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3-Hydroxyglutaric acid fails to affect the viability of primary neuronal rat cells

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Glutaric aciduria type I (GA I) is an autosomal recessive inherited metabolic disorder caused by deficiency of glutaryl-CoA dehydrogenase (GCD) resulting in the accumulation of 3-hydroxyglutaric acid (3OHG), glutaric acid and glutaconic acid in body fluids. GA I is characterized by a specific age- and brain region-dependent neuropathology. Previous studies using organotypic slice cultures of rats and primary chick embryo telencephalon cell cultures indicated that death of neurons is a consequence of an excitotoxic mechanism induced by 3OHG. We used primary neuronal cells of neonatal rats as a model system to test cell viability after treatment with 3OHG. Western blot analysis was used to prove the expression of functional *N*-methyl-D-aspartate (NMDA) receptors revealing no alteration in the expression of NMDA-2a and -2b receptor subtypes in response to 3OHG. When neuronal cells cultured for 10 or 20 days were treated with 1 mM glutamate, the viability of cells was reduced by 40%. This effect could be prevented by coincubation with the NMDA receptor antagonist MK801. In contrast, incubation of cells with 3OHG for up to 24 h in concentrations of 4–8 mM did not cause increased cell death as compared with untreated control cultures. These results indicate that 3OHG is not excitotoxic in this model of neuronal rat cell cultures despite the presence of functional NMDA receptors. Therefore, alternative or additional pathomechanisms than excitotoxicity may be relevant for neurodegeneration in GA I.

Keywords: Metabolic diseases; Glutaric aciduria type I; Neurodegeneration; Organic acids; NMDA receptors

Introduction

Glutaric aciduria type I (GA I) is an autosomal recessive organic acidemia caused by mutations in the gene encoding glutaryl-CoA dehydrogenase (GCD), which is located on the short arm of chromosome 19. GCD catalyses the oxidative decarboxylation of glutaryl-CoA, an intermediate in the catabolism of tryptophane, lysine and hydroxylysine. Deficiency of GCD leads to accumulation of 3-hydroxyglutaric acid (3OHG), glutaric acid (GA) and glutaconic acid in blood, urine and CSF (Bjugstad et al., 2000; Goodman and Biery, 1996; Goodman and Kohlhoff, 1975). The incidence of this defect is estimated to be 1:40,000.

Clinical features comprise macrocephaly, dystonia/dyskinesia following acute encephalopathic episodes and "frontotemporal brain atrophy". Acute encephalopathic episodes generally only appear up to the age of 6 years (Hoffmann et al., 1996). In pathomorphological examinations, basal ganglia necrosis and, microscopically, postsynaptic vacuolization of neurons, characteristic for excitotoxicity, have been described (Hoffmann et al., 1991). Morphologically, the "frontotemporal brain atrophy" is not defined.

The pathomechanism leading to neuronal death of GA I is still not understood. The accumulation of glutaric acid and its hydroxylated derivate 3OHG are supposed to play a key role in cell damage. 3OHG and glutaric acid are both structurally related to the excitotoxic amino acid glutamate. In many neurologic disorders, injury to neurons is thought to be caused by overstimulation of *N*-methyl-D-aspartate (NMDA) receptors by excitatory amino acids (Ikonomidou and Turski, 1995; Lipton and Rosenberg, 1994). In GA, this hypothesis is supported by studies on primary neuronal cultures of chick embryo telencephalons and slice cultures of rat brains (Bjugstad et al., 2000; Flott-Rahmel et al., 1997; Kölker et al., 1999, 2000a,b; Ullrich et al., 1999). In addition, intrastriatal administration of 3OHG in rat brains induces convulsions and excitotoxic lesions which partially can be prevented by NMDA receptor antagonists (DeMello et al., 2001).

There are divergent results whether 3OHG interacts directly with NMDA receptors or produces an indirect activation of the receptor due to reduced cellular energy levels interfering with the voltage-dependent Mg^{2+}-bloc of NMDA receptors (Das et al., 2003).

Interestingly, 3OHG was not found to be excitotoxic in primary cultures from embryonic rat striatum and cortex. In this system, the neurotoxicity of 3OHG could significantly be reduced by coincubation with bFGF and IGF-1 but not with an NMDA receptor antagonist (Bjugstad et al., 2001).

