Exogenous Glutamate Concentration Regulates the Metabolic Fate of Glutamate in Astrocytes

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Abstract: The metabolic fate of glutamate in astrocytes has been controversial since several studies reported >80% of glutamate was metabolized to glutamine; however, other studies have shown that half of the glutamate was metabolized via the tricarboxylic acid (TCA) cycle and half converted to glutamine. Studies were initiated to determine the metabolic fate of increasing concentrations of [U-13C]glutamate in primary cultures of cerebral cortical astrocytes from rat brain. When astrocytes from rat brain were incubated with 0.1 mM [U-13C]glutamate 85% of the 13C metabolized was converted to glutamine. The formation of [1,2,3-13C3] glutamate demonstrated metabolism of the labeled glutamate via the TCA cycle. When astrocytes were incubated with 0.2—0.5 mM glutamate, 13C from glutamate was also incorporated into intracellular aspartate and into lactate that was released into the media. The amount of [13C] lactate was essentially unchanged within the range of 0.2—0.5 mM glutamate, whereas the amount of [13C]aspartate continued to increase in parallel with the increase in glutamate concentration. The amount of glutamate metabolized via the TCA cycle progressively increased from 15.3 to 42.7% as the extracellular glutamate concentration increased from 0.1 to 0.5 mM, suggesting that the concentration of glutamate is a major factor determining the metabolic fate of glutamate in astrocytes. Previous studies using glutamate concentrations from 0.01 to 0.5 mM and astrocytes from both rat and mouse brain are consistent with these findings. Key Words: 13C NMR spectroscopy—[U-13C]-Glutamate — Astrocytes — Energy metabolism — Lactate — Glutamine.


It is well established that astrocytes metabolize extracellular glutamate to glutamine (Yu et al., 1982; Waniekewski and Martin, 1986; Zielke et al., 1990; Farinelli and Nicklas, 1992), which, in turn, is released from astrocytes for metabolic trafficking to neurons where it can function as a precursor for neurotransmitter glutamate (Shank and Aprison, 1988; Shank and Campbell, 1982, 1984). However, several studies have also reported that a considerable proportion of the glutamate taken up by astrocytes is metabolized via the tricarboxylic acid (TCA) cycle (Yu et al., 1982; Yudkoff et al., 1988; Schousboe et al., 1993; Sonnewald et al., 1993a; Bachelard et al., 1994) and converted to deaminated products, including lactate, rather than being directly converted to glutamine. The release of lactate by astrocytes has been demonstrated by several groups (Walz and Mukerji, 1988; Larrabee, 1992; Sonnewald et al., 1993a), and considerable evidence exists that lactate is an effective substrate for maintaining energy metabolism in synaptic terminals (Schurr et al., 1988, 1989; McKenna et al., 1993, 1994).

Overall, the previous studies have yielded conflicting results regarding the relative amount of glutamate metabolized via the TCA cycle versus the amount directly converted to glutamine (Yu et al., 1982; Waniekewski and Martin, 1986; Yudkoff et al., 1988; Zielke et al., 1990; Sonnewald et al., 1993a). In addition, the interpretation of these studies is confounded by differences in the experimental conditions and species used. It is important to determine the regulation of glutamate metabolism by astrocytes because the ability to convert glutamate to pyruvate (either via malic enzyme or phosphoenolpyruvate carboxykinase) provides a mechanism whereby TCA cycle activity in astrocytes can be maintained when levels of substrates such as glucose and ketone bodies are low. Evidence for the synthetic portion of a “pyruvate recycling pathway” in astrocytes has been provided by Sonnewald et al. (1993a), who demonstrated lactate formation from glutamate. However, the conditions under which this pyruvate is recycled via the TCA cycle are unclear.

