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Short Communication

Influence of Pathological Concentrations of Ammonia on Metabolic Fate of ^{14}C-Labeled Glutamate in Astrocytes in Primary Cultures

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Abstract: Rates of glutamine formation and of carbon dioxide production (as an indication of oxidative deamination of glutamate) were determined in primary cultures of astrocytes exposed to 50 μ*M* labeled glutamate in the absence or presence of added ammonia (0.1–3 m*M*). Glutamine formation (1.7 nmol/min/mg protein) was unaffected by all concentrations of added ammonia. This probably reflects the presence of a low content of ammonia (0.1–0.2 m*M*), originating from degradation of glutamine, in the cells even in the absence of added ammonia, and it shows that pathophysiological concentrations of ammonia do not increase the formation of glutamine from exogenous glutamate. The carbon dioxide production rate was 5.9 nmol/min/mg protein, i.e., three to four times higher than the rate of glutamine formation. It was significantly reduced (to 3.5 nmol/min/mg protein) in the presence of 1 m*M* or more of ammonia. This is in keeping with suggestions by others that toxic levels of ammonia affect oxidative metabolism. **Key Words:** Ammonia—Astrocytes—Glial cells—Glutamate—Glutamine—Oxidative metabolism. **Yu A. C. H. et al.** Influence of pathological concentrations of ammonia on metabolic fate of ^{14}C-labeled glutamate in astrocytes in primary cultures. *J. Neurochem.* **42**, 594–597 (1984).

Using primary cultures of astrocytes we had previously shown that accumulated glutamate is rapidly converted not only to glutamine but, to an even greater extent via an oxidative deamination, to tricarboxylic acid (TCA) cycle constituents and carbon dioxide (Yu et al., 1982). This led to the conclusion that glutamate functions not only as a glutamine precursor but also as a metabolic substrate for astrocytes. One, functionally important, consequence of this conclusion is that a transfer of glutamine from astrocytes to neurons is not able to compensate quantitatively for the depletion of TCA cycle constituents in neurons brought about by neuronal release of glutamate and γ-aminobutyric acid (GABA) (Schousboe and Hertz, 1983). Against this conclusion could possibly be argued that the experiments were performed in the absence of added ammonia. However, this does not mean that no ammonia is present in the cells, since conditioned media contain 0.1–0.2 m*M* ammonia (A. Schousboe, A. Yu, and L. Hertz, unpublished experiments) and ammonia easily penetrates cell membranes (Benjamin, 1982). This concentration is slightly below the *in vivo* concentration of ammonia in plasma (Benjamin, 1982).

To study the possible influence of increased ammonia levels on the metabolic fluxes from glutamate to, respectively, glutamine and TCA cycle constituents plus carbon dioxide, we have in the present study incubated primary cultures of cerebral astrocytes with ^{14}C-labeled glutamate in the presence of different concentrations of ammonia and followed the incorporation of radioactivity into glutamine and carbon dioxide as well as the contents of glu-

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Abbreviations used: AOAA, Aminooxyacetic acid; GABA, γ-Aminobutyric acid; GLDH, Glutamate dehydrogenase; MEM, Minimum essential medium; PCA, Perchloric acid; TCA, Trichloroacetic acid; TCA cycle, Tricarboxylic acid cycle.

tamate and glutamine as a function of time. This procedure allows calculation of rates of glutamine synthesis and of carbon dioxide production from glutamate as previously described by Yu et al. (1982). The concentrations of added ammonia (0.1–3.0 m*M*) were such that addition of the lower concentrations yielded final concentrations corresponding to those occurring physiologically, i.e., about 0.3 m*M* (Benjamin, 1982) and that the higher concentrations correspond to those occurring under pathophysiological conditions, which are known to have profound effects on astrocytes, both morphologically and functionally (Norenberg, 1981; Benjamin, 1982).