In this study, we tested the effect of 3OHG on the viability in another cell system, namely, cultured neonatal rat neurons. 3OHG did not induce cell necrosis in this culture system. These present results indicate that excitotoxicity induced by 3OHG is dependent

Abbreviations: GA I, Glutaric aciduria type I; 3OHG, 3-hydroxyglutaric acid; NMDA, *N*-methyl-D-aspartate; MK801, dizocilpine maleate; GABA, γ-amino-butyric acid.

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on special cell culture conditions and/or that other mechanisms than excitotoxicity may lead to neuronal death in GA I.

Material and methods

Materials

The following chemicals were used: MTT (Sigma Co, Deisenhofen, Germany), blot membranes (Whatman, UK), glutamate (Sigma Co), MK801 (dizocilpine maleate, ICN Biomedicals, OH, USA), NMDA-2a- and NMDA-2b receptor antibodies (Chemicon International, Inc, Temecula, CA, USA), goat anti-rabbit IgG (Cappel, Malvern, PA, USA), ECL reagent (Pierce, Rockford, IL, USA), petri dishes (NUNC, Hamburg, Germany), START V-neuron-medium (Biochrom, Berlin, Germany), papain (Worthington Biochemical Corporation), EBSS (Invitrogen, Carlsbad, CA, USA). 3OHG was synthesized from 3-Oxoglutaric acid dimethylester (Sigma-Aldrich) by reduction with $NaBH_4$. After cleavage of the ester bonds, the product was purified by column chromatography.

Cell culture

Animal care followed official government guidelines. Primary mixed cortex cell cultures were prepared from neonatal (day 1) Wistar rat brains. After decapitation and removal of the brains, the hemispheres were separated from the brain stem and preserved in EBSS at 4 °C. After 30 min incubation in papain/EBSS (15 U/ml), cells were suspended by trituration and seeded on poly-L-lysine-coated 35 mm petri dishes (800,000 cells/ml START-V-neuron-medium which suppresses glial growth). Cells were cultured in the same medium for up to 20 days in a humidified atmosphere with 5% CO_2 at 37 °C (Banker and Goslin, 1991). The medium was replaced every second day. The cells were used at days 10–20 in vitro.

Western blot analysis

Cells were solubilized in a PBS buffer and proteins were separated by SDS-PAGE (8% acrylamide) and transferred to a nitrocellulose membrane. After blocking with a 10 mM phosphate buffer containing 1% BSA and 0.1% TWEEN 20 at room temperature for 3 h, the membranes were incubated with polyclonal rabbit anti-rat NMDA-2a- (1:1000) or NMDA-2b receptor antibodies (1:400) overnight at 4 °C. After washing, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000) for 1 h, and immunoreactive proteins were visualized by enhanced chemiluminescence (ECL).

Treatment with 3OHG

After removal of the medium, the cell cultures were incubated with or without 4–8 mM 3OHG or 1 mM glutamate in the presence or absence of 10 μmol MK801 for 1.5–24 h either in START V-neuron medium (in which the Mg^{2+} concentration is 21.54 mg/l) or a magnesia-free medium, containing 10 μM glycine (Kölker et al., 2001).

Neuronal viability

Cell viability was determined by an MTT assay as described by the manufacturer (Carmichael et al., 1987). Protein content of the cell layer per plate was determined according to Bradford (1976).

Assessment of apoptosis

DNA-laddering technique was performed to detect apoptosis. DNA from treated cultures was isolated (Laird et al., 1991) and assayed for oligonucleosomal laddering (Khan et al., 1997) as described previously.

Determination of organic acids

Medium was evaluated at various points of time during incubation for its content of organic acids, respectively, 3OHG metabolites by gaschromatography coupled with mass spectrometry (GC/MS) after derivatization with trimethylsilyl and pentafluorobenzohydroxylamine. Spectra were compared to a library of authentic substances and quantification was carried out with a pentadecanoic acid as an internal standard.

Results

Western blot analysis of NMDA receptors

Western blot analysis of extracts from 10- and 20-day-old mixed cortex neuronal cell cultures treated with or without 4 mM 3OHG for 24 h did not reveal an altered expression level of NMDA-2a and -2b receptor subtypes (Fig. 1).