In the present study we determined the metabolites...
produced by astrocytes incubated with varying concentrations of [U-13C] glutamate. The study was designed to determine if the concentration of glutamate was important in regulating the relative amount of glutamate metabolized via the TCA cycle and converted to lactate and aspartate, versus the amount directly converted to glutamine. The results of the present study demonstrate that extracellular glutamate concentration is a key factor regulating the metabolic fate of glutamate in astrocytes. Furthermore, it is important to note that the present study resolves the apparent conflict in the literature regarding the metabolic fate of glutamate in astrocytes. The results of the majority of the previous studies with both rat and mouse astrocytes are consistent with our findings (Wanienski and Martin, 1986; Yudkoff et al., 1988; Zielke et al., 1990; Farinelli and Nicklas, 1992; Wanienski, 1992; Sonnewald et al., 1993a).

MATERIALS AND METHODS

Materials

Tissue culture dishes (Nunc) were purchased from Vanguard International (Neptune, NJ, U.S.A.). Culture media (minimal essential medium with Earle's salts), nonessential amino acids, Dulbecco's phosphate-buffered saline, and fetal bovine serum were purchased from Paragon Biotech (Baltimore, MD, U.S.A.). Nylon screening cloth (Nitex) was obtained from Tetko (Elmsford, NY, U.S.A.). Timed-pregnant female rats were purchased from Zivic Miller Laboratories (Zelienople, PA, U.S.A.). Amino acid standards for HPLC, o-phthalaldehyde, and lactate oxidase reagent were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Solutions for the Pierce bicinchoninic acid microreagent protein assay were purchased from Pierce (Rockford, IL, U.S.A.). All reagents and chemicals were of the highest analytical grade. The stable isotope [U-13C] glutamate (99% enriched) was from Cambridge Isotopes (Andover, MA, U.S.A.).

Cell preparation

Astrocytes were prepared from newborn rat brain as described by Zielke et al. (1990), by a method based on a procedure by Bochor and Sensenbrenner (1972) with some alterations. Cerebral hemispheres were removed, placed in media with 10% fetal bovine serum, cleaned of meninges, and trimmed to retain the neopallium. The isolated neopallia were transferred to a 100-mm-diameter dish containing fresh media with 90—95% humidity. The culture medium was replaced at a density of three brains per four flasks. The cells were aggregetated by vortex-mixing (60 s) to destroy most cells, was seeded in 175-cm² Falcon plastic T-flasks vessels and aggregated cells. The cell suspension, enriched with literature values, was obtained using a 30° pulse angle. An acquisition time of 1.049 s and an additional relaxation delay of 3 s were used. The number of scans was typically 4,000 for PCA extracts and 800 for the media. To avoid nuclear Overhauser effects, some spectra were also broadband decoupled only during acquisition. From the two sets of spectra, correction factors were obtained and applied to all spectra. Inversion recovery experiments were performed to obtain relaxation (T₁) values. Correction for saturation was not necessary. The total amount of 13C in the resonance of a particular metabolite was calculated using dioxane as an internal concentration standard. Percent incorporation was calculated, after subtraction of natural abundance, as described by Barany-Goffler et al. (1990). A line broadening of 5 KHz was used. Chemical shifts are reported relative to the dioxane peak at 67.4 ppm. Assignments were made by comparison with literature values (Barany et al., 1985) and by comparison with the spectra of glutamine, glutamate, lactate, and aspartate obtained under similar conditions. Cells from three or four culture flasks were pooled for spectra of PCA extracts. The recovery of 13C ranged from 75 to 83%.

Statistical analysis

The data obtained from incubations with each glutamate concentration were analyzed by ANOVA and the Student—Newman—Keuls multiple comparisons test to determine significant differences (Snedecor and Cochran, 1967). The data in Tables 1—3 are from five different experiments with dif-
different preparations of astrocytes, so that each concentration could be repeated several times. The samples incubated with 0.3 and 0.4 mM glutamate were done in duplicate, and these data were combined for data analysis because they did not provide additional information.

