent adventitial tissue was removed, and the proximal segment of peak activity, ~15–40 mm from the aortic origin, was sliced into four horizontal segments, each ~6 mm long and of 10–14 mg wet wt. Each segment was bisected vertically, and the resulting hemisegments were incubated separately to compare a control and an experimental hemisegment at a given level of the aorta by applying the t-test of paired comparisons for evaluation of statistical significance. Comparable hemisegments from two or more rats were pooled when necessary to provide sufficient tissue for analysis. Hemisegments were suspended in 2.5 ml of a standard Krebs-Ringer bicarbonate (KRB) incubation medium of the following composition (mM): 118 NaCl, 4.7 KCl, 2.0 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, and 10 glucose. Appropriate additions to the medium included unlabeled l-glutamine or l-leucine precursor and 0.3 μCi/ml l-[U-14C]leucine (specific radioactivity 306 mCi/mmol; Amersham), to study l-glutamate formation. Tissue suspensions were generally shaken for 60 min at 37°C in an atmosphere of 5% CO2-95% O2. Thereafter, tissues were removed, blotted to remove adherent medium, weighed rapidly, frozen in vials immersed in an ethanol-dry ice bath, and maintained at ~15°C until assayed. When appropriate, the endothelium was denuded before incubation of the segments, as previously described (1, 36).

**Preparation of plasma amino acid mix.** To approximate in vivo conditions more closely, tissues were also incubated in the foregoing KRB medium prepared to contain amino acids at their normal concentrations in rat plasma (2). A stock l-amino acid mix (AA mix) was prepared and added to yield the following concentrations (mM) in the incubation medium: 0.184 arginine, 0.06 alanine, 0.008 aspartate, 0.068 asparagine, 0.005 citrulline, 0.017 cystine, 0.302 glycine, 0.106 glutamate, 0.063 histidine, 0.101 isoleucine, 0.203 leucine, 0.318 lysine, 0.064 methionine, 0.083 phenylalanine, 0.374 proline, 0.20 serine, 0.37 threonine, 0.082 tryptophan, 0.123 tyrosine, and 0.228 valine. Where appropriate, l-leucine was excluded in some media and replaced with unlabeled L-glutamine to glutamate for greater sensitivity, because endogenous tissue α-ketoglutarate makes their assay nonspecific. To inhibit transamination of endogenous α-ketoglutarate to glutamate, 2 mM (aminooxy)acetate, a general transaminase inhibitor, is included in the reaction. This concentration completely inhibits conversion of added α-ketoglutarate to glutamate. For the assay a 50-μl aliquot of tissue extract, or a suitable dilution thereof, is placed in each of two wells of a 96-well microtiter plate with filters for excitation at 530 nm and emission at 590 nm. The increment in fluorescence owing to the action of l-glutamate oxidase was linear with glutamate concentration, i.e., a reduction of ~5% at 20 μM. Background fluorescence in the absence of l-glutamate oxidase was generally ~10% of the total fluorescence. Recoveries of added glutamate ranged from 94.7% to 102.9% (mean 98.9%), and duplicate samples were repeatable within 5.8–6.2%. l-Glutamine yielded negligible fluorescence in the assay, including after heat treatment of tissue segments, unless exposed to exogenous glutaminase (see below). Incubation media containing glucose and amino acids were assayed for glutamate by plating aliquots directly, without heating to 100°C, since heating such media produced unacceptable levels of H2O2 as background.

**Estimation of [14C]glutamate.** An isotope dilution method was used to quantify the [14C]glutamate formed from [14C]leucine in tissue extracts. Total radioactivity in the tissue extract was quantified by liquid scintillation spectrometry. To 100 μl of tissue extract carrier l-glutamate was added to a final concentration of 10 mM, and this mixture was resolved on a column (2-ml bed volume) of Dowex 1X8–200 anion exchange resin (Sigma). The resin had been thoroughly washed with deionized water, and the column was packed and similarly washed further. After the loaded column was washed with 1 ml of 0.5 M acetic acid and its glutamate content and radioactivity were quantified as described above. Total cpm of [14C]glutamate in the tissue extract was calculated from the specific radioactivity of the eluted glutamate fraction and the quantity of carrier glutamate added to the original tissue extract. [14C]glutamate formation (expressed as nmol/g or μmol/g wet wt of tissue) was calculated from the total cpm of [14C]glutamate and the specific radioactivity of an acetyl group of the precursor [U-14C]leucine. Stoichiometric studies of aortic seg-

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ments (36) have shown that one two-carbon moiety of leucine is incorporated per glutamate formed.

