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Alteration of amino acid metabolism in neuronal aggregate cultures exposed to hypoglycemic conditions

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Abbreviations used

BCAA, branched-chain amino acids; ChAT, choline acetyltransferase; GAD, glutamic acid decarboxylase; LDH, lactate dehydrogenase; PAG, phosphate-activated glutaminase; PBS, phosphate-buffered saline; TCA, tricarboxylic acid.

Abstract

The neuronal effects of glucose deficiency on amino acid metabolism was studied on three-dimensional cultures of rat telencephalon neurons. Transient (6 h) exposure of differentiated cultures to low glucose (0.25 mM instead of 25 mM) caused irreversible damage, as judged by the marked decrease in the activities of two neuron-specific enzymes and lactate dehydrogenase, one week after the hypoglycemic insult. Quantification of amino acids and ammonia in the culture media supernatants indicated increased amino acid utilization and ammonia production during glucose-deficiency. Measurement of intracellular amino acids showed decreased levels of alanine, glutamine, glutamate and GABA, while aspartate was increased. Added lactate (11 mM) during glucose deficiency largely prevented the changes in amino acid metabolism and ammonia production, and attenuated irreversible damage. Higher media levels of glutamine (4 mM instead of 0.25 mM) during glucose deprivation prevented the decrease of intracellular glutamate and GABA, while it further increased intracellular aspartate, ammonia production and neuronal damage. Both lactate and glutamine were readily oxidized in these neuronal cultures. The present results suggest that in neurons, glucose deficiency enhances amino acid deamination at the expense of transamination reactions. This results in increased ammonia production and neuronal damage.

Key Words

Hypoglycemia, glutamine, lactate, alanine, aspartate, aggregate cell cultures from rat telencephalon neurons.

Running Title

Hypoglycemic neuronal amino acid metabolism.

Glucose is regarded as the predominant, if not sole, energy fuel of the brain. Nevertheless, accumulating evidence indicates that amino acids play an essential role as substrates for energy metabolism and biosynthetic activities (Bradford et al., 1978; Tildon and Roeder, 1988; Hassel et al., 1997; Zielke et al., 1998; Plaitakis and Shashidharan, 2000). In particular neurons were shown to depend on the supply of amino acids to replenish their tricarboxylic acid (TCA) cycle, as they lack key anaplerotic enzymes (Hertz et al., 1999, 2000). Glutamine and branched-chain amino acids (BCAA) were shown to serve as energy substrates in the brain. Glutamine is continuously synthesized by astrocytes (Norenberg and Martinez-Hernandez, 1979) and released into the extracellular space, where it is present at high levels (Hagberg et al., 1985). BCAA, on the other hand, are essential amino acids that are efficiently transported through the blood-brain barrier (Oldendorf, 1971; Smith et al., 1987). The utilization of amino acids as TCA cycle substrates requires cleavage of the amino group(s), involving either deamination or transamination reactions. Deamination gives rise to ammonium, which in the brain is used mainly by astrocytes for the synthesis of glutamine. The fixation of ammonia is largely dependent on the activity of glutamine synthetase. Under hyperammonemic condition, some glutamine may be formed by reductive amination of α-ketoglutarate via glutamate dehydrogenase. Transamination reactions have been shown to play an important role in brain nitrogen homeostasis (for review, Hertz et al., 2000). They are critical for the extracellular trafficking of amino acids between neurons and astrocytes as well as for the intracellular flux of metabolites between the cytosolic and mitochondrial compartments via the malate-aspartate shuttle. While the extracellular trafficking plays a crucial role in the maintenance of the nitrogen balance between neurons and astrocytes, the « pseudo-malate-aspartate shuttle » (according to Hertz et al., 2000) is instrumental for the synthesis of neurotransmitters such as glutamate, GABA and aspartate. Several cycles have been proposed for the shuttling of amino acids between neurons (in particular glutamatergic neurons) and astrocytes, notably a glutamate-glutamine cycle (Benjamin and Ouastel, 1975;

Westergaard et al., 1995; Sibson et al., 2001), a glutamate-leucine cycle (Yudkoff et al., 1996a,b; Yudkoff, 1997; Hutson et al., 1998), a glutamate-lactate cycle (Pellerin and Magistretti, 1994; Pellerin et al., 1998), and a combined glutamate-glutamine/lactate-alanine cycle (Waagepetersen et al., 2000).

Glucose deficiency in the brain appears to increase the utilization of amino acids as alternative energy substrates, as indicated by a rise of brain ammonia levels (Cooper and Plum, 1987). Ammonia accumulation as well as energy depletion and the release of excitotoxic amino acid neurotransmitters have been shown to contribute to the rapid functional and structural deterioration of the hypoglycemic brain (for review, Cooper and Plum, 1987; Butterworth, 1998; Siesjö, 1988; Choi and Rothman, 1990). However, the neurotoxic potential of these processes and their temporal relationships remain to be elucidated.

The present work was focused on the effects of glucose deficiency in neurons, and made use of the brain cell aggregate culture system (Honegger and Monnet-Tschudi, 2001). These three-dimensional cultures are prepared from embryonic rat telencephalon, and grown in chemically defined medium until a high degree of cellular maturation (Honegger and Pardo, 1999). Besides their histotypic cellular organization and maturation, these free-floating cultures offer the possibility to use multiple culture replicates of relatively high cell density, allowing a high reproducibility of measures and the direct quantification of extra- and intracellular amino acid levels. In contrast to in vivo studies which do not distinguish between neurons and glial cells, we took the advantage of growing neuron-enriched aggregate cultures, thus allowing to assess the specific neuronal changes induced by hypoglycemia, particularly on amino acid metabolism. Furthermore, it was examined whether increased availability of glutamine, or the presence of lactate, another putative energy substrate (McIlwain, 1953), may modify the glucose deprivation-induced changes in amino acid metabolism and their neurotoxic effects.