MATERIALS AND METHODS

Cell cultures

Cultures of astrocytes were prepared as described by Hertz et al. (1982): the parts of the cerebral hemispheres above the lateral ventricles were dissected out of the brains from newborn Swiss mice and grown for 2 weeks in a tissue culture medium [modified Eagle's minimum essential medium (MEM)] with horse serum and subsequently for another 2–3 weeks in the additional presence of 0.25 m*M* dibutyryl cyclic AMP, a procedure known to evoke a pronounced morphological differentiation of the cells. Such cultures constitute an excellent model for astrocytes *in situ* (Hertz et al., 1982).

Determination of pool sizes and specific radioactivity of amino acids

Each culture was incubated for 2–30 min in a medium with 1.3 μCi/ml, 50 μ*M* [U-^{14}C]L-glutamate (specific radioactivity 250 mCi/mmol, New England Nuclear), 0.5 m*M* glutamine, and no serum in an atmosphere of 5% carbon dioxide in air. After the incubation the cultures were rinsed three times with ice-cold buffered saline and subsequently another two times with Tris solution (1 m*M*; pH 7.4), scraped off the culture dishes in 1 ml of either trichloroacetic acid (TCA; 0.5 *M*), perchloric acid (PCA; 1 *M*), or 70% ethanol, left in the solution at room temperature for at least 30 min, and centrifuged for 2 min at 11,000 × *g*. Protein was determined in the pellets using the conventional Lowry technique (Lowry et al., 1951) and supernatants from TCA or PCA extracts were neutralized. The contents and specific radioactivities of glutamate and glutamine in the extracts or in diluted media were determined by HPLC after precolumn derivatization with *o*-phthaldialdehyde essentially as described by Lindroth and Mopper (1979) and Yu et al. (1984), employing a Waters HPLC system (Waters Associates, Toronto, Ontario) consisting of one M-45 and one 6000A pump, a model 720 system controller, a U6K sample injector, a data module M730, a model M420 fluorescence detector, and a Resolve 5μ Bondapak C18 column. The mobile phase was 0.1 *M* potassium acetate (pH 5.7) and methanol. Initially the methanol concentration was increased from 25% to 70% in a single linear step (flow rate 0.8 ml/min during 15 min), then it was maintained at 70% for 10 min and finally reversed (over 5 min) to equilibrate the column with 25% methanol. Effluents corresponding to glutamate and glutamine were collected for subsequent determination of radioactivities.

Determination of CO_2 production

For determination of $^{14}CO_2$ production rates from purified [1-^{14}C]L-glutamate (specific radioactivity 59 mCi/mmol from New England Nuclear), individual culture dishes without lids were placed in a gas-tight chamber (Yu et al., 1982). At the beginning of the experimental period, the radioisotope, diluted in medium, was added to the cultures by injection and at its end hyamine hydroxide (2 ml) was injected into a suspended beaker and 0.4 *M* PCA (500 μl) into the culture dishes to acidify the medium. After quantitative trapping of CO_2 the radioactivity in the hyamine hydroxide was determined. This value, together with the specific activity of glutamate in the media (used to calculate that in the cultures), and the protein content in the cultures, were used to calculate the carbon dioxide production per milligram protein.

RESULTS AND DISCUSSION

Contents of glutamate and glutamine

The contents (pool sizes) of glutamate and glutamine in the cultured astrocytes after incubation with 50 μ*M* labeled glutamate for 2–30 min in the presence or absence of 3 m*M* ammonia are shown in Fig. 1, top. It can be seen that the glutamate content increased during exposure to exogenous glutamate and that the contents of glutamate were slightly affected by ammonia: at all time periods the contents are larger in the presence of 3 m*M* ammonia that in its absence. This difference is statistically significant ($p < 0.01$) when the averages are treated as paired data. No such increase was observed in the presence of 0.1 or 0.3 m*M* ammonia (results not presented). In contrast, the contents of glutamine were not altered by ammonia (Fig. 1, top).