Neuronal viability

Mixed cortex cell cultures of different ages (10 or 20 days) were incubated for 1.5–24 h with different concentrations of 3OHG and

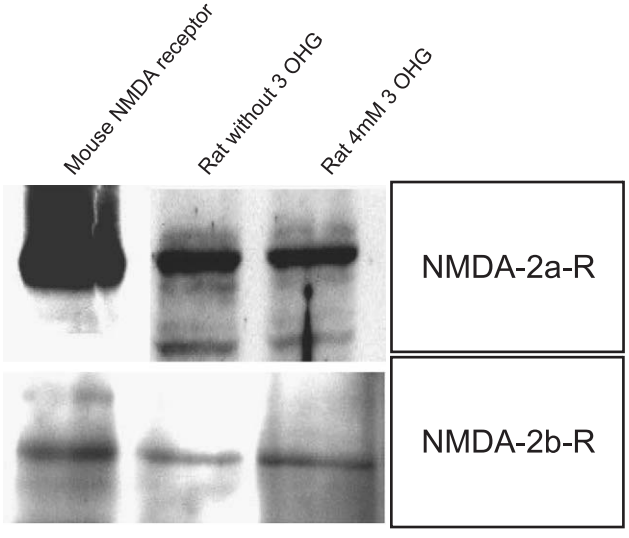

Fig. 1. Expression of NMDA-2a- and -2b receptor in primary rat cortex cultures. Expression of both receptor systems is demonstrated by Western blot analysis in the absence (lane 2) or presence (lane 3) of 4 mM 3OHG (10-day-old cell cultures, incubation time 24 h). Lane 1 gives the NMDA receptors in mouse brain cell suspension as a positive control.

glutamate and were tested for viability by the MTT procedure. The conversion of MTT correlated directly to the number of viable cells (data not shown, range 400,000–1,200,000 cells).

Treatment of 10-day-old mixed cortex cell culture with 1–4 mM 3OHG for 1.5 h in a short-term incubation trial (Fig. 3) and for 24 h in a long-term incubation trial did not affect the viability of the cell cultures (Fig. 2). This result was independent of the presence of magnesia. Higher concentrations of 3OHG (8 mM) lead to the same result. In addition, the content of total protein/dish as well as the activity of β-hexosaminidase activity released into the culture medium were not affected by treatment of cells with 3OHG for 24 h (data not shown).

In contrast, addition of 1 mM glutamate for 1.5 h (short-term incubation) to 10-day-old cell cultures reduced the viability of the cells by ca. 40% which could be prevented by coincubation with the NMDA receptor antagonist MK801 (Fig. 3). Further it was checked that MK801 itself did not alter the effect of 3OHG on the viability of cells (Fig. 3).

Assessment of apoptosis

DNA from culture cells was extracted and assayed for oligonucleosomal laddering as described previously (Khan et al., 1997; Laird et al., 1991). Results for cells incubated with 3OHG for 24 h did not reveal any indication for fractionated DNA as a sign of apoptosis (data not shown).

Determination of organic acids

The concentration of 3OHG was unchanged up to 96 h of incubation and showed no significant differences in comparison to cell-free media, indicating the stability of 3OHG in the medium (data not shown).

Discussion

In the present study, the effect of 3OHG on the viability of primary neuronal cells of neonatal rats was investigated. In a first step, the expression of both NMDA receptor subtypes (NMDA-2a and -2b) was demonstrated, confirming previous reports (Monyer et al., 1994; Wenzel et al., 1997). Densitometric evaluation of the Western blots revealed that incubation of cells with 3OHG did not alter the expression of NMDA-2a- and -2b receptor subtypes. To assess receptor integrity, we applied glutamate to the medium, which was described to initiate an excitotoxic pathway. Indeed, it could be observed that the cell viability declined by approximately 40% vs. control. This effect could be prevented by addition of the NMDA-receptor antagonist MK801.

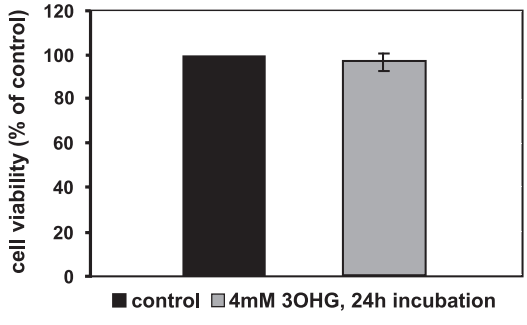

Fig. 2. Primary rat cortex cells: long-term exposition to 4 mM 3OHG. Ten-day-old cell cultures were incubated with 4 mM 3OHG for 24 h. Cell viability was tested using the MTT assay ($n = 17$).