Estimation of plasma l-glutamine and l-glutamate. Plasma l-glutamine was estimated as the increment in l-glutamate liberated on treatment with E. coli glutaminase. Heparinized blood samples obtained on exsanguination were centrifuged, and the plasmas were separated. Plasmas were diluted 1/10 with deionized water, and ultrafiltrates were obtained by centrifugation in Centricon YM-10M Filter Devices (Millipore). A 20-μl aliquot of ultrafiltrate was plated into each of 4 wells in a 96-well microplate. Two wells were used to estimate glutamate as described above. To each of the remaining two wells were added 0.01 U of E. coli glutaminase (Sigma, grade V) and acetate buffer pH 5 to a final concentration of 15 mM and a volume of 40 μL. The plates were incubated at 37°C for 1 h and neutralized with 10 μL of 0.02 N NaOH, and glutamate was quantified as described above. Standards of glutamate and glutamine (12.5, 25, and 50 μM) were carried through the procedure.

Estimation of nitrate content as index of NO formation. The following were purchased from Sigma-Aldrich (St. Louis, MO): l-arginine, l-glutamic acid, l-glutamine, α-KIC (4-methyl-2-oxopentanoic acid) Na⁺ salt, α-ketoisovalerate (3-methyl-2-oxobutanoic acid) Na⁺ salt (α-KIV), l-leucine, N⁶-nitro-l-arginine methyl ester (l-NAME), l-phenylephrine hydrochloride; l-valine, and 3-(aminopropyl)1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5).

Materials. The following were purchased from Sigma-Aldrich (St. Louis, MO): l-arginine, l-glutamic acid, l-glutamine, α-KIC (4-methyl-2-oxopentanoic acid) Na⁺ salt, α-ketoisovalerate (3-methyl-2-oxobutanoic acid) Na⁺ salt (α-KIV), l-leucine, N⁶-nitro-l-arginine methyl ester (l-NAME), l-phenylephrine hydrochloride; l-valine, and 3-(aminopropyl)1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5).

Results

l-Arginine

Table 1. Effects of l-glutamine and l-leucine on net synthesis of glutamate and on glutamate content of aortic segments incubated in vitro

<table>
<thead>
<tr>
<th>Compound(s) Tested</th>
<th>n</th>
<th>Hemisegment</th>
<th>Glutamate Content, nmol/g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tissue</td>
<td>Tissue + Medium</td>
</tr>
<tr>
<td>Plasma amino acid mix*</td>
<td>12 (10)</td>
<td>Control (KRB alone)</td>
<td>776±52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ amino acid mix</td>
<td>792±62</td>
</tr>
<tr>
<td>l-Glutamine (0.5 mM)</td>
<td>6 (6)</td>
<td>Control (KRB + amino acid mix)</td>
<td>781±109</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ l-glutamine</td>
<td>1,286±159</td>
</tr>
<tr>
<td>l-Leucine (0.2 mM)</td>
<td>14 (12)</td>
<td>Control (KRB + amino acid mix)</td>
<td>844±84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ l-leucine</td>
<td>1,045±114</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of aortic hemisegment pairs (no. of rats). The area 15–40 mm distal to the aortic ring was excised and cut into 4 horizontal rings, each ~6 mm long and of 10–14 mg wet wt. Each ring was bisected vertically to provide a control and an experimental hemisegment for comparison at the same aortic level. Hemisegments were incubated for 1 h at 37°C under 5% CO₂-95% O₂ in standard Krebs-Ringer bicarbonate (KRB) medium containing 10 mM D-glucose (MATERIALS AND METHODS) without or with compounds. Glutamate content of 24 hemisegments prepared similarly from 3 rats but not incubated was 793 ± 147 nmol/g wet wt. *The plasma amino acid mix lacking l-glutamine and l-leucine consisted of 19 L-amino acids (MATERIALS AND METHODS). These were present in the incubation medium at their respective concentrations in normal rat plasma. †These media contained 0.2 mM L-leucine + 0.3 μCi/ml of l-[1-14C]leucine. On incubation the [14C]glutamate formed (quantified as described in MATERIALS AND METHODS) was 340 ± 43 nmol/g and thus comparable to the leucine-dependent increments in total (tissue + medium) glutamate, 300 ± 83 nmol/g.