MATERIALS AND METHODS

Materials

The chemicals used were of the highest purity available. Culture media were prepared from powdered Dulbecco's Modified Eagles medium deficient in glucose, glutamine, bicarbonate and phenol red (GibcoBRL, Life Technologies AG, Basel, Switzerland). D-glucose and the putative alternative energy substrates L-glutamine, L-alanine and L-lactate were added at the concentrations indicated in the text. Bicarbonate (44 mM) was added to media used under normal culture conditions in an atmosphere of 10% CO_2 / 90% air. For metabolic studies where the formation of radiolabeled CO_2 was measured, bicarbonate was omitted and replaced by HEPES buffer (20 mM final concentration, pH 7.4) and NaCl for the correction of osmolarity. All media and buffers used for cultures were controlled for pH (pH 7.3-7.4) and osmolarity (330-340 mOsm). The culture media were all supplemented with 0.8 µM insulin (Sigma), 30 nM triiodothyronine (Sigma), 20 nM hydrocortisone-21-phosphate (Sigma), 1 µg/ml transferrin (Sigma), 4 µM biotin (GibcoBRL, Life Technologies AG, Basel, Switzerland), 1 µM vitamin B_{12} (Fluka AG, Buchs, Switzerland), 10 µM linoleate (Sigma), 1 µM lipoic acid (Sigma), 10 µM L-carnitine (Fluka), trace elements (Honegger et al., 1979; Honegger and Monnet-Tschudi, 2001) and 25 µg/ml gentamicin sulfate (Sigma).

The radiochemicals used to measure oxidative metabolism were: [U-^{14}C]-D-glucose (261 mCi/mmol, Du Pont-NEN, Bad Homburg, Germany); [U-^{14}C]-L-glutamine (234 mCi/mmol, Moravec Biochemicals, Brea, CA); [U-^{14}C]-L-lactate (150 mCi/mmol, American Radiolabeled Chemicals Inc., St. Louis, MO); [U-^{14}C]-L-alanine (182 mCi/mmol, Du Pont-NEN); [U-^{14}C]-L-leucine (292 mCi/mmol, Moravec Biochemicals). The radiochemicals used to measure enzymatic assays were: [1-^{14}C]-DL-glutamic acid (44.8 mCi/mmol, Du Pont-NEN and Moravec Biochemicals); [1-^{14}C]-acetylcoenzyme A (4 mCi/mmol, Du Pont-NEN and Moravec Biochemicals).

Cell culture

Sprague Dawley rats (OFA/Ico/Ibm strain) were obtained from Biological Research Laboratories, Füllinsdorf, Switzerland. Aggregating cell cultures were prepared from mechanically dissociated telencephalon of 16 day rat embryos as described previously (Honegger and Pardo, 1999; Honegger and Monnet-Tschudi, 2001). Cultures were initiated and grown in serum-free, chemically defined medium under constant giratory agitation (80 rpm), at 37^{o}C and in an atmosphere of 10% CO_2 / 90% humidified air. Neuron-enriched aggregate cultures were obtained by the treatment of the regular mixed-cell cultures at days 1 and 2 with cytosine arabinoside (0.4 µM) to eliminate the proliferating glioblasts. The neuron-enriched cultures contained > 90% neurons (Honegger and Pardo, 1999). Previous work has shown that such cultures contain less than 8% astrocytes and very few oligodendrocytes (Honegger and Pardo, 1999). Furthermore, immunocytochemical investigations showed similar proportions of GAD- and ChAT-positive neurons in these aggregate cultures (Pardo and Honegger, 1999), and the present finding of high intracellular levels of glutamate suggests the presence of an important population of glutamatergic neurons in these cultures. Culture media were replenished by the exchange of 5 ml of medium (of a total of 8 ml per flask) every 3rd day until day 14, and every other day thereafter. Neuron-enriched cultures received conditioned medium taken from mixed-cell (neuron-glia) sister cultures (which never received cytosine arabinoside) and diluted 1:1 with fresh medium. After 22 days of maturation in vitro, the cultures were used for experimentation. For each experiment, the aggregates from several culture flasks were pooled, washed and resuspended in minimal medium (containing the media components common to all treatments), and aliquoted to obtain replicate cultures. Aliquots (1.2 ml) of the aggregate suspension were transferred to 25-ml Erlenmeyer flasks preincubated with 2.8 ml of minimal medium supplemented with the components required for the individual treatments.

Assays for neuronal marker enzymes

Day 22 neuron-enriched aggregates were treated for 6 h with different concentrations of glucose, glutamine and lactate, then transferred to flasks containing the normal culture medium, maintained for 7 more days under normal culture conditions, and finally harvested at day 29. The aggregates of each culture replicate were washed twice with 5 ml of ice-cold PBS and homogenized in 0.4 ml of 2 mM potassium phosphate buffer (pH 6.8) containing 1 mM EDTA, using glass-glass homogenizers (Bellco, Vineland, NJ). The homogenates were briefly sonicated, divided into aliquots for the different assays and stored at -80^{o}C. The activities of glutamic acid decarboxylase (GAD, EC 4.1.1.15) and Acetyl-CoA:choline O-acetyltransferase (choline acetyltransferase, ChAT, EC 2.3.1.6) were determined by radiochemical assays as described in more detail elsewhere (Honegger and Pardo, 1999; Honegger and Monnet-Tschudi, 2001). ChAT activity was corrected for nonspecific choline transferase activity. Lactate dehydrogenase (LDH, EC 1.1.1.27) was assayed by spectrophotometry (Howell et al., 1979). Enzyme activities were expressed as pmol/min/culture replicate.

