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Glutaric aciduria I: creatine supplementation restores creatinephosphate levels in mixed cortex cells from rat incubated with 3-hydroxyglutarate

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Abstract

The pathogenesis of neurological sequelae in glutaric aciduria I (GA I) is still unclear. Some evidence exists for compromised energy generation in the brain of patients with GA I resulting in 'slow-onset' excitotoxicity. Previously, we have shown a reduced activity of the mitochondrial ATPsynthase in cultured mixed cortex cells from neonatal rats incubated with 2–4 mM 3-hydroxyglutarate (3-OH glut) for 24 h [1]. In the present study we measured cellular contents of high energy phosphate compounds (creatinephosphate CP, ATP, and ADP) in this model after a 24 h incubation period with 2–4 mM glutarate (glut) or 3-OH glut. 3-OH glut specifically led to a reduction of CP content in a dose-dependent manner, whereas concentrations of ATP, ADP, and AMP remained unchanged. The drop in CP-concentration could be prevented by preincubation with the non-competitive NMDA-receptor antagonist MK 801 or coincubation with 1 mM creatine. NMDA-receptor associated ion channels may be opened due to a lack of energy inside the neurons caused by a reduction of CP. This is followed by membrane depolarization which could impair electrogenic creatine transport into the cell.

Keywords: ATP; Creatine; Creatinephosphate; Excitotoxicity; Glutaric aciduria; Hydroxyglutarate; Mitochondria; Neurons; Respiratory chain

Introduction

The pathogenesis of cerebral symptoms in glutaric aciduria I (GA I) is still obscure. Neuromorphologic changes include external hydrocephalus, swelling, and necrosis of basal ganglia, hence damage of brain cells with a high rate of aerobic (mitochondrial) metabolism. These findings and increased excretion of lactate and dicarboxylic acids in urine during 'metabolic crises' may point to mitochondrial dysfunction in GA I [2]. Compromised function of mitochondria can lead to an activation of the 'slow-onset' neurotoxicity cascade [3]: Energy depletion results in a break-down of the membrane potential which relieves the Mg-block of voltage-dependent NMDA-receptor associated ion channels followed by an influx of sodium and calcium ions into neurons. This may result in cell swelling on one hand—as has been shown previously [1]—and cellular dysfunction due to the 'second messenger' calcium on the other hand.

GA I is caused by the deficiency of glutaryl CoA-dehydrogenase. Mitochondrial damage may be mediated by metabolites accumulating proximal to the deficient enzyme like glutaric (glut), 3-hydroxyglutaric (3-OH glut), and glutaconic acid. In a previous study, we examined activities of respiratory chain enzymes in mixed cortex cells from rat brain incubated with these substances. We found a significantly decreased activity of the mitochondrial ATPsynthase (complex V) in these cells after incubation with 3-OH glut [1]. This prompted us to look at the levels of 'high energy' phosphate compounds (ATP, creatinephosphate CP, and ADP) in mixed cortex cultures after incubation with glut and 3-OH glut.

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Materials and methods

Mixed cortex cultures from neonatal rat brains (age 1 day) were prepared according to Goslin and Banker [4] and incubated for 10 days in Start V-medium (Biochrom) on 3 cm-petri dishes (Falcon) pretreated with polylysine in an atmosphere containing 5% CO_2. Neurons were incubated for 24 h in the presence of different metabolites (glut, 3-OH glut, 2 and 4 mM final concentration) and without additions (control). In some experiments 1 mM creatine was present in the medium in an attempt to prevent CP-depletion.

Cells were washed twice with a Hepes buffer containing (mM) NaCl 96, KCl 5, KH_2PO_4 1.2, $CaCl_2$ 1, Hepes 10, glucose 10, pH 7.4, and then incubated for 20 min in 1 ml of this buffer supplemented with the respective metabolites.

We coincubated the cell cultures with the possibly toxic substances (glut and 3-OH glut) and the non-competitive NMDA-receptor antagonist MK 801 (20 μM in the incubation medium) to test the involvement of NMDA-receptors. Activation of NMDA-receptor associated ion channels is discussed in the pathogenesis of slow-onset neurodegeneration.

Cells were broken by sonication (3×7 s) using a BANDELIN probe sonicator set at 20 W of power with single pulses of 0.3 s duration. Time between pulses was 0.7 s. An aliquot of the cell homogenate was placed in an equal volume of ice-cold DMSO (dimethylsulfoxide) as described by Ouhabi [5]. 'High energy' phosphate compounds were measured via bioluminescence in a Bioorbit-luminometer using a luciferin/luciferase assay. ATP and creatinephosphate were assayed according to Ronner [6]. ADP and AMP using pyruvate kinase and myokinase as coupling enzyme, respectively, were measured as described elsewhere [7].

