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

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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-glutamate 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+ (10), 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 L-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 50001) 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 AAAA-4794.

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-[14C]glutamic acid (radioactivity activity 306 cpm/μmol; Amersham), to study [14C]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 its isoform was added to others. The total osmolarity of the medium containing AA mix was adjusted to 305 mosM by small (~1.5%) reductions of the NaCl concentration.

Preparation of ultrafiltrate of rat plasma partially depleted of glutamine. Aortic segments were incubated in this preparation to determine whether L-glutamine at its concentration in normal rat plasma can increase tissue glutamate content. Rat plasma obtained by decapitation was partially deprived of glutamate 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 with glutamate eluted further. After the loaded column was washed with 10 ml of 20 mM l-leucine, the glutamate fraction was eluted with 3 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-
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, 25, and 50 μM) were carried through the procedure.

Estimation of nitrate content as index of NO formation. Rats were fasted for 20 h, and thereafter three sequential, 1-cm segments of the proximal thoracic aorta were prepared, halved vertically into hemisegments, and incubated for 1 h at 37°C in KRB medium containing 200 μM L-arginine. The control hemisegment was incubated in the absence and the experimental hemisegment in the presence of 0.5 mM L-glutamine, and thereafter total nitrate contents of tissues and media were estimated by cadmium/copper-mediated reduction to nitrite with a kit and directions supplied by BioAssay Systems (Hayward, CA). Estimation of tissue cGMP. After incubation, tissues were weighed, immediately added to 0.1 ml of 0.1 N HCl in Eppendorf tubes, and frozen in a dry ice-ethanol bath. Thawed samples were homogenized with a polytron homogenizer at setting 5 for 30 s, and centrifuged as described above to yield aqueous extracts, which were acetylated and assayed for cGMP by competitive enzyme immunoassay with a kit and directions supplied by Assay Designs.

Contractile responses of aortic rings. The procedure and apparatus described previously (36) were used with minor modifications. The standard incubation medium described above (see Preparation and incubation of aortic slices) contained 100 μM L-arginine and was equilibrated with 5% CO2-95% O2 and recirculated at a rate of 32 ml/min from a 250-ml reservoir through a water-jacketed 50-ml chamber maintained at 37°C. Four aortic rings were cut from the thoracic aorta, 15–40 mm from the aortic valve ring, and each was mounted via stainless steel hooks to record isometric tension via a force transducer linked to a Macintosh computer using MacLab software. Segments were mounted within 10–15 min after the death of the animal, and the initial tension was adjusted to 1.0 g and so maintained for 90–120 min. Thereafter, the incubation medium was replaced, the segments were precontracted with 1 μM phenylephrine, and when plateau tension was attained test compounds were added. In some experiments the endothelium was denuded as described previously (36) before the aortic rings were mounted. 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, Nω-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). Statistical significance (P < 0.05) was evaluated by the t-test of paired comparisons or by ANOVA. Unless indicated otherwise, values are expressed as means ± SE and tissue concentrations are listed per gram wet weight of tissue.

RESULTS

L-Glutamine and L-leucine as precursors of tissue L-glutamate in vitro. The glutamate content of 24 aortic hemisegments prepared as described in MATERIALS AND METHODS and not incubated was 793 ± 147 nmol/g. Table 1 lists the glutamate values resulting from the subsequent incubation of hemisegments in various media. When hemisegments were incubated in standard KRB-10 mM D-glucose alone, tissue glutamate was not significantly different from that of nonincubated segments.

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>Control (KRB alone)</td>
<td>Tissue</td>
</tr>
<tr>
<td>Plasma amino acid mix*</td>
<td>12 (10)</td>
<td>+ amino acid mix</td>
<td>776 ± 52</td>
</tr>
<tr>
<td>L-Glutamine (0.5 mM)</td>
<td>6 (6)</td>
<td>+ L-glutamine</td>
<td>792 ± 62</td>
</tr>
<tr>
<td>L-Leucine (0.2 mM)</td>
<td>14 (12)</td>
<td>+ L-leucine‡</td>
<td>844 ± 84</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 each 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 test 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/min 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 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 glutamate (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 < 0.001).

**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 < 0.001); 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-[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,

![Fig. 1. Formation of L-glutamate from L-glutamine by aortic hemisegments incubated in vitro. Hemisegments pairs were prepared and incubated as described in MATERIALS AND METHODS and Table 1. Each pair comprised a control not treated with L-glutamine and an experimental hemisegment treated with a given concentration of L-glutamine, and 5 pairs from 5 rats were used to test each concentration. Values are mean ± SE differences between control and experimental hemisegments. Note the large difference between the vertical scale for the tissue glutamate values (left) and the vertical scale for the ambient medium and tissue + medium values (right).](http://ajpcell.physiology.org/)

### Table 2. Effects of carrier L-leucine concentration on content of tissue glutamate, net synthesis of glutamate, and synthesis of [¹⁴C]glutamate from [¹⁴C]leucine