RESULTS

Astrocytes metabolized [U-13C] glutamate to several products; however, the amount and presence of several of these metabolites were dependent on the concentration of glutamate in the media. As can be seen from the 13C NMR spectra of the cell extracts, astrocytes incubated with 0.1 mM [13C] glutamate contained primarily [13C] glutamate intracellularly (Fig. 1, bottom), whereas in astrocytes incubated with concentrations of glutamate of ≥0.2 mM 13C-labeling was also present in intracellular aspartate (Fig. 1, top). Uniformly labeled aspartate was the only isotopomer observed, indicating that a substantial portion of the oxaloacetate formed from glutamate was directly converted to aspartate rather than remaining in the TCA cycle. Furthermore, as the external glutamate concentration increased from 0.2 to 0.5 mM the amount of aspartate formed increased two- to threefold (Table 1). The aspartate levels are somewhat lower than those reported by Farinelli and Nicklas (1992) and slightly higher than those reported by Yudkoff et al. (1990).

The 1,2,3-13C3-isotopomer (J2,3 = 34.5 Hz) of glutamate (glutamate doublet) was present under all experimental conditions (Fig. 1 and Table 1), indicating that the intracellular glutamate pool also contained glutamate that had been metabolized via the TCA cycle and converted back to glutamate. The amount of the [1,2,3-13C3] glutamate isotopomer increased two- to threefold as the extracellular glutamate concentration increased from 0.1 to 0.5 mM. However, the percent doublet decreased from 24 to 10% under these conditions owing to the fact that the total intracellular [U-13C] glutamate level increased eightfold. This significant increase in total intracellular glutamate concentration was in direct correlation with the increase in extracellular glutamate concentration, in agreement with an earlier report by Farinelli and Nicklas (1992). The intracellular glutamate concentrations observed in the present study are considerably higher than those reported by Schousboe et al. (1993) but are in reason-
able agreement with the concentrations found by other investigators using astrocytes from rat brain (Zielke et al., 1990). This finding is consistent with other reports that glutamate oxidation is more active in astrocytes from mouse brain than in those from rat brain (Waniewski and Martin, 1986). It is interesting that the total intracellular glutamine concentration remained constant despite the increase in extracellular and intracellular glutamate concentration. The intracellular glutamine concentrations are comparable to those reported by Yudkoff et al. (1990) in astrocytes incubated with 0.9 mM glutamate.

Analysis of the media demonstrated that much of the [13C]glutamate was converted to glutamine and lactate, which were subsequently released by the astrocytes (Fig. 2 and Table 2). The amount of [13C]glutamate released into the media increased with extracellular glutamate concentration. Only uniformly labeled glutamine was observed in the media. The total glutamine concentration in the media almost doubled (Table 2). The amount of lactate produced by the astrocytes was not affected by the extracellular glutamate concentration but was essentially constant throughout the study (Table 2, data in parentheses). The 1,2,3-13C isotopomer (J2,3 = 36.6 Hz) of lactate (lactate doublet; Fig. 2, top) was present in the media of astrocytes incubated with 0.2–0.5 mM glutamate (Table 2). However, as can be seen from the 13C NMR spectra, there was no lactate doublet in the media from cells incubated with 0.1 mM glutamate (Fig. 2, bottom) or lower concentrations (data not shown). Thus, astrocytes incubated with glutamate concentrations of ≥0.2 mM produced lactate from both glycolysis and glutamate metabolism, whereas astrocytes incubated with ≤0.1 mM concentrations of glutamate produced lactate only via glycolysis. There was no evidence that the differences in metabolism observed were due to depolarization of the astrocytes at the higher glutamate concentrations because there was no difference in the mobilization of Ca2+ in cells incubated with 0.1 and 0.2 mM glutamate (M. C. McKenna and J. P. Kao, unpublished data).

Alanine content increased in the media with increasing glutamate concentration; however, no NMR-detectable 13C was found in alanine in any of the experiments, indicating that the carbon skeleton of alanine was derived from glycolysis rather than from the metabolism of glutamate. This finding suggests that the pool of pyruvate synthesized from the [13C]glutamate did not exchange with the pool of pyruvate available for alanine biosynthesis in astrocytes.

