Vol. 115, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1983 Pages 174-179

[^{15}N] LEUCINE AS A SOURCE OF [^{15}N] GLUTAMATE IN ORGANOTYPIC CEREBELLAR EXPLANTS

Marc Yudkoff, Itzhak Nissim, Seung Kim, David Pleasure, Klaus Hummeler and Stanton Segal

Division of Biochemical Development and Molecular Disease
The Children's Hospital of Philadelphia and the Departments
of Pediatrics and Medicine, the University of Pennsylvania School of Medicine,
Philadelphia, PA 19104

Received July 11, 1983

Approximately 26.0% of the [^{15}N] glutamate and [alpha ^{15}N] glutamine formed in organotypic cerebellar explants was derived from [^{15}N] leucine. Approximately 14.0% of the $^{15}NH_3$ and [amide ^{15}N] glutamine synthesized came from leucine nitrogen. Another 4.0% of the alpha nitrogen of both glutamate and glutamine was derived from [^{15}N] valine. These results suggest that branched-chain amino acids, particularly leucine, may be important for the synthesis of glutamic acid by the brain.

Several compounds are precursor to the carbon of glutamic acid in the brain. Glucose and glutamine are prominent in this regard (1). Less is known of the sources of the nitrogen in cerebral glutamate. Reductive amination of alpha-ketoglutarate occurs, but the steady state equilibrium of the glutamate dehydrogenase reaction may favor deamination of glutamate (2). Transamination reactions constitute another important source of glutamate nitrogen. In skeletal muscle transamination of the branched-chain amino acids, especially leucine, provides much of the nitrogen incorporated into pyruvate to form alanine, with glutamate an intermediate in the reaction sequence (3)(4)(5). Since leucine crosses the blood brain barrier more readily than most other amino acids (6), it might be particularly important for brain glutamate synthesis as well.

In this study we utilized gas chromatography-mass spectrometry to measure the fraction of glutamate nitrogen derived from [^{15}N] leucine and [^{15}N] valine in organotypic cerebellar explants. We found that more than one-fourth the glutamate nitrogen in this culture system was derived from leucine alone.

0006-291X/83 $1.50

MATERIALS AND METHODS

[^{15}N] leucine and [^{15}N]valine (95 atom % excess) were purchased from KOR Isotopes. Culture media were from Gibco, Inc. Trifluoroacetic anhydride and butanol/3M HCl were from Regis Chemical Co. Glutaminase was purchased from Sigma and glutamate dehydrogenase from Boehringer-Mannheim.

Organotypic explants of newborn mouse cerebella were prepared and maintained as described previously (7). The cultures were all well myelinated at the time of use, some 14-21 days in vitro, as judged by phase contrast light microscopy. Glass coverslips containing the organotypic explants were suspended in 3 ml of Eagles's MEM in 18mm x 150mm glass tubes. Each tube contained 1 coverslip on which were a total of two individual explants. Following a 15 min pre-incubation at 37°C, 15 microliters of 7.63 mM [^{15}N] leucine or [^{15}N] valine were added to start the experiment. After incubation for the times indicated below, the medium was withdrawn and the cells were washed rapidly with 3 ml. of cold, sterile 0.9% saline. Three ml of .01N HCl were then added to each tube and the cells were frozen and thawed three times to liberate intracellular amino acids.