<table>
<thead>
<tr>
<th>L-[¹⁴C]Leucine Concentration, μM</th>
<th>Increment in Tissue Glutamate, nmol/g</th>
<th>Tissue Final Glutamate Content, nmol/g</th>
<th>Tissue Ratio [¹⁴C]Glutamate/Total Glutamate, %</th>
<th>Tissue + Medium Final Glutamate Content, nmol/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>130 ± 32</td>
<td>−151 ± 97</td>
<td>439 ± 112</td>
<td>31.0 ± 3.4</td>
</tr>
<tr>
<td>100</td>
<td>603 ± 84</td>
<td>551 ± 178</td>
<td>1,628 ± 325</td>
<td>36.1 ± 3.0</td>
</tr>
<tr>
<td>250</td>
<td>757 ± 106</td>
<td>354 ± 86</td>
<td>1,436 ± 261</td>
<td>54.7 ± 4.9</td>
</tr>
<tr>
<td>1,000</td>
<td>770 ± 133</td>
<td>13 ± 118†</td>
<td>1,276 ± 244</td>
<td>61.0 ± 3.6*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Aortic hemisegments pairs were prepared as described in Table 1 and MATERIALS AND METHODS. Five pairs prepared from 5 rats were used to test each leucine concentration. The control hemisegment of each pair was incubated in standard KRB-10 mM d-glucose medium and the experimental hemisegment in the same medium plus L-[¹⁴C]leucine as indicated. Incubations were for 1 h at 37°C under 5% CO₂-95% O₂. *For the 15 hemisegment pairs used to test leucine at 100–1,000 μM, the final mean values for total (tissue + medium) glutamate for the control (no leucine) and experimental hemisegments were 1,560 ± 93 and 1,981 ± 176 nmol/g, respectively. These values differ significantly (t-test of paired comparisons of hemisegment pairs, t = 2.98, P < 0.005) and indicate leucine-dependent net formation of glutamate. † Δ Tissue glutamate is the difference between the control hemisegment incubated without and the experimental hemisegment incubated with leucine. The value at 1,000 μM leucine is significantly less than that at 100 μM (P < 0.025). § P < 0.001 for difference of % observed at 100 μM and 1,000 μM leucine.
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 0.85 and 0.55 mM leucine were 603 and 655 nmol/g, respectively. Thus the [14C]glutamate values of the untreated hemisegment were similar, 534 and 511 nmol/g, respectively.

Effects of L-glutamine on the synthesis of [14C]glutamate from L-[U-14C]leucine by aortic hemisegments. In each of 5 experiments aortas from 2 rats were excised, 4 hemisegment pairs were prepared from each aorta (MATERIALS AND METHODS), and corresponding hemisegments were pooled for incubation. Each concentration of L-glutamine was tested with 5 hemisegment pairs incubated for 1 h at 37°C in the standard Krebs-Ringer bicarbonate (KRB)-10 mM D-glucose medium containing 100 µM L-[U-14C]leucine. Values are means ± SE for 5 hemisegments treated with each L-glutamine concentration. The value at 0 L-glutamine is the mean ± SE of the 20 control hemisegments.

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, P = 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 [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 are shown in Table 4.
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-[1-14C]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-[1-14C]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, \( F = 8.91, P < 0.01 \)).

Fig. 3. Effects of 0.5 mM L-glutamine on the time course of metabolism of L-[U-14C]arginine by aortic hemisegments. Data are means ± SE for the following numbers of hemisegment pairs (no. of rats) tested at 15, 30, and 60 min, respectively: 4 (3 rats), 8 (6 rats), and 5 (5 rats). For each time point control hemisegments were incubated in the absence and experimental hemisegments in the presence of 0.5 mM L-glutamine in KRB-10 mM D-glucose containing the plasma L-amino acid mix (AA mix) (minus L-glutamine). AA mix contains 184 μM L-arginine (MATERIALS AND METHODS), and tracer L-[U-14C] arginine was added. Incubations were at 37°C under 5% CO2-95% O2; Two-way ANOVA for the effects of L-glutamine yielded \( F = 8.91, P < 0.01 \).

The effects of the nitric oxide (NO) donor 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5) on the oxidative decarboxylation of [1-14C]κ-ketoglutarate by aortic segments. Aortic hemisegment pairs 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 medium containing 0.2 mM L-[1-14C] 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 2 (\( F = 54.37 \)); \( P < 0.02 \) 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 hemisegments were examined, and the results are listed in Table 5. NOC-5 significantly decreased [14C]glutamate formation, with a 50% inhibition concentration of ∼210 μM. Tissue total glutamate and the percentages of [14C]glutamate in 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 incubated in the absence and presence of the indicated concentrations of NOC-5 in the standard KRB-10 mM D-glucose medium prepared to contain 0.1 mM L-[1-14C]κ-ketoglutarate for 40 min at 37°C. Values for the decarboxylation and release of 14CO2 are means ± SE for 21 control hemisegments (no NOC-5) and for 5, 5.5, and 11 experimental hemisegments treated with 0.05, 0.25, and 1.25 mM NOC-5, respectively. One-way ANOVA for the effect of NOC-5 yielded \( F = 42.4, P < 0.0001 \).