**DISCUSSION**

A considerable body of evidence confirms that astrocytes are the major site for the uptake (Schousboe et al., 1977; Erecinska and Silver, 1990) and metabolism (Shank and Campbell, 1982; Yudkoff et al., 1988, 1992; Zielke et al., 1990; Farinelli and Nicklas, 1992) of glutamate. The release of neurotransmitter glutamate, uptake and conversion to glutamine by astrocytes, and subsequent release of glutamine by astrocytes for utilization by neurons for neurotransmitter biosynthesis constitutes the “glutamate–glutamine cycle” (Hertz, 1979; Shank and Aprison, 1981). It is now well established that this cycle is not stoichiometric (Shank and Aprison, 1988) and that astrocytes may...
FIG. 2. $^{13}$C NMR spectra of metabolites from the media of cultured cerebral cortical astrocytes incubated for 2 h in the presence of 0.5 mM [U-$^{13}$C]glutamate (top) and in the presence of 0.1 mM [U-$^{13}$C]glutamate (bottom). GLN, glutamine; GLU, glutamate; LAC, lactate.

provide many other substrates to neurons and synaptic terminals for energy and neurotransmitter biosynthesis (Shank and Aprison, 1988; McKenna et al., 1989, 1990; Auestad et al., 1991; Peng et al., 1991; Sonnewald et al., 1993a,b; Westergaard et al., 1993). Nevertheless, the role of astrocytes in the removal of glutamate from the synaptic cleft and metabolism of glutamate is fundamental to maintaining overall homeostasis in brain (Yudkoff et al., 1992).

The data presented in the figures and tables demonstrate that at concentrations of <0.2 mM, the majority of label from the metabolism of [U-$^{13}$C] glutamate was found in glutamine, whereas at concentrations of ≥0.2 mM, label from [13C] glutamate was also found in lac-

### TABLE 2. Metabolites in the media from astrocytes incubated with [U-$^{13}$C]glutamate

<table>
<thead>
<tr>
<th>[U-$^{13}$C]Glutamate concentration (mM)</th>
<th>[U-$^{13}$C]Glutamine (nmol/mg of protein)</th>
<th>Glutamine (% enrichment)</th>
<th>[1,2,3-$^{13}$C]Lactate (nmol/mg of protein)</th>
<th>Alanine (nmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 mM</td>
<td>211.8 ± 83.5</td>
<td>15.3</td>
<td>ND</td>
<td>(134.1 ± 47.9)</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>138.9 ± 13.2</td>
<td>20.3 ± 2.4</td>
<td>ND</td>
<td>(60.9 ± 6.5)</td>
</tr>
<tr>
<td>(653.9 ± 49.6)</td>
<td></td>
<td></td>
<td>(5,479 ± 930)</td>
<td>(73.7 ± 3.6)</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>241.8 ± 37.9</td>
<td>35.9 ± 4.1</td>
<td>(4,881 ± 226)</td>
<td>(84.0 ± 10.4)</td>
</tr>
<tr>
<td>(631.1 ± 43.3)</td>
<td></td>
<td></td>
<td>(74.0 ± 11.1)</td>
<td></td>
</tr>
<tr>
<td>0.3–0.4 mM</td>
<td>344.4 ± 42.2</td>
<td>53.9 ± 7.6</td>
<td>(5,177 ± 183)</td>
<td>(4,613 ± 279)</td>
</tr>
<tr>
<td>(637.5 ± 43.3)</td>
<td></td>
<td></td>
<td>(74.6 ± 15.6)</td>
<td>(56.8 ± 14.5)</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>279.6 ± 35.7</td>
<td>51.9 ± 4.7</td>
<td>(5,479 ± 930)</td>
<td></td>
</tr>
<tr>
<td>(543.4 ± 66.0)</td>
<td></td>
<td></td>
<td>(74.6 ± 15.6)</td>
<td></td>
</tr>
</tbody>
</table>

Astrocytes were incubated with [U-$^{13}$C]glutamate (concentration given at the beginning of the experiment) for 2 h at 37°C as described in Materials and Methods. Data are mean ± SEM values for five to seven experiments, except for 0.05 mM, which is the average of data from two or three experiments. ND, not detected. The C-3 resonance was used for NMR quantification. Numbers in parentheses indicate total pool size determined by HPLC and assay as described in Materials and Methods.