Isotopic enrichment in $^{15}NH_3$ was determined in an aliquot of the .01N HCl supernatant according to a method we have described elsewhere (8). Enrichment in [^{15}N] leucine and [^{15}N] GABA was measured in the n-butyl-N-trifluoroacetyl (N-TFA) derivative (9), formed after drying an aliquot of the .01N HCl supernatant. To determine isotopic abundance in [^{15}N]glutamate, [amide ^{15}N] glutamine and [alpha ^{15}N] glutamine, glutamate and glutamine were isolated by taking an aliquot of the .01N HCl supernatant to dryness under nitrogen and redissolving the residue in 1 ml. of 0.5M imidazole-citrate-Tris buffer, pH 7.0. This was applied to a 3.5 x 0.5 cm. column of AG-1 resin, 100-200 mesh, in the chloride form. The eluate, containing glutamine, was saved together with a 2 ml water wash. The glutamate fraction was then eluted from the column with 3.5N HCl and the N-TFA derivative was prepared after drying the fraction under nitrogen (9). To determine separate ^{15}N isotopic abundance in the amide and alpha nitrogens of glutamine, 1 ml of a slurry of AG-1 (OH-) resin, 100-200 mesh, X-8, was added to the glutamine fraction. After vortexing for 15 seconds, the resin was allowed to settle and the supernatant was discarded. The glutamine, which was quantitatively bound to the resin (10), was eluted by adding 3 ml. of 3.5N HCl. The samples were then placed in a lyophilizer for a minimum of 24 hours and the residue was dissolved in 1 ml of 0.2 M sodium acetate buffer, pH 5.0. After incubation with glutaminase for 90 minutes at 37°C, a total of 20 drops of AG-1 (OH-) resin was added. After vortexing for 15 seconds, the supernatant, containing ammonia derived from the enzymatic hydrolysis of glutamine, was analyzed for ^{15}N according to a method described elsewhere (10). The alpha-amino glutamine nitrogen, now present in glutamate formed subsequent to the enzymatic hydrolysis, was bound to the AG-1 resin from which it was eluted with 3 ml of 3N HCl. After drying under nitrogen, this was derivatized according to Roach and Gehrke (9).

Gas chromatography-mass spectrometry was done on the n-butyl-N-trifluoracetyl amino acid derivatives on a Finnigan 4021 instrument on line with the INCOS data system. Instrument conditions have been described elsewhere (8,10). The m/e 198/199 fragments were monitored for determination of isotopic enrichment in glutamate, whether representing glutamate itself or the amide or alpha-nitrogen of glutamine or ammonia which were converted to glutamate. The m/e 182/183 fragments were monitored for determining ^{15}N in leucine and GABA. Fragments at m/e 166 and m/e 167 were followed for determination of [^{15}N]valine enrichment. For determination of [^{15}N] alanine and [^{15}N] aspartate we used ions at m/e 140/141 and m/e 184/185, respectively. Isotopic enrichment was determined according to the formula of Biemann (11).

RESULTS

As shown in Figure 1, steady-state (2 hour) intracellular enrichment in leucine and valine was 11.0-13.0 atom % excess. With [^{15}N] leucine as precursor, isotopic

