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Original Article

Protective effect of β-hydroxybutyrate on glutamate induced cell death in HT22 cells

Ying Wang[1,2], Ning Liu[3], Weiwei Zhu[1], Kaihui Zhang[3], Jianping Si[2,4], Meirong Bi[1], Xin Lv[3], Jiwen Wang[2,5]

[1]Department of Pediatrics, Jinan Central Hospital Affiliated to Shandong University, Jinan 250013, China; [2]Department of Neurology, Children's Medical Center, Qilu Hospital of Shandong University, Brain Science Research Institute, Shandong University, Jinan 250012, China; [3]Institute of Pediatric Research, Qilu Children's Hospital of Shandong University, Jinan 250022, China; [4]Department of Pediatrics, The People's Hospital of Guangrao, Dongying 257300, China; [5]Department of Neurology, Shanghai Children's Medical Center, School of Medicine, Shanghai Jiaotong University, No. 1678, Dongfang Road, New Pudong District, Shanghai 200127, China

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Abstract: A ketogenic diet is a high-fat, adequate-protein, low-carbohydrate diet used in the treatment of paediatric epilepsy. However, little is known about the mechanism underlying its antiepileptic effect. Ketone bodies contain about 70% β-hydroxybutyrate (BHB), a metabolic intermediate. Here, we sought to explore the protective effect of BHB on glutamate-induced toxicity in hippocampal neuronal HT22 cells and the underlying mechanisms. HT22 cells were pretreated with BHB, then exposed to glutamate. We examined cell viability, morphological characteristics of apoptosis, intracellular reactive oxygen species (ROS) levels, lipid peroxidation, and activation of the mitogen-activated protein kinase (MAPK) signal pathway. BHB significantly reduced the glutamate-decreased cell viability and inhibited HT22 cell death. Furthermore, BHB decreased ROS generation and alleviated lipid peroxidation. Exposure to glutamate strongly promoted the phosphorylation of c-Jun N-terminal kinase (JNK) and p38, and BHB reduced the glutamate-phosphorylated JNK and p38. MAPK signal pathways are essential for the neuroprotective effect of BHB on glutamate-induced toxicity in HT22 cells.

Keywords: β-Hydroxybutyrate, glutamate, HT22 cells

Introduction

The ketogenic diet (KD) is a high-fat, adequate-protein, low-carbohydrate diet that mimics the effects of starvation-induced ketosis to alleviate seizures [1]. It was initially developed by Wilder, in 1921 [2], and numerous studies have confirmed its antiepileptic effect, particularly in children and adolescents with intractable seizures [3-6]. The possible mechanisms of KD in convulsions are neuronal membrane potential, neuronal excitability, and antioxidant effects [7]. However, the specific mechanisms have not been fully elucidated.

Ketone bodies are important metabolic substrates produced from fatty acids in the liver under a KD and act as an alternative energy source of glucose for the brain [8]. β-Hydroxybutyrate (BHB), acetoacetate, and acetone are the major ketone bodies. Elevated levels of ketone bodies were observed in the blood and urine of patients on a KD [9]. Therefore, ketone bodies are an attractive candidate to investigate in studies of the KD anticonvulsant mechanism. Acetoacetate was first found to have anticonvulsant properties in 1935 [10]. In the past several years, acetoacetate and acetone have been found to have neuroprotective and antiepileptic properties [11-13].

Ketone bodies contain about about 70% BHB, a metabolic intermediate [14]. Prior studies have suggested that BHB significantly prolongs the latency to seizure onset induced by pilocarpine in mice, and the level of blood BHB is associated with seizure reduction [15, 16]. In addition, BHB protects neurons against Aβ-induced toxicity [17]. However, the association of BHB and the anticonvulsive capability has not been established.

We aimed to explore whether BHB has a neuroprotective effect in glutamate-induced oxidative stress and the underlying mechanisms. We used HT22 cells, which have been used as an *in vitro* hippocampal cholinergic neuronal mode to study the mechanism of oxidative glutamate toxicity [18]. Here, we investigated the effect of BHB on reactive oxygen species (ROS) and malondialdehyde (MDA) production and the mitogen-activated protein kinase (MAPK) signal pathway, all mediators related to glutamate-induced cell death, in glutamate-induced toxicity in HT22 cells.

Materials and methods

Main reagents

Dulbecco's modified Eagle's medium was from GIBCO (Munich, USA). BHB, 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Hoechst 33258 were from Sigma-Aldrich (Munich, Germany). Mouse monoclonal antibodies for c-Jun N-terminal kinase (JNK), phospho-JNK, p38, phospho-p38, and horseradish peroxidase-conjugated anti-rabbit IgG were from Cell Signaling Technology (Boston, USA). ROS and MDA assay kits were from Nanjing Jiancheng Bioengineering (Nanjing, China).