Determination of extracellular and intracellular amino acid levels

Extracellular and intracellular amino acid levels were measured on day 22 neuron-enriched aggregates treated for 6 h with different concentrations of glucose, glutamine and lactate. For extracellular levels, the corresponding control media, incubated in the absence of neuronal aggregates, were assayed in parallel. The concentration differences between culture and control media were regarded as changes in the extracellular concentrations caused by neuronal activity. The differences were related to the protein content of the respective aggregates, and expressed as net uptake (negative difference) or release (positive difference). The free amino acids from the culture media (extracellular) or from lysed aggregates (intracellular) were prepared according to Slocum and Cummings (1991). For the quantification of extracellular amino acids, culture media were collected individually, and filtered (Gelman Acrodisc 0.2

µm) to remove floating cells and debris. Aliquots (0.2 ml) of the media were then deproteinated by addition of a 5-sulfosalicylic acid solution (32 g/l final concentration) containing D-glucosaminic acid (250 µM final concentration) and (S)-2-aminoethyl-l-cysteine (250 µM final concentration) as internal standards, incubated for 10 min at room temperature, and centrifuged (10 min at at 4°C, 15600 g). The supernatants were then used for the amino acid determinations (Beckman 6300 amino acid analyzer). For the intracellular amino acids, the aggregates of each culture replicate were washed three times with 5 ml of ice-cold PBS by gravity sedimentation. After the last sedimentation, the supernatant PBS was carefully removed and the aggregate pellet immediately frozen in liquid nitrogen. To each frozen pellet, an aliquot (0.25 ml) of ice-cold PBS containing 5-sulfosalicylic acid (32 g/l), as well as D-glucosaminic acid (250 µM) and (S)-2-aminoethyl-l-cysteine (250 µM) as internal standards, was added. For lysis, the aggregate suspension was shaken for 15 minutes at room temperature, and then subjected to a second freeze-thaw cycle. The cell lysates were again shaken for 15 min at room temperature, and finally centrifuged (20 min at 4°C, 15600 g). The supernatants were used for the amino acid determinations, and the pellets were used to measure the protein content. The amino acid levels determined were related to the average protein content of the culture replicates, and expressed as nmol/mg protein. To measure the protein content, each pellet was resuspended in 0.8 ml of 0.1 M NaOH / 1% SDS, and assayed immediately by the bicinchoninic acid method (Smith et al., 1985). No significant difference of protein content was found between the aggregate cultures exposed for 6 h to the different treatments.

Determination of extracellular ammonia levels

Ammonia was measured on a Cobas FARA II automate (Roche), using the UV Enzymatic Ammonium Kit (Biomérieux, N° 61025).

Determination of the rate of oxidative metabolism

Replicate cultures of neuron-enriched aggregates were prepared in culture medium lacking bicarbonate. During the period of measurements, the cultures were kept at 37^{o}C under constant gyratory agitation, in flasks closed with an air-tight rubber stopper. The CO_2 produced by the cultures was absorbed in 0.5 ml of Hyamine-OH 10X (Packard), contained in a 1-ml tube together with a Whatmann GF/A glass fiber filter. The reaction was started by the addition of the [U-^{14}C]-labeled tracer (1 µCi/flask). The formation of $^{14}CO_2$ was measured during 3 successive intervals of 90 min, 120 min and 120 min. After each interval, the CO_2 absorber was removed for scintillation counting, and a fresh absorber was introduced into the flask. Of each CO_2 absorber collected, fluid and glass fiber filter were transferred to a scintillation vial, and the accumulated radioactivity was measured by liquid scintillation counting (Tri-Carb 2300TR, Canberra Packard, Meriden, CT). At the end of the total incubation period of 330 min, the aggregates of each replicate culture were transferred to 15-ml conical plastic tubes in ice, and the reaction stopped by the addition of 4 ml of ice-cold phosphate-buffered saline (PBS). The aggregates were then washed four times with 4 ml of PBS by gravity sedimentation. The final pellet was resuspended in 0.2 ml of 2 mM potassium phosphate buffer pH 6.8, subjected to a freeze-thaw cycle, and then sonicated to homogeny. Aliquots (40 µl) of the homogenates were taken for protein determination by a modification of the Folin phenol method (Lowry et al., 1951). CO_2 formation was expressed as nmol/min/mg protein, taking into account the specific radioactivity of the substrate used.

Statistical analyses

The statistical significance of the variations between two different series was evaluated using the unpaired Student's *t*-test. Differences with $p < 0.05$ were regarded as significant.

RESULTS

Neurotoxicity of glucose deficiency, attenuated by lactate and aggravated by high glutamine

The irreversible damage induced by transient glucose deficiency, as well as the effect of glucose substitution by lactate or glutamine as potential alternative substrates, were determined by measuring the activity of the neuron specific enzymes GAD and ChAT and of the ubiquitous cytosolic enzyme LDH. By reducing the glucose concentration to 0.25 mM, at least 4 h of exposure were required to obtain significant effects on the neuron-specific enzyme activities (not shown). Table 1 shows that glucose deficiency during 6 h caused a significant reduction in the activities of GAD (-56%), ChAT (-61%) and LDH (-20%) (medium B *vs.* medium A). Addition of lactate protected the cultures deprived of glucose from the reduction of LDH activity, and attenuated the decrease in activity of GAD (-18% *vs.* -56%) and ChAT (-15% *vs.* -61%) (C *vs.* B). Furthermore, high glutamine combined with high glucose did not affect these enzyme activities (D *vs.* A), while high glutamine combined with low glucose enhanced the effect of glucose deprivation on GAD (-83% *vs.* -56%) and LDH (-26% *vs.* -20%) activities (E *vs.* B).

Alterations in extracellular amino acids and ammonium

Extracellular levels of amino acids and ammonium were measured in the supernatants recovered from cultures incubated for 6 h in different concentrations of glucose, glutamine and lactate (Table 2). For the sake of clarity, only amino acids with changes > 5 nmol/h/mg protein are shown in Table 2. This criterion exluded proline, cystine, histidine, phenylalanine and tyrosine, which were present in the medium at the start of the treatment and taken up by the aggregates, as well as amino acids absent from the initial medium but found at low concentrations after treatment, comprising glutamate (media concentration < 1 µM),

asparagine, proline and ornithine. Aspartate, GABA, cystathionine and citrulline were absent from the initial medium and not detectable at the end of the treatment. Finally, the data for glutamine are not shown because of their high variability probably due to the extreme media concentrations used.