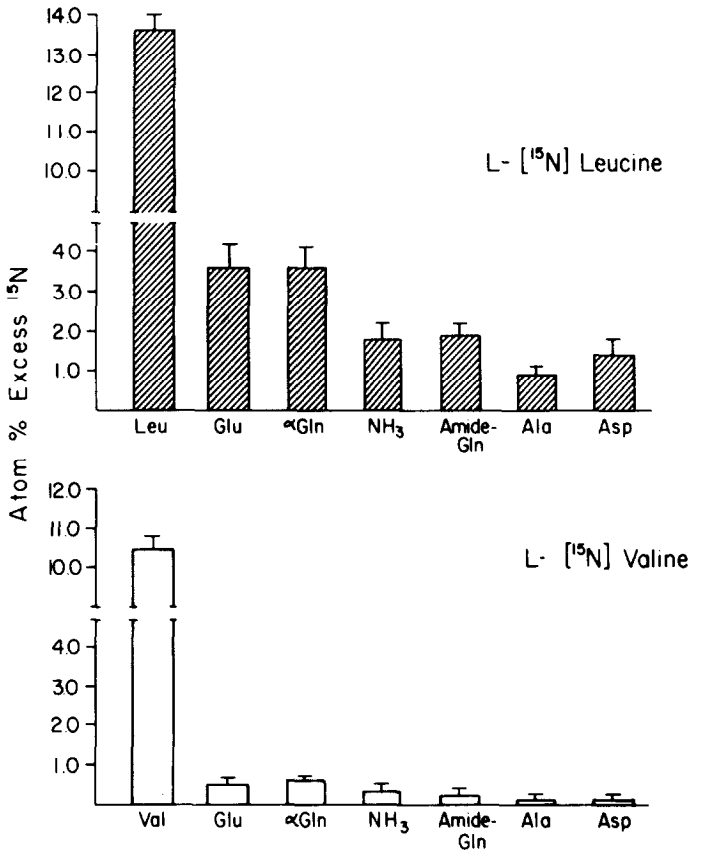

Figure 1: Isotopic abundance (atom % excess ^{15}N) in leucine, valine, glutamine (alpha and amide N), glutamate, ammonia, alanine and aspartate. Top: after addition of [^{15}N] leucine to organotypic cerebellar explants. Bottom: after addition of [^{15}N] valine. Enrichments determined from steady-state (2 hr) observations. Each value represents mean ± SD of 6 experimental observations.

abundance in glutamate, glutamine, ammonia, aspartate and alanine was considerably greater compared with [^{15}N] valine. Thus, enrichment in [^{15}N] glutamate was 3-4 atom % excess when labelled leucine was added to the incubation medium and only 0.4-0.5 atom % excess when [^{15}N] valine was added.

From the steady-state intracellular enrichments shown in Figure 1, we calculated the ratio of either [^{15}N] glutamate/[^{15}N] leucine or[alpha ^{15}N] glutamine/[^{15}N] leucine, defining the fraction of the alpha nitrogen of glutamate or glutamine derived from leucine. As indicated in Table 1, this ratio was approximately 26%. In contrast, only 4% of the alpha nitrogen of either glutamate or glutamine appeared to have been derived from valine nitrogen.

TABLE 1

FRACTION (%) ^{15}N PRODUCT FORMED FROM [^{15}N] LEU OR [^{15}N] VAL

PRODUCT	[^{15}N] LEU*	[^{15}N] VAL*
[^{15}N] Glutamate	26.3 ± 4.3	4.3 ± 1.4
[Alpha ^{15}N] Gln	25.8 ± 3.7	3.7 ± 1.1
[Amide ^{15}N] Gln	13.8 ± 1.8	3.4 ± 1.2
$^{15}NH_3$	13.5 ± 2.6	2.4 ± 0.6
[^{15}N] Alanine	6.8 ± 1.6	TR
[^{15}N] Aspartate	10.2 ± 2.3	TR

*Mean ± SD of 6 observations.

Similarly, about 14% of either [amide ^{15}N] glutamine or $^{15}NH_3$ was formed from [^{15}N] leucine and only 2-4% from [^{15}N] valine. Only trace amounts of alanine and aspartate nitrogen were synthesized from valine nitrogen and 7-10% from leucine.

To determine whether glutamic acid carbon as well as nitrogen was derived from leucine in the organotypic explants, L [2,3,6 - ^{13}C]leucine was added to the incubation medium to attain a [^{13}C] leucine enrichment of 15.0 atom % excess. After incubation for 2 hours, no enrichment of glutamate carbon could be detected. The analysis was based on isotopic abundance in the n-butyl-N-trifluoroacetyl glutamate derivatives at ions m/e 198 and m/e 201. These fragments contain all glutamate carbon except the carboxyl carbon.

DISCUSSION

The sources of brain glutamate are of clinical and biochemical importance because of the roles glutamate occupies as a neurotransmitter and a precursor to both GABA and glutamine. We found that essentially no glutamate carbon is derived from leucine, but that approximately 25% of glutamate nitrogen is formed from leucine nitrogen in organotypic cerebellar explants. Another 4.0% of glutamate nitrogen appeared to have been derived from valine. Although [^{15}N] isoleucine was not available for study, even if the contribution of this amino acid to glutamate nitrogen were no greater than that of valine, approximately one-third of glutamate nitrogen would have been formed from the three branched-chain amino acids. Branched chain amino acid transaminase activity is known to be high

in heart and skeletal muscle (12), but less is known about this reaction in brain. In skeletal muscle a single enzyme appears to mediate transamination of all three branched chain amino acids, but our data indicate that the leucine contribution to glutamate nitrogen is greater than that of valine (Fig. 1). Whether this implies a specific leucine aminotransferase or another mechanism favoring flux of leucine nitrogen into glutamate can not be determined from the present data.