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but total (tissue + medium) glutamate (1,119 ± 75 nmol/g) exceeded that of nonincubated segments (P < 0.01), indicative of net formation of glutamate in vitro. Hemisegments incubated in the standard KRB plus AA mix lacking L-glutamine and L-leucine yielded tissue and total (tissue + medium) glutamate values similar to those tested in KRB alone. Hence the exogenously added 19 amino acids of the AA mix did not enhance net glutamate formation over that supported by endogenous tissue precursors. (Endogenous sources could include amino acids, e.g., L-leucine, serving as precursors for glutamate synthesis and proteins yielding glutamate via proteolysis.) By contrast, addition to the AA mix of either 0.5 mM L-glutamine or 0.2 mM L-leucine significantly increased the tissue glutamate levels (P < 0.0025 and P < 0.025, respectively) and the total (tissue + medium) values (P < 0.001 and P < 0.0025, respectively), indicative of increased net synthesis.

Whether L-glutamine could influence aortic glutamate content under normal plasma conditions was explored further by incubating aortic hemisegments in rat plasma ultrafiltrate depleted of glutamine (MATERIALS AND METHODS). Hemisegments so incubated, under the conditions described in Table 1, without and with 0.5 mM L-glutamine yielded tissue glutamate levels of 1,218 ± 62 and 1,426 ± 117 nmol/g, respectively (16 hemisegment pairs from 9 rats; paired t-test, P < 0.01).

Precursor L-glutamine concentration and tissue glutamate levels. The effects of L-glutamine were examined with hemisegments in which one hemisegment was incubated without and the other with a concentration of L-glutamine from 0.1 to 10.0 mM. The results of five experiments are shown in Fig. 1. The normal plasma level, 0.5 mM, again increased both the net formation (paired t-test, P < 0.001) and the tissue content (paired t-test, P < 0.005) of glutamate. With increasing precursor concentrations tissue glutamate levels approached a plateau of ~1.4 μmol/g. In additional experiments (data not shown) five sequential segments from the aortic ring were tested with exogenous L-glutamine, and the net increments in tissue glutamate showed a pattern similar to that observed previously with precursor L-leucine (36), i.e., highest values in the segment 15–40 mm from the aortic root and diminishing peripherally. Denuding the endothelium of the peak area segments before incubation with L-glutamine decreased the tissue glutamate levels by ~54%. The bulk of the glutamate formed from L-glutamine by intact or endothelium-denuded segments was in the ambient medium (Fig. 1).

L-Glutamine and L-glutamate plasma concentrations of 28 rats used in our studies were estimated. Plasma L-glutamine levels (μM; means ± SE) of 11 rats aged 50–80 days and 17 rats aged 81–185 days were 592 ± 19 and 497 ± 28, respectively (P < 0.01); corresponding L-glutamate values were 106 ± 9 and 108 ± 7. The plasma L-glutamine values are similar to those previously reported (32) for male Sprague-Dawley rats.

Precursor L-leucine concentration and tissue glutamate levels. The effects of L-[U-14C]leucine concentrations from 50 to 1,000 μM on aortic hemisegment pairs were examined similarly, i.e., by comparison of each untreated control with its treated hemisegment, and the results are listed in Table 2. Leucine concentrations of 100–1,000 μM increased the total (tissue + medium) glutamate content significantly (P < 0.005), indicating net glutamate synthesis. The mean quantities of [14C]glutamate formed were comparable to the leucine-dependent increases in net synthesis. For example,
Hence at leucine concentrations of 100 μM tissue total glutamate increased from 36% to 61% (KRB-10 mM D-glucose medium containing 100 μM L-[U-14C]leucine incubated for 1 h at 37°C in the standard Krebs-Ringer bicarbonate medium). Values are means ± SE for 5 hemisegments treated with each L-glutamine concentration. The value at 0 μM leucine is the mean ± SE of the 20 control hemisegments.

at 100 μM leucine the mean values for [14C]glutamate formed and for the increase in total (tissue + medium) glutamate were 603 and 655 nmol/g, respectively. Thus the leucine-dependent increment in glutamate was mainly via the Leu→Glu pathway (36). The net formation of glutamate owing to L-glutamine was completely eliminated by denuding the endothelium before incubation (9 hemisegment pairs, 3 rats) in accord with the endothelial localization of the Leu→Glu pathway reported previously (35, 36).

Formation of [14C]glutamate increased with L-leucine concentration to a plateau level, with an apparent half-saturation concentration of ~60 μM. Tissue glutamate content, however, did not increase correspondingly; the glutamate increment at 100 μM leucine, 551 nmol/g, decreased to 13 nmol/g at 1,000 μM leucine (P < 0.025), and the percentage of [14C]glutamate/tissue total glutamate increased from 36% to 61% (P < 0.001). Hence at leucine concentrations of 100 μM and higher a tissue glutamate plateau of ~1.3–1.6 μmol/g was reached, comparable to the plateau observed with L-glutamine. That both of the exogenous amino acid precursors yielded similar maximal glutamate levels suggested tissue regulation and possible interaction of the formation pathways.