Glucose deficiency caused a significantly increased net uptake into the aggregates, of leucine, isoleucine, valine, arginine, serine and lysine (Table 2, B *vs.* A). Alanine, which was not present in the fresh medium, and methionine showed a significantly decreased net release with glucose deprivation. In parallel, a marked increase of ammonium was observed in the culture supernatants. The changes caused by glucose deprivation were either prevented or attenuated by the addition of 11 mM lactate (C *vs.* B, and C *vs.* A, respectively). In addition, supplementation of lactate under low glucose significantly increased the net release of alanine (C *vs.* A). High concentrations of glutamine in the presence of high glucose caused a decreased net uptake (or an increased net release, depending on the amino acid) of most of the amino acids listed, while ammonium release was slightly increased and alanine remained unaffected (D *vs.* A). High concentration of glutamine in the presence of low glucose reduced the utilization of leucine and isoleucine, and increased the net release in the medium of most amino acids (E *vs.* B). In contrast to the effects of lactate supplementation (C *vs.* B), glutamine did not prevent the glucose deprivation-induced increase in ammonia production, nor the decrease in alanine net release (E *vs.* B). Because of the scatter of glutamine measurements (see above) it was not possible to determine to which extent glucose deficiency increased the consumption of glutamine.

Alterations in intracellular amino acids

Changes in intracellular concentrations of amino acids were measured in cultures of neuron-enriched aggregates incubated for 6 h under glucose deficiency or under lactate or glutamine supplementation in low glucose (Table 3). Only amino acids showing significant changes

between the different treatments are listed in Table 3. The results show that glucose deficiency caused a significant decrease in the intracellular levels of glutamine, alanine and serine, but did not significantly affect the levels of leucine, isoleucine, valine and lysine (B *vs.* A), which showed an increased net uptake from extracellular medium (see Table 2). Furthermore, glucose deprivation caused a drastic increase in the intracellular aspartate concentration, while the levels of intracellular glutamate, GABA and glycine were significantly decreased. Supplementation of lactate during glucose restriction reverted practically all changes observed under glucose restriction (C *vs.* B and A, respectively), in good agreement with the data obtained for the extracellular amino acids. High extracellular glutamine in the presence of high glucose greatly increased the intracellular glutamine levels (D *vs.* A). It also significantly increased the intracellular levels of aspartate, while it slightly decreased the intracellular levels of leucine, isoleucine, arginine, lysine, threonine and methionine. Under glucose deficiency, high glutamine further increased the intracellular aspartate concentrations, and prevented the decrease in glutamate and GABA levels, but not that of alanine, serine and glycine (E *vs.* B and A, respectively). It also slightly decreased the intracellular levels of arginine, lysine and threonine, as was observed previously in the presence of high glucose.

Oxidative metabolism of glucose and some alternative energy substrates

The oxidative metabolism of glucose and of the putative alternative substrates glutamine, lactate, alanine and leucine was analysed as a function of glucose concentration by measuring the rate of $^{14}CO_2$ formation from their respective [U-^{14}C]-labeled tracers in neuron-enriched aggregate cultures. First, glucose oxidation was measured at three different glucose concentrations (0.14, 0.8, and 5.5 mM) in culture medium containing equal concentrations (0.8 mM) of the 4 presumptive energy substrates lactate, glutamine, alanine, and leucine. As shown in Fig. 1, the rate of glucose oxidation increased gradually between the first and the

third intervals of time. This slow equilibration of tracer in the oxidative pathway of glucose is a well-known characteristic of neurons. Similar amounts of radiolabeled CO_2 accumulated at glucose media concentrations of 0.8 mM and 5.5 mM, while lower levels were found at 0.14 mM of glucose, indicating glucose deficiency at the latter concentration.

The oxidative metabolism of the four presumptive energy substrates glutamine, lactate, alanine and leucine was analysed in neuron-enriched cultures placed in equal concentrations (0.8 mM) of the four substrates, and exposed to glucose concentrations of 5.5 mM and 0.14 mM. In these instances, it was found that in each case $^{14}CO_2$ attained steady rates already after the first 90 min interval of incubation (data not shown), in contrast to the results obtained with glucose (Fig. 1). Therefore, comparisons were made between the oxidation rates measured during the second interval (between 90 and 210 min of incubation; Fig. 2). Fig.2 shows relatively high oxidative metabolism of glutamine and lactate as compared to alanine and leucine. Furthermore, during glucose deprivation, the oxidation of all four substrates was significantly increased. This response was most pronounced for lactate (99% increase; $p < 0.001$) as compared to alanine (84% increase; $p = 0.001$), leucine (38% increase; $p = 0.001$) and glutamine (12% increase; $p < 0.02$).

DISCUSSION

Alterations of neuronal integrity upon low glucose exposure

Transient glucose deficiency in neuron-enriched aggregate cultures caused irreversible neuronal damage and cell death, as shown in this study by the decrease in GAD, ChAT, and LDH activities taken as indicators for neuronal cell loss, and in agreement with previous observations in mixed-cell aggregate cultures (Pardo and Honegger, 1999). In addition, it was found here that the presence of high glutamine exacerbated the deleterious effect of glucose deprivation, whereas lactate prevented the decrease in LDH activity and attenuated the decline of GAD and ChAT activities. Interestingly, glucose deprivation affected LDH relatively little as compared to the activities of the neurotransmitter-synthesizing enzymes GAD and ChAT, suggesting that neuronal damage was most pronounced in synaptic terminals. However, of the two known forms of GAD (i.e. GAD 65 and GAD 67), one (GAD 67) is not restricted to the synaptic region, and the selective protection of other neuronal subtypes cannot be excluded. Since glutamate media levels remained very low (< 20 µM under high glutamine) throughout the 6 h glucose deprivation period, the implication of this amino acid in the neurotoxic effects could be excluded. In contrast, a good correlation was found between the toxic effects of glucose deficiency and the increase in extracellular ammonia concentration. In agreement with these findings, there is a wealth of data from observations in vivo and in vitro showing that during hypoglycemia, amino acids serve as alternative energy substrates, and that increased amino acid oxidation results in the accumulation of brain ammonia (Siesjö, 1988; Pulsinelli and Cooper, 1989; Clarke et al., 1989), to which neurons are particularly vulnerable (for review, Cooper and Plum, 1987; Butterworth, 1998).

