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Acetoacetate Protects Neuronal Cells From Oxidative Glutamate Toxicity

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Glutamate cytotoxicity contributes to neuronal degeneration in many central nervous system (CNS) diseases, such as epilepsy and ischemia. We previously reported that a high-fat and low-carbohydrate diet, the ketogenic diet (KD), protects against kainic acid-induced hippocampal cell death in mice. We hypothesized based on these findings that ketosis resulting from KD might inhibit glutamate cytotoxicity, resulting in inhibition of hippocampal neuronal cell death. Therefore, we investigated the role of ketone bodies [acetoacetate (AA) and β-hydroxybutyrate (β-OHB)] both in a mouse hippocampal cell line (HT22) and in rat primary hippocampal neurons. As a result, we found that pretreatment with 5 mM lithium AA and 4 mM Na β-OHB protected the HT22 hippocampal cell line and primary hippocampal neuronal culture against 5 mM glutamate toxicity and that up to 2 hr of pretreatment with 5 mM AA had a protective effect against 5 mM glutamate toxicity in the HT22 cell line. Pretreatment with 5 mM AA decreased ROS production of HT22 cell line at 2 and 8 hr exposure of glutamate, and it decreased the appearance of annexin V-positive HT22 cells, which are indicative of an early stage of apoptosis, and propidium iodide-positive HT22 cells, which are indicative of necrosis.

Key words: acetoacetate; β-hydroxybutyrate; glutamate; ketogenic diet; HT22

Glucose is the main energy source in the brain; however, during prolonged fasting, as well as in immature animals during suckling, the brain utilizes other energy sources, such as ketone bodies [β-hydroxybutyrate (β-OHB), acetoacetate (AA), and acetone]. Under normal conditions, blood levels of ketone bodies are maintained below 0.5 mM (Sokoloff, 1973), but, during fasting or a high-fat, low-protein, and low-carbohydrate diet, blood levels of ketone bodies become elevated (referred to as *ketosis*); these ketone bodies are produced from liver and used as an energy substrate (Casazza et al., 1984; Nordli et al., 1992).

The ketogenic diet (KD) is a high-fat, low-protein, and low-carbohydrate diet used to treat refractory epilepsy in children. The elevation of blood ketone bodies is a prominent result of a KD. Accordingly, several reports have suggested that the antiepileptic effect of a KD may be due to the direct action of ketone bodies (Pan et al., 1999; Thio et al., 2000). It remains unclear, however, whether ketosis is a critical factor in the antiepileptic effect of KD. Previous reports have suggested that, during starvation or administration of ketone bodies, the ketone bodies exert a neuroprotective effect in models of hypoxia/ischemia, Alzheimer's deisease, and Parkinson's disease (Marie et al., 1990; Kashiwaya et al., 2000; Suzuki et al., 2001; Masuda et al., 2005). Consistently with these observations, we reported that a KD reduced kainic acid-induced hippocampal cell death by decreasing several apoptosis-related genes, such as caspase-3 (Noh et al., 2003). Furthermore, Sullivan et al. (2004) suggested that a KD might exert a neuroprotective effect in the presence of the ATP synthase inhibitor oligomycin (used to maximize mitochondrial membrane potential), by diminishing reactive oxygen species (ROS) production in the mouse hippocampus, which is largely dependent on mitochondrial membrane potential. Recent studies have suggested that oxidative stress is an important consequence of glutamate activation and excitotoxicity and plays a critical role in epileptic brain damage (Sudha et al., 2001; Patel, 2004). Two pathways for glutamate toxicity have been

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described: receptor-initiated excitotoxicity (Choi, 1988) and nonreceptor-mediated oxidative glutamate toxicity (Tan et al., 1998). High concentrations of extracellular glutamate (>200 μM) have been shown to cause glutamate-mediated oxidative stress by preventing cystine uptake into cells through a glutamate/cystine antiporter, followed by depletion of intracellular cystine and loss of glutathione (GSH; Schubert et al., 1992). With a diminishing supply of GSH, excess ROS accumulate, leading ultimately to cell death.

Oxidative glutamate toxicity has been most extensively studied in HT22 cells. This immortalized mouse hippocampal cell line lacks ionotrophic glutamate receptors, and glutamate in this system inhibits cystine transport and leads to GSH depletion (Morimoto and Koshland, 1990). Subsequently, ROS are produced by mitochondria, which cause an increase in Ca^{2+} influx and finally cell death (Maher and Davis, 1996). Therefore, this system was used to test the protective efficacy of ketone bodies against glutamate-mediated toxicity. We show that ketone bodies, especially AA, protect HT22 cells and rat primary hippocampal neurons from oxidative stress caused by glutamate.