### L-Glutamine inhibition of Leu→Glu pathway
To explore a possible interaction of the pathways, the effects of L-glutamine on the endothelial synthesis of [14C]glutamate from L-[U-14C]leucine were examined. As shown in Fig. 2, L-glutamine concentrations of 0.5–10.0 mM inhibited [14C]glutamate formation via the Leu→Glu pathway significantly (1-way ANOVA, F = 8.01, P < 0.001). The mean reductions in [14C]glutamate owing to 0.5 and 2.0 mM L-glutamine were 21.2% and 37.3%, respectively; the corresponding levels of tissue total glutamate, 0.85 ± 82 and 1.08 ± 89 nmol/g, respectively, were not significantly different. Reductions in tissue uptake of L-[U-14C]leucine owing to L-glutamine did not account for the decreases in [14C]glutamate. Tissue uptake of the L-leucine precursor was not changed significantly by 0.5 mM L-glutamine, and whereas L-glutamine concentrations of 2 and 10 mM decreased L-leucine uptake by 18.8% and 40.5%, respectively, the corresponding reductions in [14C]glutamate formation were considerably greater, 37.3% and 66.5%.

The effects of L-glutamine on the synthesis of [14C]glutamate from L-[U-14C]leucine were further tested as described above in the standard KRB medium containing the plasma AA mix (MATERIALS AND METHODS). Addition of 0.5 mM L-glutamine decreased the [14C]glutamate values of the untreated hemisegment controls, 496 ± 21 nmol/g, to 350 ± 36 nmol/g (P < 0.01, paired t-test of 5 hemisegment pairs from 5 rats); the corresponding values for tissue uptake of precursor L-[U-14C]leucine were similar, 534 and 511 nmol/g, respectively.

Exploratory studies to determine the mechanism of the inhibition owing to L-glutamine showed that blocking NOS activity with L-NAME eliminated the inhibition (Table 3). Without L-NAME the treatments with 0.5 and 2.0 mM L-glutamine inhibited [14C]glutamate formation by 20.3% (P < 0.025) and 45.8% (P < 0.025), respectively. L-NAME eliminated this inhibition without affecting the control values of [14C]glutamate formation. The results point to NO mediation of the inhibition owing to L-glutamine.

### L-Glutamine increases NO formation
The effect of incubation with 0.5 mM L-glutamine on the nitrate content of aortic segments was examined (MATERIALS AND METHODS) as an index of NO formation in vitro. Values of total (tissue + medium) nitrate in the absence versus presence of 0.5 mM L-glutamine

<table>
<thead>
<tr>
<th>Experiment</th>
<th>L-Glutamine Concentration, mM</th>
<th>No L-NAME</th>
<th>L-NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>340±51</td>
<td>443±72</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>271±44</td>
<td>381±47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P &lt; 0.025*</td>
<td>P = NS</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>396±67</td>
<td>367±65</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>215±28</td>
<td>348±75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P &lt; 0.025*</td>
<td>P = NS</td>
</tr>
</tbody>
</table>

Values are means ± SE. To test each concentration of L-glutamine 10 aortic hemisegment pairs were prepared from 10 rats. Five pairs were used to test the effects of L-glutamine in the absence and 5 pairs in the presence of 2.0 mM Nω-nitro-L-arginine methyl ester (L-NAME). Each pair consisted of a control (without L-glutamine) and an experimental (with L-glutamine) hemisegment incubated in standard KRB-10 mM D-glucose medium containing 0.1 mM L-[U-14C]leucine for 1 h at 37°C. *P values for effects of L-glutamine were calculated by t-test of paired comparisons.