The net uptake of BCAA is increased in glucose deficiency combined to glutamine restriction

Glucose deficiency in conjunction with low glutamine caused an increase in the net uptake from media, without intracellular accumulation, of several amino acids and particularly the branched-chain amino acids (BCAA) leucine, isoleucine and valine, indicating enhanced consumption. In agreement with these findings, it has been shown that neurons use BCAA (or the corresponding keto acids, respectively) for oxidative metabolism (Chaplin et al., 1976; Yudkoff et al., 1996a,b; Hutson et al., 1998), and that synaptosomes possess specific uptake systems for BCAA (Tan et al., 1988; Rao et al., 1995).

Low glucose shifts glutamine metabolism from transamination to deamination

High extracellular glutamine was found to significantly reduce the use of BCAA and other amino acids both at high and low glucose. This may be explained by the high oxidative metabolism of glutamine found in neuronal aggregate cultures, where the rate of $^{14}CO_2$ formation from [U-^{14}C]-glutamine was of the same order of magnitude as that from [U-^{14}C]-glucose. Glutamine is oxidized by neurons (Bradford et al., 1978; Tildon, 1983), and it was shown in synaptosomes that a high proportion of glutamate (or glutamine, for that matter) taken up by neurons is used for energy production, and that this pathway is accelerated in conditions of low glucose (Erecinska et al., 1988, 1993; McKenna et al., 1993). Our study shows however that glucose deficiency caused only a slight increase in the rate of glutamine oxidation, while ammonia production was greatly increased. This discrepancy may indicate an alteration of the *route* rather than the *rate* of glutamine metabolism. It has been shown that in neurons, glutamine is readily deaminated by the phosphate-activated glutaminase (PAG) (Hogstad et al., 1988; Hertz et al., 2000), an enzyme localized at the outer side of the inner mitochondrial membrane (Laake et al., 1999). The resulting glutamate is then converted to α-ketoglutarate, either by transamination or by oxidative deamination by glutamate

dehydrogenase. Since only deamination liberates ammonia, the present findings suggest that glucose deficiency caused a change in the metabolic pathway, i.e. a shift from transamination to deamination reactions. This conclusion is supported by the fact that glutamate dehydrogenase activity is regulated by the energy charge of the cell. It has been shown recently (Plaitakis and Shashidharan, 2000) that under high extracellular glutamate and/or low intracellular ATP/ADP ratio, neuronal glutamate metabolism shifts from transamination to oxidative deamination. This may also explain the observation by McKenna et al. (1993) that in synaptosomes, exogenous glutamine enters the TCA cycle via glutamate dehydrogenase and not via transaminase. Furthermore, it has been shown that increased brain ammonia inhibits the activity of transaminases such as GABA-transaminase, alanine aminotransferase, and aspartate aminotransferase, as well as key metabolic enzymes such as glutaminase, α-ketoglutarate dehydrogenase, and glutamate dehydrogenase (Rao and Murthy, 1993 ; Butterworth, 1998). The present finding that high glutamine aggravated the effects of glucose deprivation in GABAergic neurons (GAD activity) as compared to cholinergic neurons (ChAT activity) may be explained by a higher rate of glutamine deamination in GABAergic neurons, since cerebral GABAergic neurons exhibit relatively high PAG activity (Larsson et al., 1985; Hogstad et al., 1988).

Low glucose increases aspartate and decreases alanine intracellularly

The view that glucose deficiency perturbed neuronal transamination reactions is supported by our finding that neuronal cultures exposed to low glucose increased their intracellular concentration of aspartate and decreased their intracellular concentration of alanine, two amino acids involved in pivotal transamination pathways. In accord with the latter finding, previous reports showed increased aspartate formation in hypoglycemia (Engelsen et al., 1986) and in synaptosomes under glucose-free conditions (Erecinska et al., 1988; Waagepetersen et al., 1998). These changes in alanine and aspartate levels were most likely

the consequences of a decreased glycolytic flux and the limitation of intermediates such as pyruvate and acetyl-CoA. It can be expected that at low glycolytic activity, alanine formation by transamination of pyruvate would be reduced. Furthermore, acetyl-CoA deficiency would block the initial step of the TCA cycle, i.e. the formation of citrate from acetyl-CoA and oxaloacetate, and therefore cause the accumulation of oxaloacetate and aspartate, the transamination product of oxaloacetate. Interestingly, the opposite reaction can also occur: in synaptosomes, accumulation of acetyl-CoA derived from ketone bodies diverts oxaloacetate towards citrate synthesis, while the rate of aspartate transamination decreases (Daikhin and Yudkoff, 1998). Aspartate participates in the malate-aspartate shuttle required for the transport of reducing equivalents (NADH) from cytosol to mitochondrial matrix (Cooper and Meister, 1985; Palaiologos et al., 1988; Daikhin and Yudkoff, 1998). In this shuttle, the exchange of cytosolic malate against α–ketoglutarate across the inner mitochondrial membrane is coupled with the exchange of glutamate against aspartate (formed by transamination from oxaloacetate). It has been proposed that, at least in glutamatergic neurons, the malate-aspartate shuttle can be replenished by exogenous glutamine, giving rise to glutamate by the PAG-catalyzed reaction, and that this « pseudo-malate-aspartate shuttle » is crucial for the production of neurotransmitters such as glutamate, aspartate and GABA (Hertz et al., 2000). In agreement with this view, the present results show that in glucose deficiency, high glutamine prevented the decrease of intracellular glutamate and GABA, and further raised the intracellular aspartate levels. In addition, there was practically no cellular release of aspartate, as indicated by the absence of detectable levels of aspartate in media supernatants. It appears most likely that during glucose deprivation, aspartate accumulated in the mitochondria due to the lack of reducing equivalents in the cytosol and the low energy charge of the cell.