Some caution should be exercised before extrapolating from the results of this in vitro investigation to an in vivo role for leucine in glutamate synthesis. The current experiments were conducted under artificial conditions, since the incubation medium contained no non-essential amino acids, which undoubtedly are a critical source of glutamate nitrogen, and the medium leucine concentration (398 μM) was much greater than would be expected to occur in the blood. Notwithstanding these caveats, however, our data do suggest a possible physiological role for leucine as an important nitrogen donor in the brain.

Leucine would be well suited for such a role because of the felicity with which this amino acid traverses the blood-brain barrier (6). It has been suggested (13) that blood leucine is an important source of the glutamate utilized for synthesis of glutamine from glutamate and ammonia, this reaction being an important mechanism of cerebral ammonia disposition. It is of interest that the infusion of branched-chain amino acids has been reported to have a salutary clinical effect in the management of hepatic coma (14)(15), in which the brain becomes exquisitely sensitive to the toxic effects of ammonia (16). To the extent that hepatic encephalopathy is referable to ammonia toxicity, the favorable clinical effect of leucine infusions may relate to an enhancement of ammonia detoxification by making available more glutamate for glutamine synthesis, since we have found this pathway to be a prime route of ammonia disposition in cerebellar explants (17).

Acknowledgement: Supported by grants HD 08536 and NS 17752 from the NIH.

REFERENCES

1. Hamberger, A.C., Chiang, G.H., Nylen, E.S., Scheff, S.W. and Cotman, C.W. (1979) Brain Res. 168:513-530.
2. Yu, A.C., Schousboe, A. and Hertz, L (1982) J. Neurochem. 39:954-960.

3. Odessey, R., Khairallah, E.A. and Goldberg, A.L. (1974) J. Biol. Chem. 249: 7623-7629.
4. Garber, A.J., Karl, I.E., and Kipnis, D.M. (1976). J. Biol. Chem. 251:836-843.
5. Chang, T.W. and Goldberg, A.L. (1977) J. Biol. Chem. 253:3677-3684.
6. Oldendorf, W.M. (1971) Am. J. Physiol. 221:1629-1639.
7. Pleasure, D and Kim, S.U. (1976) Brain Res. 104: 193-196.
8. Nissim, I., Yudkoff, M., Yang, W., Terwilliger, T. and Segal, S. (1981) Anal. Bioch. 114:125-130
9. Roach, D. and Gehrke, C.W. (1969) J. Chromatog. 44:269-278.
10. Yudkoff, M., Nissim, I. and Segal S. (1982) Clin. Chim. Acta 118:159-166.
11. Biemann, K. (1962) Mass Spectrometry, in Organic Chemical Applications, Biemann K., ed., McGraw-Hill, New York, pp. 223-227.
12. Ichihara A., Noda, C., and Tanaka, K. (1981). Oxidation of Branched Chain Amino Acids with Special Reference to Their Transaminase, in Metabolism and Clinical Implications of Branched Chain Amino and Ketoacids, eds. Walser, M., and Williamson, J.R., Elsevier North Holland, New York, pp. 227-231.
13. Cooper, A.J.L., McDonald, J.M., Gelbard, A.S., Gledhill, R.F., and Duffy, T.E. (1979). J. Biol. Chem. 254:4982-4992.
14. Fischer, J.E., Funovics, J.M., Aguirre, A. et al. (1975) Surgery 78:276-290.
15. Fischer, J.E., Rosen, H.M., Ebeid, A.M., James, J.H., Keane, J.M. and Soeters, P.B. (1976) Surgery 80:77-91.
16. Ehrlich, M., Plum, F., and Duffy, T.E. (1980) J. Neurochem. 34:1538-1542.
17. Yudkoff, M., Nissim, I., Kim, S.U., Pleasure, D. and Segal, S. J. Neurochem, in press.