Additional evidence pointing to regulation of the glutamate content came from studies of the effects of L-valine and its deamination product, α-KIV, compounds that inhibit the Leu→Glu pathway (36) and the BCDC. Aortic segments were incubated with 100 μM L-[U-14C]leucine and various concentrations up to 5 mM of these compounds. L-valine and α-KIV inhibited [14C]glutamate formation similarly, by ~80%, with half-inhibition concentrations of 0.85 and 0.55 mM, respectively. The comitant effects on tissue glutamate content, however, differed: L-valine resulted in no significant change, whereas 0.5 mM α-KIV decreased glutamate content by ~45% (P < 0.001). L-valine inhibition of [14C]glutamate formation is apparently compensated by regulatory mechanisms that utilize its -NH2 group to form glutamate, e.g., by transamination of α-ketoglutarate, whereas α-KIV, lacking the -NH2, cannot be used similarly.
Table 4. Effects of L-glutamine in vitro on aortic tissue metabolism of L-[U-14C]arginine

<table>
<thead>
<tr>
<th>L-Arginine, mM</th>
<th>L-Glutamine, mM</th>
<th>Tissue Arginine Metabolite, mmol/g</th>
<th>Ratio (cpm tissue metabolite/total tissue cpm), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>0</td>
<td>16.2 ± 1.6</td>
<td>9.9 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>20.0 ± 2.4</td>
<td>15.4 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.05*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>0</td>
<td>29.1 ± 3.2</td>
<td>8.3 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>34.9 ± 5.9</td>
<td>13.2 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.001*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. Ten aortic hemisegment pairs prepared from 10 rats were used at each concentration of L-[U-14C]arginine. Control and experimental hemisegments were incubated for 1 h at 37°C, without and with 0.5 mM L-glutamine, respectively, in KRB containing 10 mM D-glucose and L-[U-14C] arginine as indicated. L-glutamine also decreased tissue uptake of the precursor L-[14C]arginine, and the final ratios of tissue [14C]arginine metabolite/tissue total 14C) are shown. *P values for effects of L-glutamine were calculated by t-test of paired comparisons.

were 484 ± 128 and 729 ± 135 nmol/g, respectively (P < 0.025, t-test of paired comparisons of 9 hemisegment pairs from 9 rats).

The effects of L-glutamine on the conversion of L-[14C]arginine to [14C]citrulline and NO by aortic NOS were also examined with a modification described previously (1) of the method of Bredt and Snyder (7). The results in Table 4 show that L-glutamine increased the metabolism of 0.1 and 0.25 mM L-arginine by 23.4% (P < 0.05) and 19.9% (P < 0.01), respectively. Additional experiments explored the effects of added 0.5 mM L-glutamine on the time course of metabolism of 0.184 mM L-[U-14C]arginine by aortic hemisegments incubated in KRB medium containing the plasma AA mix depleted of L-glutamine. The results in Fig. 3 show L-arginine metabolism to be linear with time for 60 min of incubation and a rate increase of ~30% owing to the addition of 0.5 mM L-glutamine (2-way ANOVA, F = 8.91, P < 0.01).

Table 5. Effects of nitric oxide donor NOC-5 on formation of [14C]glutamate from L-[U-14C]leucine by aortic hemisegments in vitro

<table>
<thead>
<tr>
<th>NOC-5, mM</th>
<th>n</th>
<th>[14C]glutamate (a)</th>
<th>Total glutamate (b)</th>
<th>ab, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>28</td>
<td>265 ± 16</td>
<td>567 ± 33</td>
<td>42.8 ± 1.2</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td>242 ± 28</td>
<td>607 ± 61</td>
<td>39.6 ± 1.3</td>
</tr>
<tr>
<td>200</td>
<td>5</td>
<td>141 ± 19</td>
<td>459 ± 55</td>
<td>30.5 ± 1.8</td>
</tr>
<tr>
<td>300</td>
<td>5</td>
<td>117 ± 24</td>
<td>444 ± 89</td>
<td>27.1 ± 2.6</td>
</tr>
<tr>
<td>400</td>
<td>5</td>
<td>73 ± 23</td>
<td>398 ± 62</td>
<td>16.7 ± 3.3</td>
</tr>
<tr>
<td>1,500</td>
<td>5</td>
<td>12 ± 7</td>
<td>318 ± 74</td>
<td>2.4 ± 1.3</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of incubation vessels. Aortic hemisegment pairs were prepared as described in MATERIALS AND METHODS, and 3 corresponding hemisegments from 3 rats were pooled for each vessel. Control [no 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5)] and experimental (treated with indicated NOC-5 concentration) hemisegments were incubated in KRB-10 mM D-glucose containing 0.2 mM L-[U-14C] leucine for 30 min at 37°C under 5% CO2-95% O2. P values calculated by 1-way ANOVA for effects of NOC-5; P < 0.0001 for both [14C]glutamate formation (F = 17.08) and ab (F = 54.37); P < 0.02 for effects on total glutamate content (F = 3.03).