An alanine-glutamine shuttle between neurons and astrocytes ?

Alanine was found to be continuously formed and released by the neuron-enriched aggregate cultures, in accordance with findings by Waagepetersen et al. (2000) in glutamatergic neurons. It therefore appears that neuron-derived alanine formed by transamination of pyruvate could function as conveyor of nitrogen from neurons back to astrocytes, to balance the nitrogen flux via glutamine from astrocytes to neurons. Such an alanine-glutamine shuttle could function between astrocytes and any type of neuron requiring glutamine as metabolic substrate. It is reminiscent of the well-known alanine-glucose cycle functioning between muscle and liver cells. While in the liver alanine is converted mainly to glucose and urea, astrocytes may use alanine primarily for the formation of pyruvate and glutamate. Glutamate can then serve as substrate for the synthesis of glutamine by making use of the ammonia produced by neurons, while pyruvate may serve as substrate for oxidative metabolism or gluconeogenesis, depending on the energetic charge of the astrocyte. Therefore, in addition to its role in nitrogen homeostasis, this hypothetical alanine-glutamine shuttle could provide astrocytes with a feedback signal from neurons, able to modulate astroglial activity and the flux of metabolites from astrocytes to neurons. Increased neuronal glycolytic activity would thus increase the flux of alanine from neurons to astrocytes and subsequently accelerate the flux of essential metabolic substrates from astrocytes to neurons.

Neurons are protected by lactate during glucose deficiency

Neurons were protected by lactate, at least partially, from the detrimental effects of glucose deficiency, as indicated by the unchanged LDH activity, and the significant recovery of GAD and ChAT activities. Lactate also prevented the increased amino acid consumption due to glucose deprivation and the concomitant rise in ammonia production, as well as the decline of intracellular glutamate and GABA levels, in accord with previous studies (Schousboe et al., 1997; Bakken et al., 1998; Waagepetersen et al., 1998). Lactate oxidation greatly increased

under glucose deficiency in our neuronal cultures, attaining a metabolic rate comparable to that of glucose. This is in good agreement with data showing that lactate, formed predominantly in astrocytes, is metabolized preferentially by neurons where it is able to substitute glucose as energy substrate (for review, McIlwain, 1953; Schurr et al., 1988; Tsacopoulos and Magistretti, 1996; Waagepetersen et al., 1998; Hertz et al., 2000). In accordance with this view, lactate prevented the glucose deprivation induced increase of intracellular aspartate and concomitant decrease of intracellular alanine, in keeping with the notion that glucose deficiency affected critical transaminase reactions in neurons.

Conclusions

Our study, focused on neuron-enriched aggregate cultures, allowed us to demonstrate that:

(i) Transient glucose deficiency causes irreversible neuronal damage and cell death, which are exacerbated in the presence of high glutamine, and prevented, at least partially, in the presence of lactate.

(ii) Low glucose in conjunction with low glutamine induces an increase in the consumption of the branched chain amino acids leucine, isoleucine and valine.

(iii) Glucose deficiency shifts neuronal glutamine metabolism from transamination to deamination, illustrated by the intracellular increase in aspartate and decrease in alanine levels.

Finally, the continuous release of alanine by neurons suggests the presence of an alanine-glutamine shuttle between neurons and astrocytes, providing astrocytes with a feedback signal from neurons for adapting astroglial activity to neuronal metabolism.

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TABLE 1: Irreversible changes in the activities of neuron-specific enzymes and lactate dehydrogenase in aggregate cultures exposed for 6h to low glucose: increased toxicity in the presence of high glutamine and decreased toxicity in the presence of high lactate.

Medium	Concentration of main energy substrates [mM]			Enzymatic activitiy [pmol/min/culture][a]		
	Glucose	Lactate	Glutamine	GAD	ChAT	LDH
A	25.00	-	0.25	1654±77	666±9	857±21
B	0.25	-	0.25	723±29***	262±18***	683±65***
C	0.25	11.00	0.25	1361±86*	567±53*	863±89
D	25.00	-	4.00	1544±10	669±54	825±79
E	0.25	-	4.00	273±27***	280±51***	503±12***

[a] Cultures were assayed at day 29, 7 days after exposure for 6 h to media containing different levels of energy subtrates (media A to E). Data are the mean ± S.D. of 3 replicate cultures. GAD, glutamic acid decarboxylase; ChAT, choline acetyltransferase; LDH, lactate dehydrogenase. Significant differences compared to cultures kept in medium A are shown: *** $p < 0.001$; * $p < 0.05$ (Student's *t*-test).

TABLE 2: Changes in the levels of amino acids and ammonia in the media of aggregate cultures exposed for 6 h to different concentrations of energy substrates [a].