In preliminary experiments, glut and 3-OH glut as well as creatine were added to the cell homogenate after sonication in order to exclude interference of these incubation substances with the assay.

Protein was assayed according to Bensadoun and Weinstein [8].

Total creatinekinase-activity (EC 2.7.3.2.) was measured bioluminometrically following ATP consumption [9].

Cell viability was assessed by measurement of lactic dehydrogenase (LDH, EC 1.1.1.27)-release from the neurons [10].

Materials

Tissue culture materials were from Biochrom (Berlin, Germany), FALCON 3001 tissue culture dishes came from Becton Dickinson (Heidelberg, Germany). All chemicals used including glut were from Sigma–Aldrich (Taufkirchen, Germany). NMDA and MK 801 were from Calbiochem (Bad Soden, Germany), enzymes were from Boehringer (Mannheim, Germany). 3-OH-glut was provided by Dr. R. Fingerhut, Hamburg, Germany. BIOORBIT test kits for luminometric measurements were obtained from Labsystems (Frankfurt, Germany).

Statistical analysis

Data are expressed as mean ± SD. Under each condition measurements in each cell line were carried out three times, variation of these triplicate measurements was about 5%. Results from cell cultures incubated under different metabolic conditions were compared using Student's *t* test for paired data. Cells from one cell line were incubated under different metabolic conditions on different petri dishes and were compared to 'control' (basal) conditions where no additions were made to the incubation medium of cells from this cell line. Thus each cell line had its own 'control' value.

Results

In preliminary experiments LDH-release from neurons was measured in order to judge cell integrity. Results are shown in Fig. 1. Both 100 μM NMDA and 4 mM 3-OH glut led to a rise in LDH-release. When cells were preincubated with 20 μM MK 801 addition of 4 mM 3-OH glut had no effect on enzyme release. MK 801 on its own did not influence enzyme activity significantly. When untreated cells were broken by sonication a total intracellular LDH-activity of 75 nmol/min per mg protein was recovered in the cell homogenate ($n = 5$ preparations, results not shown).

The contents of ATP, ADP, AMP, and CP in the cell preparations are summarized in Table 1. Concentrations of ATP and ADP were not significantly changed by incubation with glut or 3-OH glut. Glut had no effect on CP-levels, however CP-contents were significantly reduced in the presence of 4 mM 3-OH glut.

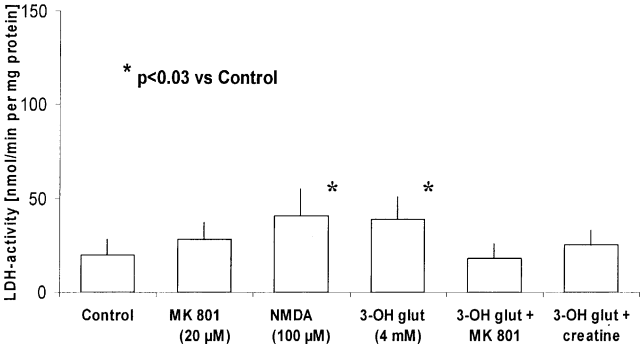

Fig. 1. LDH-activity in the medium after 24-h incubation period, means ± SD $n = 5$. Control, no addition; creatine, 1mM creatine.

Table 1
Cellular contents of ATP, CP (creatinephosphate), ADP, AMP under different incubation conditions; mean ± SD, (*n*): number of petri dishes

Incubation condition	ATP	CP	ADP	AMP
	(nmol/mg protein)			
Control	19 ± 8(18)	10 ± 8(15)	5 ± 2(18)	2 ± 1(14)
4 mM 3OH-glut	17 ± 11(16)	5 ± 4(13)[a]	4 ± 3(14)	1 ± 0(9)
2 mM 3OH-glut	16 ± 8(5)	6 ± 3(5)[a]	5 ± 3(5)	2 ± 0(5)
4 mM glut	17 ± 11(5)	10 ± 6(5)	6 ± 4(5)	1 ± 0(5)
4 mM 3OH-glut + 20 μM MK 801	15 ± 7(15)	9 ± 8(18)	3 ± 2(18)	1 ± 0(12)
4 mM 3OH-glut + 1 mM creatine	17 ± 9(12)	9 ± 7(12)	5 ± 2(12)	1 ± 1(12)

[a] $p < 0.05$ vs control.