Effects of NO donor on Leu→Glu pathway. The effects of the NO donor NOC-5 on the formation of [14C]glutamate from [14C]leucine by aortic hemisegment pairs were examined, and the results are listed in Table 5. NOC-5 significantly decreased [14C]glutamate formation, with a 50% inhibition concentration of ~210 mM. Tissue total glutamate and the percentages of [14C]glutamate/total glutamate were also decreased significantly.

To define the site of NO inhibition of the Leu→Glu pathway, we focused on the BCDC. Aortic hemisegments were...
tested with 0.1 mM [1-14C]α-KIC (MATERIALS AND METHODS) and various concentrations of NOC-5, and the results shown in Fig. 4 demonstrate marked inhibition of BCDC activity by NOC-5 (ANOVA, \( F = 42.4, P < 0.0001 \)), with a 50% inhibition concentration of \( \sim 110 \) μM.

**L-glutamine and contractility responses in vitro.** In view of the foregoing results demonstrating that L-glutamine at its normal plasma concentration increases aortic glutamate content and NO formation, the contractile responses to L-glutamine of aortic rings precontracted with phenylephrine were examined and compared with those elicited by L-glutamate. Pharmacological concentrations of L-glutamate (10–20 mM) exhibit a biphasic response consisting of an initial brief contraction followed by a prolonged relaxation (Fig. 5, F and G). Treatment with 2 mM L-glutamine (Fig. 5C) yielded a similar response pattern but greater relaxation than that after 20 mM L-glutamate. The relaxation response to various concentrations of L-glutamine was examined systematically and expressed as a relaxation rate, i.e., the decrease in isometric tension per gram of tissue per minute (Fig. 6), or as a percentage of the phenylephrine contraction. Both parameters of relaxation were increased by all concentrations of L-glutamine from 0.5 to 10.0 mM. Relaxation rates for controls versus rings treated with 0.5 mM L-glutamine were 1.47 ± 0.19 vs. 2.84 ± 0.45 g·g⁻¹·min⁻¹, respectively (\( P < 0.005 \)). The corresponding percentages of the phenylephrine contractions were 24.7 ± 3.5 vs. 54.0 ± 5.6% (\( P < 0.005 \)). The relaxation responses were preceded by brief contractile responses only at higher L-glutamine concentrations, 2–10 mM, but not after 0.5 mM.

Additional experiments support the conclusion that L-glutamine evokes relaxation responses by increasing endothelial formation of NO. Removal of the endothelium of aortic rings before treatment with 10 mM L-glutamine decreased the resulting relaxation rate from the value in intact rings of 14.31 ± 1.80 g·g⁻¹·min⁻¹ to 4.08 ± 0.63 g·g⁻¹·min⁻¹ in denuded rings (\( P < 0.01; 8 \) intact vs. 8 denuded rings from 4 rats). Treatment with 2.0 mM L-NAME decreased the relaxation responses to 10 mM L-glutamine from 12.05 ± 2.39 g·g⁻¹·min⁻¹ in controls to 2.43 ± 0.70 g·g⁻¹·min⁻¹ in L-NAME-treated segments (\( P < 0.01; 8 \) control vs. 8 treated rings from 4 rats).

Inasmuch as relaxation responses to NO are mediated by increases in cGMP (22), the effects on aortic segment cGMP of incubation with 5 mM L-glutamine for 1 h at 37°C were examined. The cGMP content in untreated controls, 62.9 ± 5.7 pmol/g, was increased to 111.5 ± 12.7 pmol/g by L-glutamine (\( P < 0.001; 19 \) control vs. 19 treated segments from 6 rats). Repetition of these studies using segments denuded of endothelium yielded cGMP values of 3.6 ± 1.5 and 2.9 ± 0.9 pmol/g in control and L-glutamine-treated segments, respectively (6 segments per group from 2 rats), i.e., no effect of L-glutamine.

**DISCUSSION**

The foregoing experiments in vitro support the working hypothesis of plasma L-glutamine regulation of the aortic content of tissue glutamate and thereby of the contractility of the underlying vascular smooth muscle. Data in Table 1 demonstrate that of 21 plasma amino acids added exogenously only L-glutamine and L-leucine at their physiological plasma concentrations were effective precursors in vitro for net glutamate.

**Fig. 5.** Contractile responses of precontracted aortic rings evoked by L-glutamine and L-glutamate. Results of 2 experiments, each with an aorta from 1 rat, are shown. Sequential rings A–D and E–H were prepared from the area 15–40 mm from the aortic origin and tested as described in MATERIALS AND METHODS. Tension was adjusted to 1.0 g, and rings were contracted with 1 μM L-phenylephrine at the onset. Control segments A, D, E, and H were followed without further treatment. As shown by arrows, rings B and C were treated with the indicated concentrations of L-glutamine and rings F and G with L-glutamate.