	Main substrate concentrations of media A to E								
	A	B		C			D		E	
Glucose [mM]	25.00	0.25		0.25			25.00		0.25	
Glutamine [mM]	0.25	0.25		0.25			4.00		4.00	
Lactate [mM]	0.00	0.00		11.00			0.00		0.00	
			B *vs.* A		C *vs.* B	C *vs.* A		D *vs.* A		E *vs.* B
Leucine	-12.1±1.1	-20.9±1.4	**	-7.3±6.1	*	n.s.	-4.7±3.5	*	-2.4±2.4	***
Isoleucine	-15.4±1.7	-24.0±1.1	**	-11.6±6.5	*	n.s.	-8.1±4.0	*	-5.8±2.4	***
Valine	-6.9±1.8	-13.9±1.5	**	-5.5±4.9	*	n.s.	-1.3±0.9	**	1.1±1.5	***
Serine	-4.9±1.2	-6.8±1.7	*	-2.2±2.9	n.s.	n.s.	-0.9±1.0	*	1.2±1.1	**
Arginine	-4.6±0.9	-8.0±1.2	*	-2.8±2.8	*	n.s.	-1.8±0.8	*	1.1±1.2	**
Threonine	-5.6±3.1	-8.9±2.2	n.s.	2.1±5.2	*	n.s.	1.1±1.5	*	3.2±1.5	**
Lysine	-1.9±0.9	-6.7±0.9	**	4.8 ±3.4	**	*	3.9±1.2	**	7.8±1.9	***
Methionine	4.0±0.5	2.4±0.5	*	6.0±1.8	*	n.s.	6.1±1.0	*	7.0±0.5	***
Glycine	-5.9±1.1	-5.8±0.6	n.s.	-1.7±3.1	n.s.	n.s.	-1.9±1.6	*	2.8±1.1	***
Alanine	14.9±1.5	4.2±0.2	***	23.3±3.4	**	*	19.0±2.8	n.s.	6.0±0.3	**
NH_4^+	21.6±2.5	63.7±4.1	***	22.9±1.5	***	n.s.	30.1±4.6	*	61.4±7.1	n.s.

[a] Media supernatants were assayed at day 22, after 6 h of exposure to media containing different levels of energy substrates (media A to E). Control media containing no aggregate cultures were incubated in parallel. Data, expressed as nmol/h/mg protein content of the cultures (means ± S.D) show concentration differences between culture media (samples from 3 separate cultures) and the respective control media. Negative values indicate net uptake of the compound; positive values indicate net release of the compound. Significant differences between exposure to media A to E are shown: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s., not significant (Student's *t*-test).

TABLE 3: Intracellular amino acid concentrations in aggregate cultures exposed for 6 h to different concentrations of energy substrates [a].

	Main substrate concentrations media A to E								
	A	B		C			D		E	
Glucose [mM]	25.00	0.25		0.25			25.00		0.25	
Glutamine [mM]	0.25	0.25		0.25			4.00		4.00	
Lactate [mM]	0.00	0.00		11.00			0.00		0.00	
			B *vs.* A		C *vs.* B	C *vs.* A		D *vs.* A		E *vs.* B
Leucine	6.6±0.3	6.1±0.3	n.s.	5.9±0.2	n.s.	*	5.2±0.3	**	5.1±1.0	n.s.
Isoleucine	6.4±0.4	5.8±0.3	n.s.	5.8±0.3	n.s.	n.s.	5.2±0.3	*	4.9±0.8	n.s.
Valine	6.5±0.2	4.2±2.8	n.s.	6.0±0.1	n.s.	*	5.5±0.7	n.s.	4.9±0.8	n.s.
Serine	13.3±0.7	11.4±0.8	*	13.0±0.6	*	n.s.	10.7±3.4	n.s.	8.1±1.6	*
Arginine	4.2±0.1	4.5±0.4	n.s.	4.4±0.3	n.s.	n.s.	2.8±0.3	***	2.9±0.4	**
Threonine	21.6±1.0	18.9±1.5	n.s.	21.5±1.0	n.s.	n.s.	16.0±2.2	*	13.1±2.1	*
Lysine	8.8±0.5	9.1±0.6	n.s.	8.7±0.7	n.s.	n.s.	5.8±0.8	**	6.1±0.8	**
Methionine	1.7±0.1	1.6±0.2	n.s.	1.7±0.1	n.s.	n.s.	1.3±0.2	*	1.1±0.3	n.s.
Glycine	125.7±4.9	100.0±6.3	**	110.7±6.0	n.s.	*	104.0±15	n.s.	70.0±14	*
Alanine	5.8±0.4	1.0±0.1	***	5.8±0.4	***	n.s.	5.4±1.1	n.s.	1.4±0.4	n.s.
Aspartate	10.1±1.1	55.0±3.0	***	8.0±1.5	***	n.s.	17.4±0.5	**	86.8±17	*
GABA	49.3±1.7	20.1±1.7	***	56.5±3.0	***	*	59.3±9.0	n.s.	48.6±7.2	**
Glutamate	46.3±2.0	18.1±0.7	***	43.1±2.3	***	n.s.	61.5±9.8	n.s.	52.2±9.0	**
Glutamine	5.2±0.4	1.5±0.1	***	4.9±0.1	***	n.s.	56.7±7.8	***	46.7±8.5	***

[a] Culture extracts were assayed at day 22, 6 h after exposure to media containing different levels of energy subtrates (media A to E). Data, expressed as nmol/mg protein content of the cultures are the means (± S.D) of 3 separate cultures. Significant differences between exposure to media A to E are shown: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s., not significant (Student's *t*-test).

Legends to Figures

FIG. 1. Rate of glucose oxidation in neuron-enriched aggregate cultures depending on the glucose medium concentrations and the time of incubation. Replicate cultures of neuron-enriched aggregates were assayed in triplicates. The formation of radiolabeled CO_2 was determined at different time-intervals after addition of the radiolabeled tracer [U-^{14}C]-glucose. Data are mean ± SE (bars) values of three independent experiments.

FIG. 2. Rate of oxidation of four potential energy substrates in neuron-enriched aggregate cultures at two different glucose media concentrations. Replicate cultures of neuron-enriched aggregates were assayed in triplicates. The formation of radiolabeled CO_2 was determined during a period of 120 min, starting 90 min after addition of the [U-^{14}C]-labeled tracers. Data are mean ± SE (bars) values of three independent experiments.