When 1 mM of creatine was added to the incubation medium 24 h prior to the measurement of phosphate compounds CP-levels under 3-OH glut incubation remained normal. Preincubation of the neurons with 20 μM of the non-competitive NMDA-receptor antagonist MK801 could prevent the drop of the CP-concentration induced by incubation with 3-OH glut.

Total creatinekinase-activity was not affected by 4 mM 3-OH glut, activity was 171 ± 16 vs 175 ± 28 nmol/min per mg protein ($n = 15$) in untreated cells.

Discussion

Previously, we have shown that the ATPsynthase activity is reduced when mixed cortex cultures from rat brain were incubated in the presence of 4 mM 3-OH glut [1]. In the present study we show that incubation of the cell cultures with 4 mM 3-OH glut led to a reduced content of CP, however ATP-, AMP-, and ADP-levels were not affected by 3-OH glut in the incubation medium; 4 mM glut had no effect on cellular CP levels.

Standard deviations for the 'high energy' phosphate compounds were surprisingly high. This was mainly due to large variations between different cell lines while determinations within one cell line (under the same incubation condition) differed only by 5%. Each single cell line had its own 'control' value to allow for variation between cell lines.

The mechanism for CP-reduction is not clear. We measured the activity of creatinekinase—the enzyme responsible for the synthesis of CP—in cultures incubated with 3-OH glut but did not find any difference compared to normal (control) cells. ATP as a substrate for the creatinekinase reaction remained constant. As supplementation of the incubation medium with creatine—the second substrate for the synthesis of CP—could prevent the drop of CP, transport of creatine into the cells and/or mitochondria may be impaired by 3-OH glut. Creatine is transported via an electrogenic transporter across the plasma membrane [11,12]. Membrane depolarization could impair creatine transport into the cytoplasm and mitochondria with a reduction in intracellular creatine concentration, hence a lack of substrate for the creatinekinase reaction. Opening of voltage-gated NMDA-receptor associated ion channels by the release of the magnesium block due to reduced cellular energy production could lead to an influx of cations (mainly calcium and sodium) which would further reduce the membrane potential and lead to a reduced transport rate of creatine into the cytosol. This could explain the protective effect of the NMDA-receptor antagonist MK 801. We were not able to test this hypothesis as the analytical methods were not sensitive enough to detect the tiny changes of intracellular creatine concentration. Since the creatine transporter works at half maximal rate under physiological conditions [13] the reduced transportation rate of creatine may be compensated by an elevated concentration of creatine in the incubation medium.

Alternatively, 3-OH glut could directly interact with the creatine transporter as has been described for other organic acids [14].

On the other hand the mitochondrial creatinekinase—responsible for the synthesis of CP from creatine and ATP—is bound to the inner mitochondrial membrane. Interaction of the enzyme with the membrane is electrostatic [15] and can be influenced by several ions as for example calcium [16] which lead to dissociation of the enzyme from the inner mitochondrial membrane. When the creatinekinase is dissociated from the inner mitochondrial membrane functional coupling of the creatinekinase reaction to oxidative phosphorylation is lost [15].

We measured a normal catalytic capacity of the creatinekinase reaction (under substrate saturation in vitro) after incubation with 3-OH glut. However, this does not exclude functional uncoupling of the enzyme from oxidative phosphorylation in vivo which might explain reduced CP production. This defect may be compensated by an elevated concentration of creatine in the cell and the mitochondrial compartment after creatine supplementation.

Reduced activity of the mitochondrial ATPsynthase induced by 3-OH glut may be responsible for the drop in cellular CP concentration as well. CP functions as an 'energy buffer' inside the cell. Decreased amounts of CP

may result in a drop of the energy-dependent potential of the cell membrane which could trigger 'slow-onset' neurotoxicity [3] by relieving the Mg-block of voltage-dependent NMDA-receptor associated ion channels. This could lead to cellular dysfunction and eventually cell death. Indeed, cell damage induced by 3-OH glut was demonstrated by an increased LDH-release from the cells. This could be prevented by the non-competitive NMDA-receptor antagonist MK 801 which demonstrated the involvement of NMDA-receptors.

Our findings are in agreement with results of other studies showing a protective effect of creatine in neuronal cultures from chick embryo telencephalons incubated with 3-OH glut [17].

If the changes seen in cultured neurons could be detected in patients with GA I by MRI-spectroscopy a therapeutic trial of creatine supplementation in vivo may be considered.

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