**Fig. 6.** Effects of various concentrations of L-glutamine on the rate of relaxation of precontracted aortic rings. Rings were prepared from the area 15–40 mm from the aortic origin, precontracted with 1.0 μM L-phenylephrine, and tested as described in MATERIALS AND METHODS and Fig. 5. The number of rings tested with glutamine concentrations (mM) of 0, 0.5, 1.0, 2.0 and 10.0 were 20, 4, 4, 5, and 22, respectively. Relaxation rate is the slope of the decrease in tension with time following the peak tension and is given in units of grams per gram wet weight of tissue per minute. One-way ANOVA for the effects of glutamate on relaxation rate yielded \( F = 22.78, P < 0.0001 \).
synthesis in the thoracic aortic wall. The synthetic pathways involved are illustrated in Fig. 7. Although both L-glutamine and L-leucine are effective precursors, L-glutamine can yield glutamate directly via the action of glutaminase (24), whereas prior studies of the stoichiometry of the endothelial Leu→Glu pathway (36) indicate that each molecule of L-leucine entering this pathway provides one 2-carbon acetyl group per glutamate formed and the remaining glutamate carbons come from oxaloacetate produced from glucose-derived pyruvate via pyruvate carboxylase.

It is instructive to compare the aortic pathways with corresponding processes in brain tissue for glutamate synthesis and the regulation of tissue glutamate content. A comprehensive literature (14, 24, 26, 47) documents glutamate as the major excitatory neurotransmitter in the central nervous system and describes mechanisms of its formation, regulation, and metabolism. The major amino acid precursors for brain glutamate formation are also L-glutamine and L-leucine (14). In contrast to the utilization of the L-leucine carbon atoms in the Leu→Glu pathway of the aortic endothelium, in brain leucine provides only its α-amino group, which may contribute up to half of the glutamate nitrogen in cells capable of net glutamate formation. The carbon skeleton of brain α-ketoglutarate is derived mainly via glucose metabolism. Two astrocyte-neuronal cell cycles, a glutamate-glutamine cycle and a leucine-α-KIC cycle (11), have been characterized. These regulatory mechanisms maintain and “buffer” neuronal glutamate content and ensure very low glutamate concentrations in the extracellular fluids. In the present studies the glutamate content of aortic segments was also regulated in vitro. An upper limit in the range of 1.3–1.6 μmol/g wet wt was observed (Fig. 1 and Table 2) as the concentrations of precursor L-glutamine or L-leucine were increased. Although the specific regulatory mechanisms in aorta remain to be characterized fully, one such mechanism is an interaction by which the L-glutamine precursor inhibits glutamate formation via the Leu→Glu pathway (Fig. 2). This inhibition in vitro followed treatment with 0.5 mM L-glutamine, the normal plasma concentration, both in the presence and in the absence of the AA mix of 20 other plasma amino acids. The inhibition by L-glutamine was blocked by L-NAME (Table 3), suggesting mediation by increased NO formation, and this mechanism was confirmed and further characterized by systematic studies that demonstrated that (1) 0.5 mM L-glutamine increases aortic tissue nitrate formation and the metabolism of L-[U-14C]arginine to citrulline and NO both in the presence (Fig. 3) and in the absence (Table 4) of the plasma AA mix; (2) direct treatment of aortic segments with the NO donor NOC-5 inhibits the synthesis of [14C]glutamate from L-[U-14C]leucine (Table 5); and (3) NOC-5 inhibits the oxidative decarboxylation of [14C]α-KIC (Fig. 4), pointing to the BCDC as the target site of the inhibition (Fig. 7).^2

There is considerable evidence that the BCDC is the major controlling enzyme in the metabolism of the branched-chain amino acids generally (12, 13, 18) and in the aortic Leu→Glu pathway specifically (36). Regulation of liver BCDC activity is reported to occur via covalent phosphorylation by a BCDC