Conversion of glutamate to glutamine

The specific radioactivities of glutamate and glutamine are shown in the lower part of Fig. 1. It can be seen that the specific radioactivity of glutamate increased rapidly, regardless of whether or not 3 m*M* ammonia was present, and that it reached a constant level after 10 min. The time course of this increase in specific activity is similar to that previously observed (Yu et al., 1982) and consistent with the very high uptake rate for glutamate (Hertz et al., 1978). There are no significant differences between the specific activities in the presence or absence of 3 m*M* ammonia. Nor did added ammonia concentrations of 0.1, 0.3, or 1.0 m*M* have any effect (results not presented). The level reached after 15 min (≈24 dpm/pmol) is about one-half of that measured in the medium at the same time (47.0 ± 0.8 dpm/pmol). This shows a compartmentation of the glutamate pool in the cells, as previously suggested by Yu et al. (1982) and discussed in more detail by Hertz et al. (1983). This presence of metabolic compartmentation in a single cell type is an indication that the concept of compartmentation (Balazs and Cremer, 1972; Berl et al., 1975) may reflect not only differences between different cell types but also intracellular heterogeneity. After 30 min there was a decline in the radioactivity in the cells, which was mirrored by an increased radioactivity in the medium (results not presented) and probably reflects cell damage after prolonged exposure to the serum-free medium.

The specific radioactivity in glutamine increased more slowly and approached the level in glutamate after about 15 min. Again, ammonia at a concentration of 3 m*M* (Fig. 1, bottom) or at 0.1, 0.3, and 1.0 m*M* (results not presented) had no effect. This, together with the unchanged

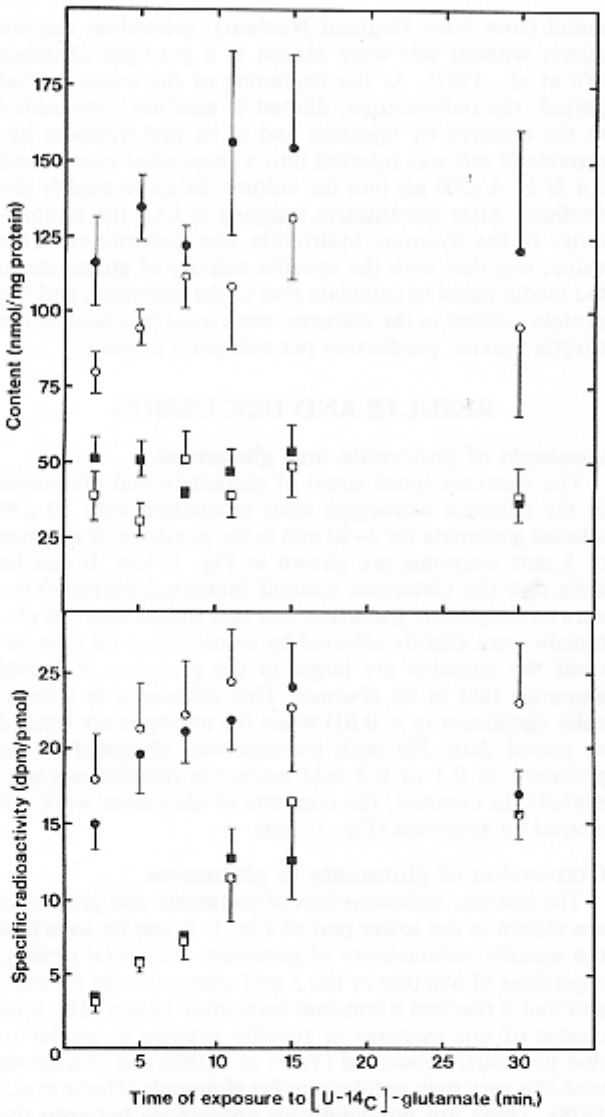

FIG. 1. Top: Contents of glutamate (○, ●) and glutamine (□, ■) in primary cultures of astrocytes incubated for 2–30 min in medium with 50 μ*M* L-[U^{14}C]glutamate and zero (○, □) or 3 (●, ■) m*M* ammonia. Results are means of three to six experiments and SEM values are shown by vertical bars if they extend beyond the symbols. **Bottom:** Specific radioactivities of glutamate (○, ●) and glutamine (□, ■) in the same cultures as above. Symbols as above.

glutamine pool in the presence of ammonia (Fig. 1, top), shows that added ammonia does not affect the rate of glutamine synthesis. From the rate of increase in specific activity during the 5–11 min period, the specific activity of glutamate during this period, and the pool size of glutamine, the glutamine synthesis rate can be calculated to be 1.7 nmol/min/mg protein (for details see Yu et al., 1982). This rate is similar to that previously determined by Yu et al. (1982). The lack of effect by added ammonia, both in the physiological range and at pathophysiological levels, may seem surprising but it should be remembered that the cells even without ammonia addition contain 0.1–0.2 m*M* ammonia. This is well above the K_m value for ammonia (0.04 m*M*) of the glutamine synthetase, determined in glioma cells (Pishak and Phillips, 1980).