Values with different superscripts are significantly different from other values in the same group by ANOVA and Student–Newman–Keuls test at p < 0.05.
LEVEL OF GLUTAMATE REGULATES ITS METABOLISM

TABLE 3. Metabolism of [U-13C]glutamate in astrocytes

<table>
<thead>
<tr>
<th>Extracellular glutamate concentration</th>
<th>% metabolized directly to glutamine</th>
<th>% metabolized via the TCA cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mM</td>
<td>84.7</td>
<td>15.3</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>65.9</td>
<td>34.1</td>
</tr>
<tr>
<td>0.3–0.4 mM</td>
<td>63.2</td>
<td>35.8</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>57.3</td>
<td>42.7</td>
</tr>
</tbody>
</table>


It is quite evident from the present studies that glutamate enters the TCA cycle and is resynthesized from TCA cycle intermediates even in astrocytes incubated with concentrations as low as 0.05 mM because the 1,2,3,13C isotope of glutamate was present under all experimental conditions. Our studies suggest that the intracellular glutamate, or TCA cycle constituents, must reach a higher concentration for aspartate and lactate biosynthesis from the glutamate carbon skeleton. The reason for this is not readily explained by the kinetics of the enzymes involved, e.g., aspartate aminotransferase, with a $K_m$ for glutamate of 0.34 mM; mitochondrial malic enzyme, with a $K_m$ for malate of 0.13 mM; glutamate dehydrogenase, with a $K_m$ for glutamate of 1.11 mM (M. C. McKenna and J. T. Tildon, unpublished data); and glutamine synthetase, with a $K_m$ for glutamate of 7 mM (Yudkoff et al., 1994). However, the formation of multienzyme complexes could influence metabolism. The amount of [13C]lactate and aspartate produced was not solely a function of the amount of glutamate entering the TCA cycle. This is evident because the amount of glutamate produced was essentially unchanged with 0.2–0.5 mM glutamate (Table 2), suggesting that a step in its synthesis via either malic enzyme (McKenna et al., 1990; Kurz et al., 1993) or phosphoenolpyruvate carboxylase (Hampecht et al., 1994) was saturated and rate limiting. In contrast, the amount of [13C]aspartate production via aspartate aminotransferase continued to increase in parallel with the increase in glutamate concentration. The percent 13C enrichment in the aspartate formed from [13C]glutamate was extremely high: 67 and 84% in cells incubated with 0.3–0.4 and 0.5 mM glutamate, respectively. These data demonstrate that there is a selective conversion of the glutamate carbon skeleton into aspartate in astrocytes consistent with the high specific activity found in aspartate in studies using [13C]glutamate (Zielke et al., 1990).

The apparent dissociation of the entry of [13C]-glutamate into the TCA cycle and the production of [13C]-aspartate suggests that the entry of glutamate into the TCA cycle is not all proceeding via aspartate aminotransferase, but that at lower glutamate concentrations other enzymes such as glutamate dehydrogenase or other transaminases might be involved in the conversion of glutamate to α-ketoglutarate. This concept is supported by earlier results by McKenna et al. (1993), demonstrating that glutamine oxidation by rat astrocytes was partially, but not completely, blocked by aminooxyacetate. Additional evidence is provided by recent studies by U. Sonnewald et al. (manuscript submitted for publication) demonstrating that aminooxyacetic acid had no effect on the entry of glutamate into the TCA cycle in mouse astrocytes.

