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Glutamate: Where does it come from and where does it go?

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ABSTRACT

Pyruvate carboxylation, the anaplerotic reaction in the brain, has been demonstrated in astrocytes but not neurons. Since anaplerosis cannot proceed without cataplerosis in a closed system such as the brain, there have to be mechanisms to degrade molecules such as glutamate, glutamine, GABA and aspartate which have more carbon atoms than pyruvate. Pyruvate recycling is a cataplerotic process which is very active in liver. It has also been demonstrated in the brain and has been shown to proceed both in astrocytes and neurons. Increasing recycling as a consequence of increasing glutamate concentration in medium has been shown in astrocytes. In the present study cerebellar granule neurons were incubated with medium containing 0.1, 0.25 or 0.5 mM [U-^{13}C]glutamate or [U-^{13}C]aspartate and pyruvate recycling in combination with tricarboxylic acid (TCA) cycle metabolism was analysed in glutamate, aspartate and malate using mass spectrometry. It could be shown that pyruvate recycling of TCA cycle intermediates as seen in glutamate increased with increasing [U-^{13}C]glutamate but not [U-^{13}C]aspartate concentration confirming compartmentation of glutamate metabolism and the importance of glutamate in cataplerosis. Partial pyruvate recycling (lactate production from the TCA cycle) was more active in astrocytes than neurons in line with the astrocytes' greater capacity for glutamate uptake.

1. Introduction

It is well established that de novo glutamate synthesis can only occur when compounds with at least four carbon atoms enter the tricarboxylic acid (TCA) cycle (***anaplerosis***). This process has to be matched by the opposite, ***cataplerosis***. Balancing these two processes, an absolute necessity, has not received much attention in the past. Anaplerosis can happen in neurons and astrocytes by entry of specific essential amino acids or uneven chain fatty acids from the diet, entering the brain via the blood–brain barrier. The main suppliers of glutamate precursors are, however, astrocytes that can convert pyruvate via pyruvate carboxylation to the TCA cycle intermediate: oxaloacetate (Patel, 1974; Shank et al., 1985; Sonnewald and Rae, 2010; Yu et al., 1983). Since most glutamate is needed in the glutamatergic neurons there has to be a link between astrocytes and neurons, which is glutamine. A constant stream of pyruvate carboxylase generated glutamine is flowing to the neurons that convert it to glutamate, which is used in neurotransmission and sent back to astrocytes that convert it to glutamine or channel it into the TCA cycle (McKenna et al., 2012). There is a flaw in this system, however, and that is the fact that the TCA cycle cannot produce molecules with three carbon atoms (i.e. pyruvate); in other words, it cannot act as a carbon sink. Anaplerosis has to be coupled to cataplerosis and lactate formation from the TCA cycle (Sonnewald, 2014) and pyruvate recycling are good candidates for this. Pyruvate recycling is a pathway for complete oxidation of glutamate (Fig. 1). It has been known to take place in the liver but was first detected in brain in 1990 (Cerdan et al., 1990). The metabolism of [1,2-^{13}C]glucose and [U-^{13}C]-3-hydroxybutyrate was studied after in vivo injection and ^{13}C MRS analysis of rat brain extracts. Quantitative analyses of the ^{13}C spectra demonstrated a cerebral pyruvate recycling system contributing maximally 17% of the pyruvate metabolism through the pyruvate dehydrogenase in brain (Cerdan et al., 1990). This recycling was thought to happen in neurons and not astrocytes because recycling was apparent in glutamate but not glutamine. In 1995, Hassel and Sonnewald reported partial pyruvate recycling in mouse brain astrocytes, demonstrated by lactate formation from TCA cycle metabolites which was detected when mice were injected with [2-^{13}C]acetate but not [1-^{13}C]glucose. The enrichment of total brain lactate from [2-^{13}C]acetate reached approximately 1% (above natural abundance) in both the C-2 and the C-3 positions in fasted mice. It was calculated that this could account for 20% of the lactate formed in the glial compartment (Hassel and Sonnewald, 1995). Pyruvate recycling in brain was also shown in other animal studies (Haberg et al., 1998; Melo et al., 2006; Morken et al., 2014; Sonnewald et al., 1996). However, pyruvate recycling was not detected in rabbit brain or by in vivo MRS studies (Duarte et al., 2011; Lapidot and Gopher, 1994). The physiological

Abbreviations: GAD, glutamate decarboxylase; GLN, glutamine; GLU, glutamate; GS, glutamine synthetase; KG, α-ketoglutarate; MRS, magnetic resonance spectroscopy; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; TCA, tricarboxylic acid.

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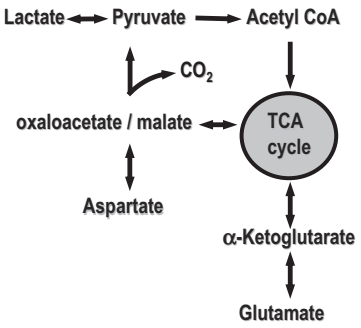

Fig. 1. Schematic presentation of partial (lactate formation) and full (re-entry into the TCA cycle) pyruvate recycling.

relevance of pyruvate recycling has been debated and a very logical explanation would be an increase during hypoglycaemia, when pyruvate content is low. Experiments to prove this have, however, failed to do so (Bakken et al., 1998b).