HT22 cell culture

HT22 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin and maintained at 37°C in a humidified incubator with 5% CO_2. HT22 cells were seeded onto 96-well plates at 5,000 cells per 100 µl growth media in each well and grown overnight before initiation of any experimental treatments. For phase-contrast microscopy, Hoechst 33258 staining and ROS measurement, HT22 cells were seeded onto 6-well plates at 100,000 cells per dish.

Cell viability assay

HT22 cells were pretreated with 4 and 8 mM BHB for 12 h, then stimulated with 5 mM glutamate for 24 h. Cell viability was estimated by measuring MTT metabolism. Briefly, 10 µl MTT solution (5 mg/ml) was added to each well of a 96-well plate and incubated for 3 h at 37°C. Subsequently, the supernatant was removed, and 100 µl dimethylsulfoxide (DMSO) was added to each well for dissolving formazan crystals. The absorbance at 490 nm was measured. Untreated cells were considered the control, and results are expressed as a percentage of the control.

ROS measurement

The level of intracellular ROS was detected by use of the non-fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA). Cells were pretreated with 4 mM BHB for 12 h, then stimulated with 5 mM glutamate for 24 h. Then the culture medium was removed, and cells were washed gently with phosphate buffered saline (PBS) 3 times. Subsequently, 10 µM DCFH-DA was added to cells and incubated at 37°C for 20 min in the dark, then cells were washed with warmed serum-free DMEM 3 times. Accumulation of intracellular ROS was observed and photographed by laser scanning confocal microscopy. Fluorescence images were quantified by use of NIH Image J and evaluated by comparing with the control.

MDA measurement

Total MDA was measured by use of a kit. Cells were pretreated with 4 mM BHB for 12 h, then stimulated with 5 mM glutamate for 12 or 24 h. Cells were rinsed with (PBS) and lysed with ice-cold RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), then centrifuged at 1600 ×g for 10 min at 4°C. Supernatant was collected for measuring MDA. Color development was monitored at 560 nm in a kinetic mode with a plate reader. There were 6 samples in each group.

Western blot analysis

HT22 cells were pretreated with 4 mM BHB for 12 h, stimulated with 5 mM glutamate for 30 min, then lysed in ice-cold RIPA protein lysis buffer with protease inhibitors. The protein concentration was determined by the Enhanced BCA Protein Assay Kit. A total of 30 µg protein was separated on 12% SDS-PAGE and transferred to a PVDF membrane. Membranes were incubated in blocking buffer (1× PBS containing 5% non-fat, dried milk and 0.1% Tween 20) for 1 h at room temperature, then incubated with antibodies against phosphorylated-JNK, total-JNK, phosphorylated-p38, and total-p38 at 1:1000 dilution for overnight at 4°C. Then membranes were washed 3 times in PBS containing 0.1% Tween 20 and incubated for 1 h with horseradish peroxidase-conjugated sec-