MATERIALS AND METHODS

Cell Culture and Experimental Treatments

HT22 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, without L-glutamine), supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin (Gibco BRL, Gaithersburg, MD). HT22 cells were seeded onto 96-well plates at 5,000 cells per 100 μl growth media in each well and grown overnight prior to initiation of any experimental treatments. For phase-contrast microscopy, HT22 cells were seeded on 60-mm tissue culture dishes at 100,000 cells per dish. AA (lithium salt; Sigma, St. Louis, MO) was dissolved in 10 mM phosphate buffer, and the pH was adjusted to 7.5 with 0.1 M HCl. D-β-OHB (sodium salt; Sigma) was dissolved in 10 mM phosphate buffer. Cells were exposed to various doses AA, β-OHB, or acetone for 24 hr. Previous studies have treated cells with ketone bodies for 24 hr to examine their effects on cell viability (Massieu et al., 2003). We also treated the cells with ketone bodies for 24 hr, based on these investigations. After this period, 5 mM L-glutamic acid (Sigma) was added to each well for another 12 hr. For the rescue experiments with AA, cells were treated with glutamate for 0 min, 10 min, 30 min, or 2 hr before AA was added.

Primary Hippocampal Cultures

Primary cultures of hippocampal neurons were prepared from Sprague-Dawley (SD) rat embryos of 18 days gestation, as described by Kashiwaya et al. (2000), with some modifications. Briefly, after dissection, hippocampi were minced and treated with 0.25% trypsin-EDTA (Gibco, Boston, MA) for 20 min. After centrifugation, cells were resuspended in a neurobasal medium (Gibco) containing 1:50 B27 (Gibco), 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum and were subsequently dissociated by repeated pipetting through a 1-ml blue Eppendorf pipette tip. The cells were then plated at a cell density of 1×10^5 cells/dish in poly-d-lysine-coated 24-well plates. On days 2 and 4, one-half of the medium was exchanged. On day 5, one-half of the medium was removed and mixed with 200 μl DMEM (without L-glutamine). β-OHB or AA was added to the mixed media, and 200 μl was replaced in the well to create a concentration of 5 mM. Twenty-four hours later, one-half of the medium was replaced with DMEM (without L-glutamine) and one of the following components: 100 μl medium only, medium containing ketones (β-OHB or AA), medium containing 5 mM glutamate (Sigma), or a combination of the latter two components. The cells were then incubated for 12 hr. Neuronal population in these cultures was 95% as determined by immunohistochemistry against microtubule-associated protein (MAP) and glial fibrillary acidic protein (GFAP), neuronal and glial markers, respectively (data not shown).

Cell Viability

Cell viability was estimated by measuring metabolism of 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), as described previously (Tan et al., 1998). Briefly, 10 μl MTT solution (5 mg/ml) was added to each well of a 96-well plate, and cells were maintained for 4 hr at 37°C. Then, 100 μl of solubilization solution [50% dimethylformamide and 20% sodium dodecyl sulfate (SDS), pH 4.8] was added to each well. After an overnight incubation, absorbance at 570 nm was measured. Cell viability was expressed as percentage of neuroprotection vs. controls set at 100%. To confirm MTT assay results, we performed trypan blue (TB) exclusion staining, followed by cell counting. TB is a vital dye excluded by viable cells with intact cell membranes. Fifty microliters of 0.4% TB was added to 50 μl of the prepared cells suspension, and we then counted the number of stained (blue) and unstained (white) cells in each of the four corner squares of the hemacytometer. Cell viability (%) was calculated according to the following equation: cell viability (%) = [number of unstained (viable) cells/total cells counted (stained + unstained)] × 100.

ROS Measurement

Time-course experiments were performed to compare ROS production in HT22 cells after glutamate exposure. ROS production was detected by using the dye 2,7-dichlorofluorescein diacetate (DCF; Sigma), as described by Tan et al. (1998). Data were collected with a FACScan fluorescence accelerated cell scanner, using the data acquisition program Cellquest (Becton-Dickinson, San Jose, CA). Visual inspection, via confocal microscopy, confirms the increase in fluorescence with lengthened exposures of the plated cells to glutamate.