The conversion of glutamate to lactate may represent a mechanism for the complete oxidation of the glutamate carbon skeleton for energy. Sonnewald et al. (1993a, b) have shown that recycling of TCA cycle constituents through pyruvate does occur in astrocytes. Such a recycling pathway might be important in maintaining energy metabolism when pyruvate or acetyl-CoA levels are low. Furthermore, the formation of lactate might constitute a mechanism for the detoxification of excess glutamate, without overproduction of glutamine. It may be particularly relevant that both the intracellular glutamine concentration and the concentration in the media were essentially constant in the presence of glutamate concentrations from 0.05 to 0.5 mM. In the present study the amino acids in the media were present in high enough concentrations to provide sufficient nitrogen for glutamine biosynthesis.

The formation of lactate would be an effective means of detoxification of glutamate because lactate levels up to 20 mM are well tolerated by brain cells (Schurr et al., 1988, 1989) and lactate is readily utilized for energy by both neuronal and glial cells (Larrabee, 1992; Schurr et al., 1988, 1989; McKenna et al., 1993, 1994). The amount of lactate produced from glutamate is only a small fraction of the total lactate production by astrocytes (Table 2); however, it represents a substantial amount of the glutamate metabo-
lized (~25%) in cells incubated with 0.2 mM glutamate. The metabolism of glutamate via the TCA cycle rather than to glutamine requires the disposal of ammonia by astrocytes and is undoubtedly influenced by the presence of compounds that can act as amino-group acceptors (Yudkoff et al., 1994). The higher concentrations of alanine in the media from astrocytes incubated with 0.2 and 0.3—4 mM, compared with those incubated with 0.1 mM, are most likely due to the availability of ammonia from glutamate metabolism. The presence of glutamine does not alter lactate formation; however, it does decrease the conversion of glutamate to glutamine (M. C. McKenna and U. Sonnewald, ongoing studies).

There was no evidence in the present study of formation of the 1,2,3-$^{13}$C-isotopomer of glutamate (glutamine doublet) even though there was considerable formation of the corresponding isotopomer of glutamate (Table 1) in the astrocytes. This finding suggests that the glutamate returned from the TCA cycle probably stays within the mitochondria because it does not mix with the pool of glutamate taken up from the extracellular milieu that is available to the cytosolic enzyme glutamine synthetase. The presence of the 1,2,3-$^{13}$C-isotopomer of glutamate (glutamine doublet) in an earlier study by Sonnewald et al. (1993a) using astrocytes from mouse brain suggests that this aspect of glutamate metabolism is different in rat and mouse astrocytes.

The results of the present study suggest that there are two distinct compartments of pyruvate in astrocytes because there was no NMR-detectable $^{13}$C enrichment of the alanine in the media. This indicates that the pool of $^{13}$C-pyruvate, originating from $^{13}$C-glutamate metabolism in the mitochondria, that gave rise to $^{13}$C-lactate did not mix with the unlabeled pyruvate from glycolysis that gave rise to the alanine in the media. It is of interest to note that Malloy’s group (Zhao et al., 1995) has demonstrated that such compartmentation exists in heart; they consistently observed that only a fraction of the lactate present mixes with the pyruvate pool that enters the TCA cycle.

In summary, the results of the present study demonstrate that glutamate concentration regulates the proportion of glutamate metabolized directly to glutamine versus the amount of glutamate metabolized via the TCA cycle. Astrocytes converted glutamate to both glutamine and lactate, two substrates that are released and readily utilized by synaptic terminals for energy. Our data provide further evidence that metabolism in astrocytes is highly compartmentalized as reported in earlier studies (McKenna et al., 1990; Schousboe et al., 1993; Sonnewald et al., 1993b). Further knowledge about the nature of this compartmentation will greatly contribute to our understanding of the regulation of energy metabolism in astrocytes and the integrated trafficking of substrates between glial and neuronal cells.

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