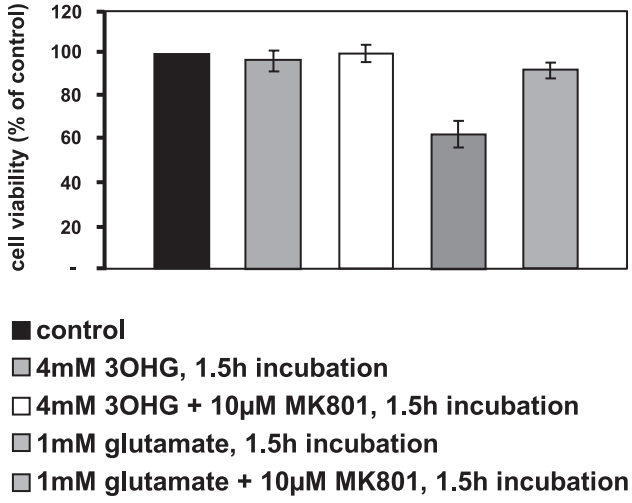

Fig. 3. Primary rat cortex cells: short-term incubation with 3OHG, glutamate and MK801. Ten-day-old cell cultures were incubated with different substances for 1.5 h. The cell viability was tested using the MTT assay ($n = 6$). Glutamate induced a significant reduction of viable cells ($P = 0.05$).

In contrast, incubation of primary neuron cultures of different ages with 3OHG in concentrations of up to 8 mM for 24 h did not induce necrosis. The results were not influenced by the magnesium content of the culture media. In addition, no apoptotic cells could be detected by DNA laddering.

These results are in contrast with former studies, including those of own group, namely, that 3OHG induces death of neurons by activation of NMDA receptors (Kölker et al., 1999; Ullrich et al., 1999).

The theory that 3OHG induces excitotoxicity thus cannot be applied to all cell cultures. The biochemical bases for the different results remain speculative. Basically different expression of carrier proteins for organic acids, of inhibitory γ-amino-butyric acid (GABA)-ergic receptors and of proteins interfering with the energy status of the cells f.e. may lead to the divergent results.

In addition to in vitro data, clinical findings let us speculate that an excitotoxic mechanism may play a role in GA I. Acute encephalopathic crises are triggered by infections (energy crises) and are associated with brain stem ganglia degeneration. Urinary excretion of lactate and decarboxylic acids during metabolic crises indicate a mitochondrial damage. In addition, a postsynaptic vacuolation of neurons, typical for excitotoxicity, has been found by morphological examination.

On the other hand, not all clinical findings can consequently be explained by excitotoxic mechanisms. This includes the development of macrocephalia, "frontotemporal brain atrophy", leucodystrophy and subdural hematoma.

Bjugstad et al. (2001) using embryonic neuron cultures from the rat showed that cell necrosis induced by 3OHG could not be prevented significantly by an NMDA receptor antagonist but by coincubation of the cells with growth factors. Preliminary results of our group show that 3OHG also interferes with the differentiation

of rat endothelial cells not expressing NMDA receptors (Freudenberg et al., 2002; Morley et al., 1998).

Thus, one could speculate that neurodegeneration of different brain areas during acute (striatum necrosis) and chronic disease (“frontotemporal atrophy”) (Amir et al., 1987; Forstner et al., 1999) are triggered by different pathological mechanisms. Age-related differences in brain structure as well as different concentrations of accumulating neurotoxic metabolites during acute and chronic disease may contribute to these different mechanisms.

In this study, it could be demonstrated that 3OHG did not induce excitotoxicity in primary neuronal cultures of the rat. The results indicate that in vitro excitotoxicity induced by 3OHG is dependent on special cell culture conditions. Let us finally summarize the current state of knowledge on excitotoxicity in GA I: 3OHG has been found to be excitotoxic in chick embryo telencephalon cells and in corticostriatal and hippocampal slice cultures from rat brain. In contrast, no excitotoxic effects have been found in embryonic rat striatum and cortex. Furthermore, there is a knockout mouse model that does not show neuronal loss in the striatum (Koeller et al., 2002). Further, we pointed out that not all clinical features appear to have any straightforward explanation in terms of excitotoxicity mediated by NMDA receptors. Our own findings show no effects of 3OHG in primary neuronal rat cell cultures. Given all the evidence, this suggests to us that different or additional mechanisms than excitotoxicity may also be important to neurodegeneration in GA I as well.

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