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2 L-Glutamate derived from L-glutamine can participate in other pathways that might influence the formation of [14C]glutamate from [U-14C]leucine. Via the branched-chain aminotransferase reaction, glutamate could lower the α-KIC concentration and thereby slow the BCDC reaction. Second, via the glutamate dehydrogenase reaction, diversion of glutamate carbon into the tricarboxylic acid cycle and conversion to pyruvate and subsequent acetyl CoA might reduce the amount of leucine-derived carbon converted to acetyl CoA. Neither of these mechanisms would be sensitive to inhibition by L-NAME and thus they are unlikely in view of the evidence for mediation by NO. Third, glutamate derived from glutamine can increase endogenous arginine formation in some cell types in the absence of exogenous arginine (30). Increases in NO owing to glutamine in aorta, however, do not depend on increased formation of endogenous arginine. The results in Table 4 and Fig. 3 show that L-glutamine increases the metabolism of exogenously supplied L-arginine to citrulline and NO. Furthermore, L-glutamate at its plasma concentration (0.5 mM) inhibited the formation of [14C]glutamate from [14C]leucine by 21.2% in the absence of exogenous arginine (Fig. 2 and text) and by 29.4% (text) in the ambient medium containing 0.184 mM L-arginine (plasma amino acid mix). Thus supplying exogenous arginine did not diminish the action of L-glutamate.
kinase to inactivate the enzyme and by dephosphorylation via a BCDC phosphatase to activate it (18). The inhibition of aortic BCDC activity by NO, therefore, could occur directly, e.g., by nitrosylation of the BCDC E1α subunit (the subunit responsible for the decarboxylation of the branched-chain α-ketoacids, including α-KIC), similar to the inhibition by nitrosylation of a number of other oxidative enzymes (40), by activation of a BCDC kinase reaction, or by inactivation of a BCDC phosphatase.

The molecular mechanisms by which L-glutamine increases NOS activity for NO formation in aortic segments remain to be characterized fully, but it is likely that one such is increase in endothelial cell glutamate (Fig. 7). In brain tissue L-glutamate stimulates neural NOS activity and NO release in neurons (7, 16) and glial cells (4). In contrast with the present studies of freshly isolated aortic segments, a number of prior studies report that endothelial cells in culture respond differentially to L-glutamine. In cultures of bovine aortic (3, 38) and venular (25) endothelial cells L-glutamine (but not L-glutamate) inhibited the conversion of L-citrulline to L-arginine (38) and decreased the release of endothelium-derived relaxing factor (20) and NO (3, 25). The inhibitory effects were not due to reductions in total intracellular arginine or endothelial NOS (eNOS) activity estimated in cell homogenates. A possible explanation of the different responses of cells in culture versus freshly isolated aortic tissues comes from studies of Wu et al. (46), who reported that glucosamine inhibits NO synthesis in endothelial cells and that the utilization of L-glutamine as a precursor for glucosamine-6-phosphate synthesis in cultured endothelial cells could explain L-glutamine inhibition of NO formation. In their studies glucosamine-6-phosphate synthesis was 10–100 times more active in cultured compared with freshly isolated endothelial cells. It is reasonable to suggest that for endothelial cell division and growth in culture L-glutamine is required for synthesis of hexosamines, which acts to decrease NO formation, whereas in the freshly isolated aortic segments studied in this report L-glutamine acts to increase eNOS activity and NO formation.

The hypothesis under investigation posits that the glutamate content of the thoracic aortic wall mediates a dynamic control of the contractility and compliance of the vascular smooth muscle and thereby of the capacitance of the elastic reservoir. Favoring this hypothesis are prior observations that inhibitors of the rat aortic Leu→Glu pathway, including inhibitors of the BCDC or of L-glutamate transporters, enhanced contractile responses of aortic rings in vitro (36). Concordantly, treatment with high concentrations of L-glutamate, ~100 times the normal plasma concentration, resulted in prolonged relaxations preceded by brief contractions. That such responses could have physiological relevance is favored in the present studies by the observations that L-glutamine at its normal concentration in plasma can increase tissue glutamate content, NO formation, and relaxation responses (Fig. 6). The relaxation responses to L-glutamine accompanied increases in aortic cGMP (Fig. 7) and were prevented by prior removal of the endothelium and by L-NAME, evidence of mediation by enhanced eNOS activity.

NO has important functions in the blood vascular system of humans and animals, including lowering arterial blood pressure levels (9), decreasing blood coagulability (33), and retarding vascular smooth muscle proliferation (39). Prior reviews of eNOS activity describe a number of substances (15, 28) and physical forces (5, 44) capable of stimulating NO formation. Normal plasma constituents that can function to regulate the basal secretion of NO, however, are not well characterized. The in vitro experiments in this report point to L-glutamine as one such plasma constituent in the rat and suggest the utility of further studies in vivo, where decreases in plasma L-glutamine accompany metabolic acidosis (32), sepsis (31), and trauma, extensive surgery, and severe exercise (6).

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