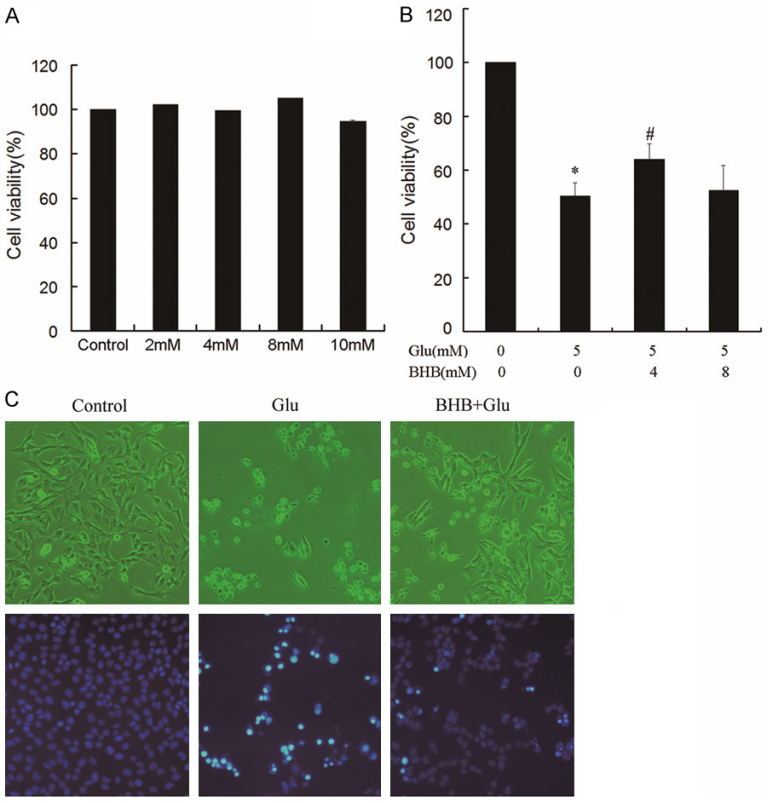

Figure 1. β-hydroxybutyrate (BHB) protects HT22 cells against glutamate-induced toxicity. A: HT22 cells were treated with 2 mM, 4 mM, 8 mM and 10 mM BHB for 24 h and cell viability was determined by MTT assay. Data are mean ± SD. B: Cells were treated with 5 mM glutamate for 24 h after 4 or 8 mM BHB pretreatment for 12 h, and cell viability was determined by MTT assay. Data are mean ± SD. *P<0.05 vs control; #P<0.05 vs glutamate. C: HT22 cells were pretreated with 4 mM BHB for 12 h, stimulated with 5 mM glutamate for 24 h, then stained with Hoechst 33258, and photographed by phase-contrast microscopy.

ondary antibodies. Protein bands were visualized by enhanced chemiluminescence (Pierce® ECL Western Blotting Substrate, Thermo Scientific).

Statistical analysis

All data are presented as mean ± SD from 3 or more independent experiments. Differences among groups were analyzed by SPSS 16.0 (SPSS Inc., Chicago, IL). Comparison between means was carried out using Paired-sample T test. P<0.05 was considered statistically significant.

Results

BHB protected HT22 cells against glutamate-induced toxicity

To determine whether BHB protected against glutamate-induced toxicity, we first investigate the effect of BHB on viability of HT22 cells with 4 different concentrations, and we found that BHB has no effect on the growth of HT22 cells (**Figure 1A**). So, in the present study HT22 cells were pretreated with 4 mM and 8 mM BHB for 12 h, then exposed to 5 mM glutamate for another 24 h, then cell viability was determined. Glutamate treatment reduced cell survival by 50%, which was increased by 14% on pretreatment with 4 but not 8 mM BHB (**Figure 1B**). Moreover, HT22 cells exposed to 5 mM glutamate for 24 h showed plasma membrane blebbing and cell shrinkage and lost their normal spindle-shaped morphology (**Figure 1C**). In contrast, cells pretreated with BHB cells maintained their normal neurite morphology, similar to cells in vehicle control cultures. We tested the chromatin condensation and DNA fragmentation of HT22 cells by Hoech-st 33258 staining (**Figure 1C**). Nuclei of glutamate-treated cells were stained bright, which suggests cell damage. These results provide evidence for glutamate-induced apoptosis in HT22 cells, and BHB protected HT22 cells against glutamate-induced damage.

BHB decreased ROS production and lipid peroxidation in HT22 cells

Oxidative stress has been associated with cell death, and ROS and lipid peroxidation are important oxidative stress indicators. Therefore, we examined ROS level after 24 h glutamate treatment in HT22 cells. An amount of 5 mM glutamate significantly increased ROS production by more than six-fold as compared with untreated cells (**Figure 2A**, **2B**). BHB significantly suppressed ROS generation, so BHB may protect HT22 cells by inhibiting oxidative stress. After 12 and 24 h treatment with glutamate, MDA level, as an indicator of lipid peroxidation, decreased significantly as compared with glutamate alone (**Figure 2C**).