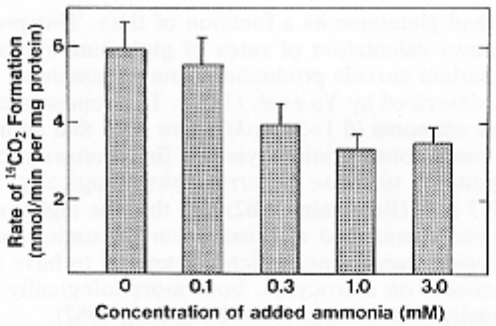

FIG. 2. Rate of $^{14}CO_2$ formation from [1-^{14}C]glutamate in primary cultures of astrocytes exposed to 0, 0.1, 0.3, 1.0, or 3.0 m*M* ammonia. Results are means of six to nine experiments and SEM values are shown by vertical bars.

Conversion of glutamate to carbon dioxide

The rate of $^{14}CO_2$ production from [1-^{14}C]glutamate over a 30-min period was calculated as previously described (Yu et al., 1982), i.e., on the basis of the specific activity of glutamate in the intracellular pool, at the different ammonia concentrations. In the absence of added ammonia the carbon dioxide production amounted to 5.9 nmol/min/mg protein (Fig. 2), i.e., a value that is slightly higher than the one previously determined (Yu et al., 1982). Addition of 0.1 m*M* ammonia, which increases the total ammonia concentration to 0.2–0.3 m*M* (see introduction), i.e., within the physiological level, had no significant effect but after addition of 0.3 and especially of 1 or 3 m*M* ammonia the carbon dioxide production was significantly reduced (Fig. 2). Five millimolar aminooxyacetic acid (AOAA), a transaminase inhibitor, had no additional effect, indicating that the initial transversion of glutamate to α-ketoglutarate, as in the absence of ammonia (Yu et al., 1982), occurs as an oxidative deamination, catalyzed by the glutamate dehydrogenase (GLDH). The increase in glutamate content in the presence of added ammonia (Fig. 1, top) may therefore suggest that the GLDH-catalyzed reaction under these circumstances to a lesser extent than under normal conditions (Yu et al., 1982) favors α-ketoglutarate formation. A similar mechanism might contribute to the ammonia-induced increase in glutamate synthesis in hippocampal slices reported by Hamberger et al. (1982). That this equilibrium can easily be altered by changes in ammonia concentration is in agreement with its equilibrium constant (Williamson et al., 1967). This might per se explain the inhibition of the formation of labeled carbon dioxide as due to a relative decrease in the amount of labeled α-ketoglutarate formed from glutamate compared with that formed in the TCA cycle. Another possibility is, however, that ammonia may have a direct inhibitory effect on oxidative decarboxylation of α-ketoglutarate (McKhann and Tower, 1961; Norenberg, 1981; Benjamin, 1982).

The present study has thus demonstrated that minor alterations in ammonia content within the physiological range have no effect on glutamate metabolism to either glutamine or carbon dioxide and it has confirmed our previous conclusion (Yu et al., 1982) that the quantitatively most important process for conversion of glutamate in astrocytes is an oxidative deamination. This is compatible with the finding by Deshmukh and Patel (1980) that glutamate is a better substrate for metabolism in nonsynaptosomal mitochondria than in synaptosomal mito-

chondria. That the direction of the α-ketoglutarate-glutamate reaction favors formation of α-ketoglutarate in at least one cell type in the brain is also in keeping with the recent observation by Plaitakis et al. (1982) that patients with deficient GLDH are less able than normal persons to degrade glutamate. Pathophysiological concentrations of ammonia were found to interfere with this process.

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