Fig. 4. Effects of the nitric oxide (NO) donor 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5) on the oxidative decarboxylation of [1-14C]κ-ketoglutarate by aortic segments. Aortic hemisegments pairs 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 medium containing 0.2 mM L-[1-14C] 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 2 (\( F = 54.37 \)); \( P < 0.02 \) for effects on total glutamate content (\( F = 3.03 \)).

![Table 4. Effects of L-glutamine in vitro on aortic tissue metabolism of L-[U-14C]arginine](image1)

<table>
<thead>
<tr>
<th>L-Arginine, mM</th>
<th>L-Glutamine, mM</th>
<th>Tissue Arginine Metabolite, nmol/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>0.50</td>
<td>0</td>
<td>20.0 ± 2.4</td>
<td>15.4 ± 1.4</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>0.25</td>
<td>0.50</td>
<td>34.9 ± 3.9</td>
<td>13.2 ± 1.4</td>
</tr>
<tr>
<td>0.50</td>
<td>0</td>
<td>28.5 ± 3.2</td>
<td>10.3 ± 0.8</td>
</tr>
<tr>
<td>0.25</td>
<td>0.50</td>
<td>38.5 ± 3.9</td>
<td>15.2 ± 1.2</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. AA mix-depleted of L-glutamine. Data are means ± SE for 21 control hemisegments (no. of incubation vessels). Aortic hemisegment pairs prepared from 10 rats were used at each concentration of L-[U-14C]arginine. Control and experimental hemisegments were incubated in KRB medium containing the plasma AA mix (AA mix) (minus L-glutamine). AA mix isopropyl-2-oxo-1-triazene (NOC-5) and experimental (treated with indicated NOC-5 concentration) hemisegments were incubated in KRB-10 mM D-glucose medium containing 0.2 mM L-[1-14C] 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 2 (\( F = 54.37 \)); \( P < 0.02 \) for effects on total glutamate content (\( F = 3.03 \)).

![Table 5. Effects of nitric oxide donor NOC-5 on formation of [14C]glutamate from L-[U-14C]leucine by aortic hemisegments in vitro](image2)

<table>
<thead>
<tr>
<th>NOC-5, μM</th>
<th>n</th>
<th>[14C]glutamate (a) Total glutamate (b)</th>
<th>ab/a, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>28</td>
<td>265 ± 16</td>
<td>567 ± 33</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td>242 ± 28</td>
<td>607 ± 61</td>
</tr>
<tr>
<td>200</td>
<td>5</td>
<td>141 ± 19</td>
<td>459 ± 55</td>
</tr>
<tr>
<td>300</td>
<td>5</td>
<td>117 ± 24</td>
<td>444 ± 89</td>
</tr>
<tr>
<td>400</td>
<td>5</td>
<td>73 ± 23</td>
<td>398 ± 62</td>
</tr>
<tr>
<td>1,500</td>
<td>5</td>
<td>12 ± 7</td>
<td>318 ± 74</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 medium containing 0.2 mM L-[1-14C] 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 2 (\( F = 54.37 \)); \( P < 0.02 \) for effects on total glutamate content (\( F = 3.03 \)).
tested with 0.1 mM [1-14C]-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 \( \sim 110 \) \( \mu \)M.

**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 relaxation rate from the value in intact rings of 14.31 \( g \cdot g^{-1} \cdot \text{min}^{-1} \), respectively \((P < 0.005)\). The corresponding percentages of the phenylephrine contractions were 24.7 \( \pm \) 3.5 vs. 54.0 \( \pm \) 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 \( g \cdot g^{-1} \cdot \text{min}^{-1} \) to 4.08 \( g \cdot g^{-1} \cdot \text{min}^{-1} \) 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 \( g \cdot g^{-1} \cdot \text{min}^{-1} \) in controls to 2.43 \( g \cdot g^{-1} \cdot \text{min}^{-1} \) 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 \( \pm \) 5.7 pmol/g, was increased to 111.5 \( \pm \) 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 \( \pm \) 1.5 and 2.9 \( \pm \) 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...
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

²L-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 endogenously supplied L-arginine to citrulline and NO. Furthermore, L-glutamine at its plasma concentration (0.5 mM) inhibited the formation of [14C]glutamate from [14C]leucine by 21.2% in the presence 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-glutamine.
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 NO activity for NO formation in aortic segments remain to be characterized fully, but it is likely that one such increase is 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 differently to L-glutamine. In cultures of bovine aortic (3, 38) and venular (25) endothelial cells L-glutamate (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 NO (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 glutosamine inhibits NO synthesis in endothelial cells and that the utilization of L-glutamine as a precursor for glutamine-6-phosphate synthesis in cultured endothelial cells could explain L-glutamine inhibition of NO formation. In their studies glutamine-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-glutamate is required for synthesis of hexosamine, which acts to decrease NO formation, whereas in the freshly isolated aortic segments studied in this report L-glutamate 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-glutamate at its normal concentration in plasma can increase tissue glutamate content, NO formation, and relaxation responses (Fig. 6). The relaxation responses to L-glutamate 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).

REFERENCES


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