Assessment of Apoptosis by Flow Cytometry

Apoptotic cells were detected by flow cytometry after staining with both fluorescein isothiocyanate-conjugated annexin V and propidium iodide (PI), with a commercially available kit (Annexin V-FLUOUS Kit; Roche Molecular

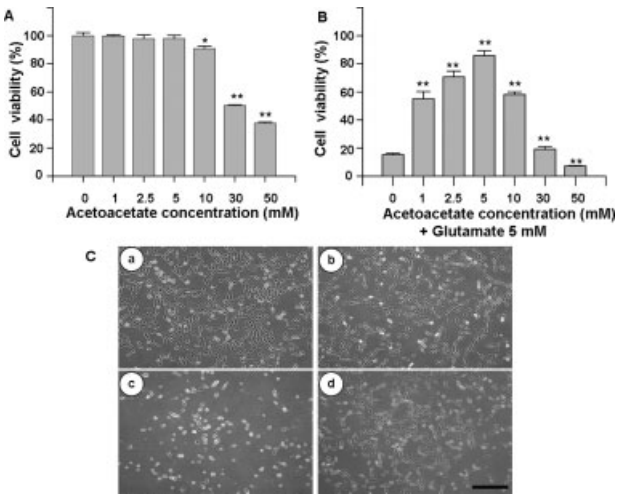

Fig. 1. Acetoacetate (AA) protects HT22 cells from glutamate-mediated toxicity. **A:** Concentration-dependent effects of AA on HT22 cell viability. Cells were treated with AA for 24 hr, and cell viability was determined by MTT assay. Cell survival was expressed as percentage of neuroprotection vs. controls set at 100% (mean ± SEM; each n = 30). **B:** Concentration-dependent neuroprotective effects of AA on glutamate treatment. Cells were treated with 5 mM glutamate for 12 hr following AA treatment for 24 hr. Asterisks denote significant differences from untreated controls (mean ± SEM; each n = 30). *$P < 0.05$, **$P < 0.0005$. **C:** Representative photomicrographs of the HT22 cells. Cells were incubated in the presence (b,d) or absence (a,c) of 5 mM AA for 24 hr. After this period, cells were treated with glutamate (5 mM) for 12 hr (c,d). Photomicrographs show phase-contrast images of representative fields of cells. Scale bar = 40.2 μm.

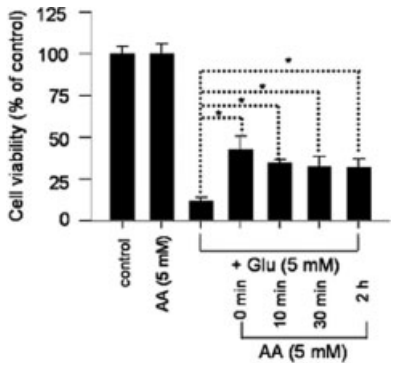

Fig. 2. Rescue experiments in which cells were treated with glutamate for 0 min, 10 min, 30 min, or 2 hr before acetoacetate (AA) was added. Asterisks denote significant differences from untreated controls (mean ± SEM; each n = 24). *$P < 0.005$, one-way ANOVA, followed by a Tukey's multiple-comparison test.

Biochemicals). To detect early and late apoptosis, both adherent and floating cells were harvested together and incubated for 10 min in Annexin V-FLUOUS labeling solution. In each experiment, 10,000 events were measured in the gate regions chosen for calculations.

Data Analysis

All data are presented as mean ± SEM from three or more independent experiments. All statistical analyses were performed via one-way ANOVA, followed by Tukey's multiple-comparison tests. Differences with a probability (P) less than 0.05 were considered statistically significant.

RESULTS

AA and β-OHB but Not Acetone Protect HT22 Cells From Glutamate-Induced Toxicity

To test the toxicity of AA itself in neuronal cells, we first treated HT22 cells with various concentrations of AA. AA concentrations up to 5 mM were tolerated by neurons without significant affect on cell viability, whereas higher concentrations of AA (>5 mM) led to cell death (Fig. 1A). To determine the effective concentration of AA, HT22 cells were exposed to 5 mM glutamate in the presence of various concentrations of AA, and cell viability was determined as described above (Fig. 1B). Glutamate treatment reduced cell survival by 15% (Fig. 1B), but this viability was increased by pretreatment with AA in a concentration-dependent manner. The calculated median effective concentration (EC_{50}) of added AA was 0.9 mM, with a maximal effect (86% protection) at 5 mM (Fig. 1B). As shown in Figure 1Cc, a 12-hr exposure to 5 mM glutamate resulted in the disappearance of neurites and shrinkage of cell bodies. However, pretreatment with 5 mM AA provided effective protection in HT22 cells (Fig. 1Cd), compared with glutamate-only-treated cells (Fig. 1Cc). Cells treated with 5 mM AA alone appeared healthy, confluent, and undamaged (Fig. 1Cb), similar to control cells (Fig. 1Ca). Interestingly, glutamate-induced cell death could be partially prevented in rescue experiments in which AA was added at various times after glutamate treatment (Fig. 2). Simultaneous application of AA and glutamate resulted in a protective effect (43% protection). AA still provided a protective effect when given after glutamate treatment, and the effect did not differ from 10 min to 2 hr.