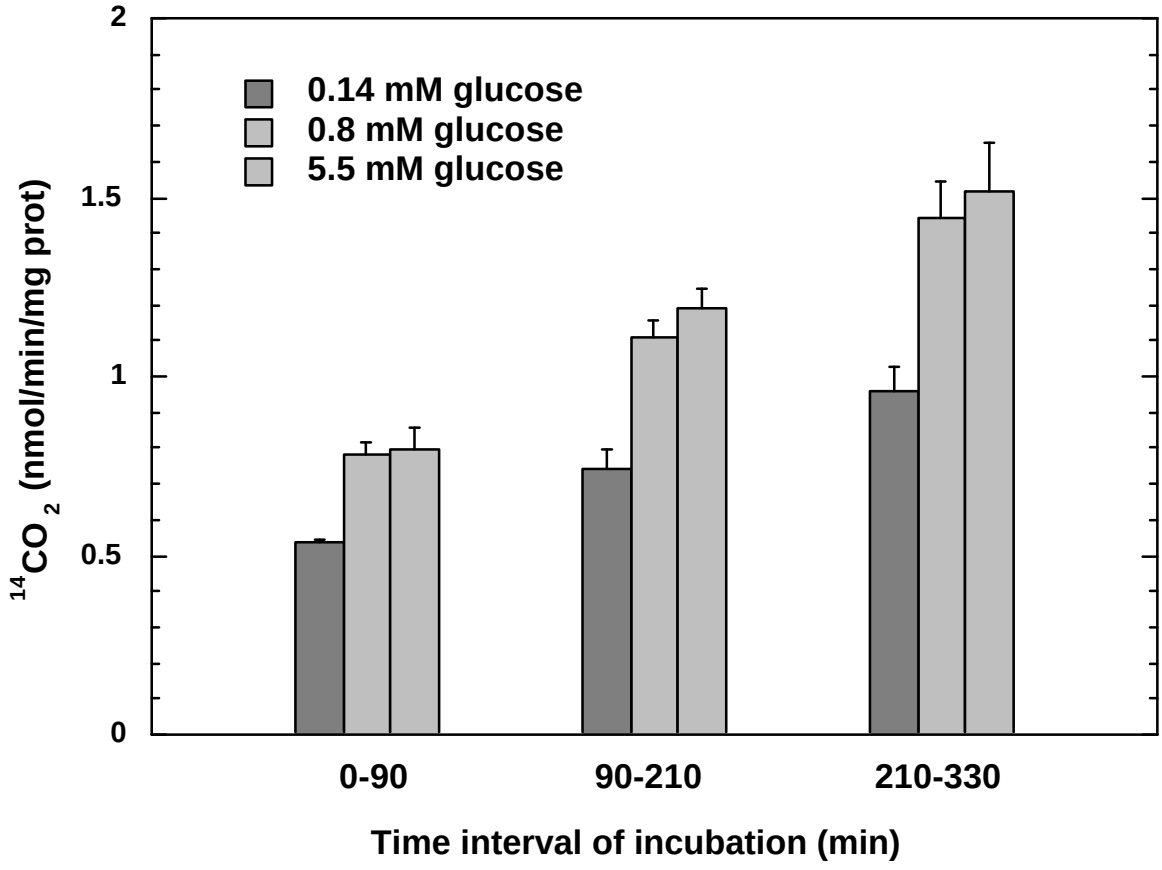
2
1.5
1
0.5
0
0.14 mM glucose
0.8 mM glucose
5.5 mM glucose
$^{14}CO_2$ (nmol/min/mg prot)
0-90
90-210
210-330
Time interval of incubation (min)

Figure 1

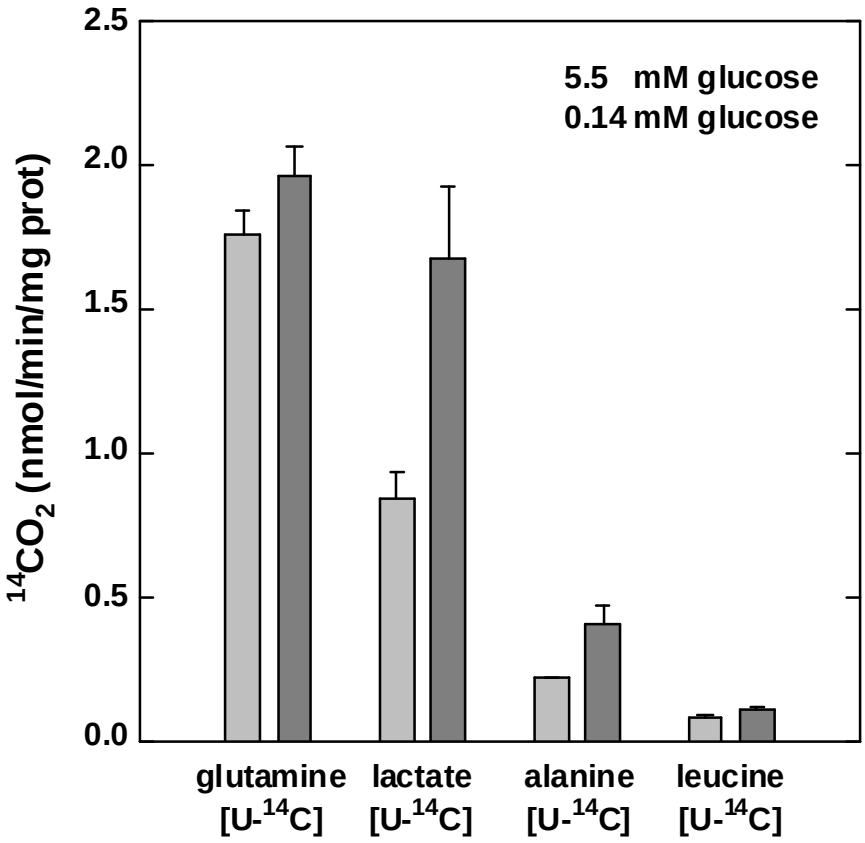
2.5
2.0
1.5
1.0
0.5
0.0
$^{14}CO_2$ (nmol/min/mg prot)
5.5 mM glucose
0.14 mM glucose
glutamine [U-^{14}C]
lactate [U-^{14}C]
alanine [U-^{14}C]
leucine [U-^{14}C]

Figure 2