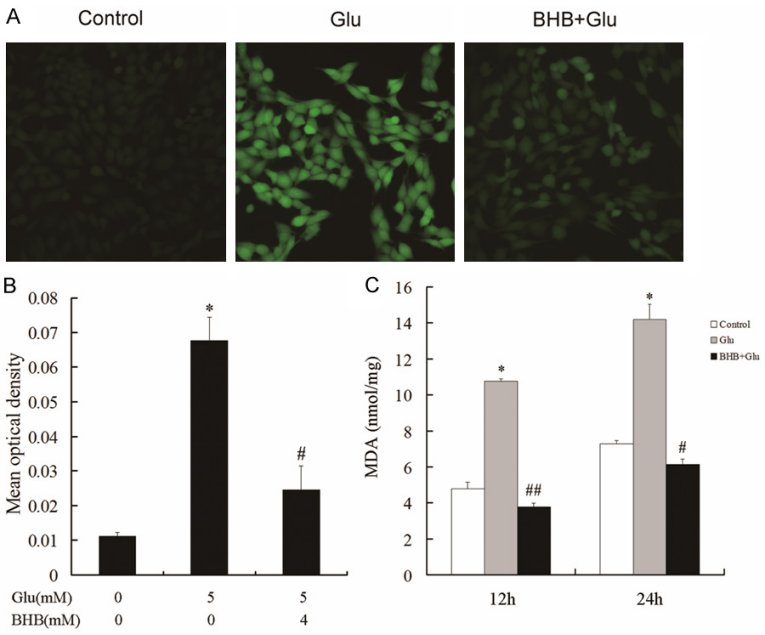

Figure 2. Effect of BHB on reactive oxygen species (ROS) production and changes in malondialdehyde (MDA) expression. Cells were incubated with or without BHB, 4 mM, for 12 h, then 5 mM glutamate was added for 24 h. A, B: Cells were loaded with 2,7-dichlorofluorescencein (DCF) to measure ROS production. C: Cells were harvested and MDA content was measured. Data are mean ± SD. *P<0.05 vs control; #P<0.05, ##P<0.01 vs glutamate.

BHB activated the p38 MAPK and JNK pathway

An amount of 5 mM glutamate significantly increased the phosphorylation of p38 and JNK, whereas BHB reduced the glutamate-induced phosphorylation of p38 and JNK (**Figure 3A**, **3B**). These results suggest that BHB has a protective effect on glutamate-induced HT22 cell death perhaps by inhibiting the MAPK signal pathway.

Discussion

KD has been used as a therapeutic approach for paediatric epilepsy [9]. Many clinical and experimental studies have been done to understand the possible therapeutic mechanism and focused on neuronal membrane potential, neuronal excitability, and antioxidant effects [7]. The effects are often associated with important metabolic changes that induce increased levels of ketone bodies, mainly BHB and acetoacetate [19]. A proposed hypothesis for the anticonvulsant actions of ketogenic diets is that the ketone bodies protect against cell death via antioxidant effects [7]. Here, we investigated the potential capacity of ketone bodies to protect against the toxic effects of high concentrations of glutamate and the underlying mechanisms. We found that BHB significantly reduced glutamate-decreased cell viability and inhibited hippocampal neuronal HT22 cell death. Furthermore, BHB decreased ROS generation and alleviated lipid peroxidation. Exposure to glutamate strongly promoted the phosphorylation of JNK and p38, which was reversed by BHB treatment. MAPK signal pathways are essential for the protective effect of BHB on glutamate-induced toxicity in HT22 cells.

BHB is a major component of the ketone bodies produced in liver, which have a broad regulatory role in metabolic disease by altering histone acetylation and gene expression, post-translational protein function, and cell surface receptor activation [14]. Earlier studies showed that BHB has neuroprotective effects and decreases the apoptosis of neuronal cells. BHB inhibited the apoptosis of PC12 cells induced by H_2O_2 through inhibiting oxidative stress [20]. BHB could recover cell viability of BV2 cells reduced by lipopolysaccharide [21]. Moreover, HT22 cell viability was significantly increased on pretreatment with BHB for 24 h, followed by exposure to 5 mM glutamate for another 12 h [22]. Here, we first pretreated HT22 cells with 4 or 8 mM BHB for 12 h, then exposed them to 5 mM glutamate. Consistent with previous reports, 4 but not 8 mM BHB significantly inhibited the decreased cell viability and apoptosis induced by glutamate. The maximal protective effects of BHB might be at 4 mM.

Accumulating evidence suggests that the initiation and progression of neurodegenerative diseases are associated with glutamate oxidative toxicity and oxidative stress [18]. Rapid ROS production is one of the initial events in oxidative glutamate toxicity and activates the downstream lipoxygenase and apoptosis-related sig-