Using cell cultures, cell type and subcellular localisation of the major enzyme involved in pyruvate recycling, malic enzyme, could be established (Cruz et al., 1998, 2001; McKenna et al., 1995, 2000, 2006). Furthermore, cerebellar astrocytes were incubated with [U-^{13}C]glutamate and pyruvate recycling was detected in intracellular glutamate, glutamine and aspartate (Olstad et al., 2007a). Approximately 25% of glutamate was from pyruvate recycling but only 10% of aspartate. Also glutamine labelling via pyruvate recycling in astrocytes was much lower than that observed in glutamate. This was due to the fact that only 15% of glutamine was derived from mitochondrial metabolism as compared with 25% of glutamate and 90% of aspartate. Detection of only a small amount of recycling in glutamine in astrocytes could explain why Cerdan et al. (1990) suggested that recycling took place in neurons, not astrocytes.

Cerebellar neurons in culture are an excellent model to study cerebral metabolism since they consist of a majority of glutamatergic with about 5% GABAergic neurons, mirroring the distribution in brain (Sonnewald et al., 2004a, 2004b). When these cultures were incubated with medium containing [U-^{13}C]glutamate, GC/MS analysis showed clearly pyruvate recycling in glutamate and aspartate (Olstad et al., 2007a). When evaluating the extent to which glutamate was derived from pyruvate recycling, it was more prominent in glutamate in astrocytes than in neurons. However, in astrocytes less glutamate is derived from the TCA cycle without further pyruvate recycling than in neurons and the pyruvate recycling/TCA cycle only ratio for glutamate derived from pyruvate recycling was similar in astrocytes and neurons (Olstad et al., 2007b). It should be noted that all pyruvate recycling has metabolism in the TCA cycle as a necessity. The abbreviation PR is used for those TCA cycle derived intermediates that continue into pyruvate recycling. The expression TCA cycle is used to refer to the metabolites which do not enter pyruvate recycling.

The present study was undertaken to analyse the dependence of pyruvate recycling in cerebellar neurons on the concentration of glutamate and aspartate. Using ^{13}C labelled compounds and ^{13}C magnetic resonance spectroscopy (MRS) and or gas chromatography mass spectrometry (GC-MS) it is possible to monitor cellular metabolism and astrocyte–neuronal interactions. Various ^{13}C labelled substrates have been used to unravel different aspects of cerebral metabolism. We incubated cerebellar neurons with medium containing 0.1, 0.25 and 0.5 mM [U-^{13}C]glutamate or [U-^{13}C]aspartate. Analysis of neuronal cell extracts by GC/MS revealed a concentration dependence of pyruvate recycling in neurons.

2. Materials and methods

2.1. Materials

Pregnant NMRI mice were obtained from Taconic M&B (Copenhagen, Denmark). Plastic tissue culture dishes were purchased from Nunc A/S (Roskilde, Denmark) and foetal calf serum from Seralab Ltd. (Sussex, UK). Incubation medium was purchased from GIBCO BRL Life Technologies A/S (Roskilde, Denmark), [U-^{13}C]aspartate and [U-^{13}C]glutamate (99% enriched) were from Cambridge Isotope Laboratories (Woburn, MA, USA). Glutamate receptor antagonists 6,7-dinitroquinoxaline-2,3-dione (DNQX) and D-2-amino-5-phosphonopentanoic acid (D-AP5) came from Sigma (St. Louis, MO, USA). The GC/MS derivatisation reagent MTBSTFA (N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide) and the t-BDMS-Cl (tert-butyldimethylchlorosilane) were purchased from Regis Technologies, Inc. (Morton Grove, IL, USA). All other chemicals were of the purest grade available from regular commercial sources.

2.2. Cell cultures

Cerebellar granule neurons were prepared from 22 mice 5–8 days old (Schousboe et al., 1989). Tissue was exposed to mild trypsinisation followed by trituration in a DNAse solution containing a soybean trypsin inhibitor. Cells were suspended ($2–3 \times 10^6$ cells/ml) in a slightly modified DMEM containing 10% (v/v) foetal calf serum and cultured in poly-L-lysine coated wells 35 mm diameter in six-well Nunc culture dishes. Cytosine arabinoside (20 μM) was added after 48 h to prevent astrocyte proliferation.

Experiments were performed on 1-week-old cultures from at least two different preparations within each group ($n = 3–6$). At the time of the experiment, culture medium was removed from the wells and replaced with 2 ml culture medium without foetal calf serum and glutamine but containing 3 mM/l glucose and 25 μM DNQX and 100 μM D-AP5 (to minimise glutamate toxicity) and 0.10, 0.25, or 0.50 mM [U-^{13}C]glutamate or [U-^{13}C]aspartate for 2 h at 37°C (experimental medium). One additional group was incubated with experimental medium containing 0.25 mM [U-^{13}C]glutamate for only 1 h.

At the end of the incubation period, the experimental medium was removed and cells were rinsed with cold phosphate buffered saline. Enzyme activity was stopped by addition of 70% ethanol (1250 μl) to the wells, the cells were scraped with a rubber policeman and removed from the wells with the ethanol solution and transferred to Eppendorf tubes which were centrifuged at 10,000 rpm for 15 min. Five hundred microlitres of the supernatant was used for GC/MS analyses.

2.3. GC/MS

Cell extracts (3.5 cm Petri dishes) were adjusted to pH 2 and dried under atmospheric air, and metabolites were derivatised with MTBSTFA in the presence of 1% *t*-BDMS-Cl (Mawhinney et al., 1986). The derivatised samples were analysed in a Hewlett Packard 5890 Series II gas chromatograph linked to a Hewlett Packard 5971A mass spectrometer. Atom percent excess (^{13}C) was determined after correcting for naturally abundant ^{13}C and silicon from silyl groups (Biemann, 1962).

2.4. Statistical analysis

Extreme outliers were removed from their respective groups, leaving three to six samples in each group. Results are given as the mean ± STD. Differences between groups were analysed statistically with one way ANOVA followed by a least significant difference post hoc test, and a p-value of <0.05 was considered significant.