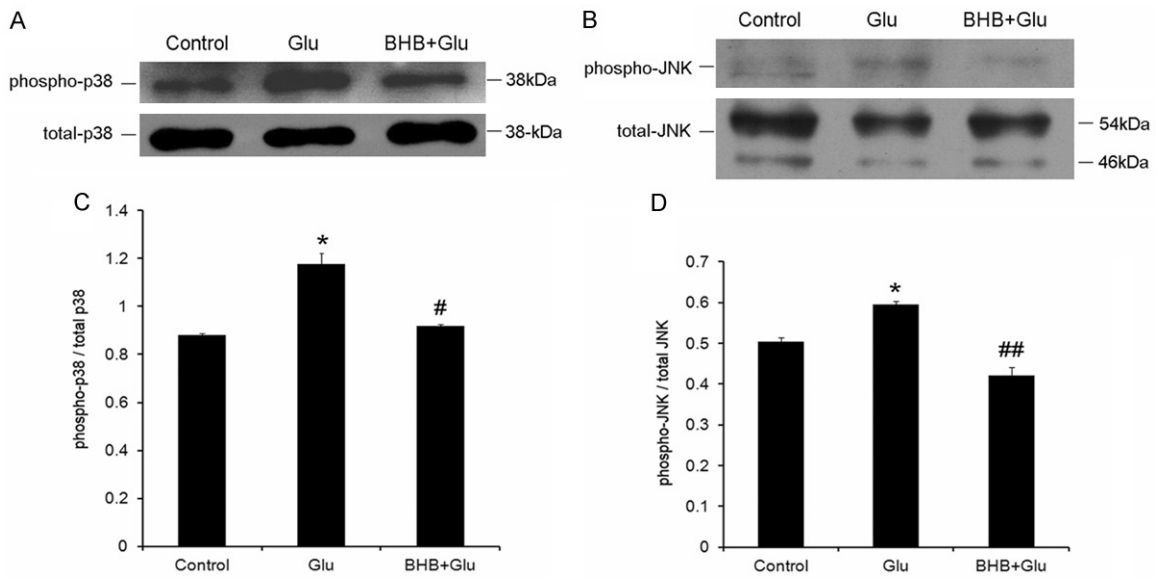

Figure 3. Representative western blot analyses of phosphorylated and total p38 and phosphorylated and total JNK expression. Cells were pretreated with 4 mM BHB for 12 h, then exposed to 5 mM glutamate for 0.5 h. Shows western blot analysis of phosphorylated and total p38 (A, B) and phosphorylated and total JNK (C, D). Data are mean ± SD. *P<0.05 vs control; #P<0.05, ##P<0.01 vs glutamate.

nal pathway [23]. Although prior study showed the neuroprotective effects of BHB on glutamate-induced toxicity in HT22 cells, the underlying mechanism was not elucidated. Here, we used a glutamate-induced oxidative toxicity model in the HT22 cell line to explore the neuroprotective effects of BHB and the mechanisms. HT22 cells lack ionotropic glutamate receptors, so glutamate-induced cell death was due to oxidative stress by a high concentration of glutamate blocking the glutamate-cystine transporter in the plasma membrane [24]. The reduced cystine uptake leads to GSH depletion and finally increased ROS production [25]. Lipid metabolism has an important role in oxidative glutamate toxicity. Lipid peroxidation assay measures the concentration of MDA as the level of lipid peroxidation [26]. We further measured the cellular levels of ROS and MDA after glutamate and BHB treatment. BHB reduced the glutamate-increased ROS production and intracellular MDA concentration. These data are consistent with earlier findings of a high-fat KD significantly decreasing ROS production in the hippocampus and BHB reducing intracellular ROS generation in Aβ-induced PC12 cells [17, 27]. In addition, KD-fed mice and humans showed increased plasma BHB levels [28]. Thus, the antiepileptic effect of BHB may be associated with the regulation of ROS and lipid peroxidation.

The MAPK pathway plays a key role in regulating cell growth, proliferation, differentiation, migration, and apoptosis [29]. The classical MAPK pathway includes extracellular signal-regulated kinase (ERK), JNK or stress-activated protein kinase and p38 MAPK. Activation of JNK and p38 can promote cell death [30], and the MAPK pathway is activitated in glutamate-induced toxicity [18]. Here, we showed that BHB significantly reversed the glutamate-induced overexpression of phosphorylated JNK and p38. This finding was consistent with the previous studies of BHB decreasing p38 MAPK phosphorylation [31]. Therefore, the p38 MAPK and JNK pathway is involved in the glutamate-induced toxic effects in neuron cells.

The results suggest the BHB protects hippocampal neuronal HT22 cells against glutamate-induced apoptosis by inhibiting ROS production, which induced the MAPK signal pathways. BHB offers protection against oxidative stress in HT22 cells, which supports its use for the treatment of epilepsy.

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Disclosure of conflict of interest

None.

Address correspondence to: Xin Lv, Institute of Pediatric Research, Qilu Children's Hospital of Shandong University, Jinan 250022, China. E-mail: lvxinetyy@163.com; Jiwen Wang, Department of Neurology, Children's Medical Center, Qilu Hospital of Shandong University, Brain Science Research Institute, Shandong University, Jinan 250012, China. E-mail: wangjiwen_99@hotmail.com

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