We next examined the effect of β-OHB on HT22 cells. To test the toxicity of β-OHB itself in neuronal cells, we treated HT22 cells with various concentrations of β-OHB. β-OHB concentrations up to 4 mM were tolerated by neurons without significant affect on cell viability, whereas concentrations greater than 4 mM led to cell death (Fig. 3A). To determine the effective concentration of β-OHB, HT22 cells were exposed to 5 mM glutamate in the presence of various concentrations of β-OHB, and cell viability was determined as described above (Fig. 3B). Cell viability was significantly increased by pretreatment with β-OHB in a concentration-dependent manner (Fig. 3B). The calculated median effective concentration (EC_{50}) of added β-OHB was 3.1 mM, and maximal protection was observed at 4 mM (52% protection). Nevertheless, this viability was lower than that for AA (Fig. 1B). In Figure 3C, photomicro-

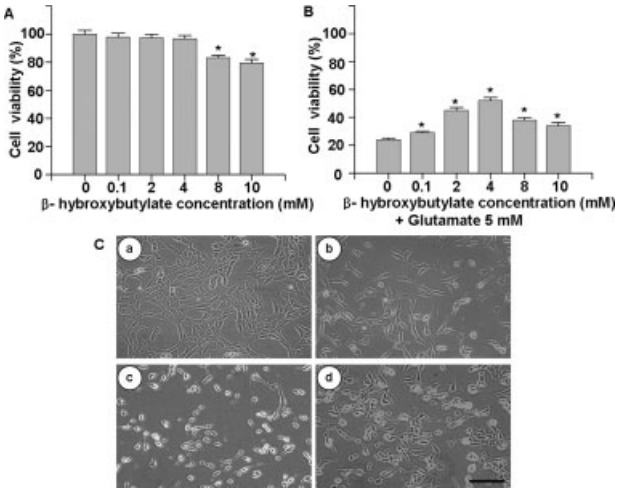

Fig. 3. **A:** Concentration-dependent effects of β-hydroxybutylate (β-OHB) on HT22 cell viability. Cells were treated with β-OHB for 24 hr, and cell viability was determined by MTT assay. Cell survival was expressed as percentage neuroprotection and compared with controls set at 100% (mean ± SEM; each n = 30). **B:** Concentration-dependent neuroprotective effects of β-OHB on glutamate treatment. 5 mM glutamate was applied to the cells for 12 hr following β-OHB treatment for 24 hr. Asterisks denote significant differences from untreated controls (mean ± SEM; each n = 24). $*P < 0.05$, one-way ANOVA, followed by a Tukey's multiple-comparison test. **C:** Representative photomicrographs of the HT22 cells. Cells were incubated in the presence (b,d) or absence (a,c) of 4 mM β-OHB for 24 hr. After this period, cells were treated with glutamate (5 mM) for 12 hr (c,d). Photomicrographs show phase-contrast images of representative fields of cells. Scale bar = 40.2 μm.

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Finally, we examined the effect of acetone on HT22 cells. To test the toxicity of acetone itself in neuronal cells, we treated HT22 cells with various concentrations of acetone (Fig. 4A). To determine the effective concentration of acetone, HT22 cells were exposed to 5 mM glutamate in the presence of various concentrations of acetone, and cell viability was determined by the MTT assay. As shown in Figure 4B, cell viability was not increased significantly by pretreatment with acetone at various doses. Therefore, we focused on the effects of AA on ketone bodies in the present study.

AA Protects Rat Primary Hippocampal Neurons Against Glutamate Toxicity

HT22 cells do not express an ionotrophic glutamate receptor. To test the role of ketone bodies in glutamate receptor-intact neurons, we applied AA, β-OHB or acetone to primary hippocampal neurons. Glutamate-induced cell death was completely protected by pretreatment with 5 mM AA (Fig. 5). Additionally, 4 mM β-OHB showed a significant protective effect compared with primary hippocampal neurons treated with glutamate alone ($P < 0.005$), but acetone did not showed protective effects.

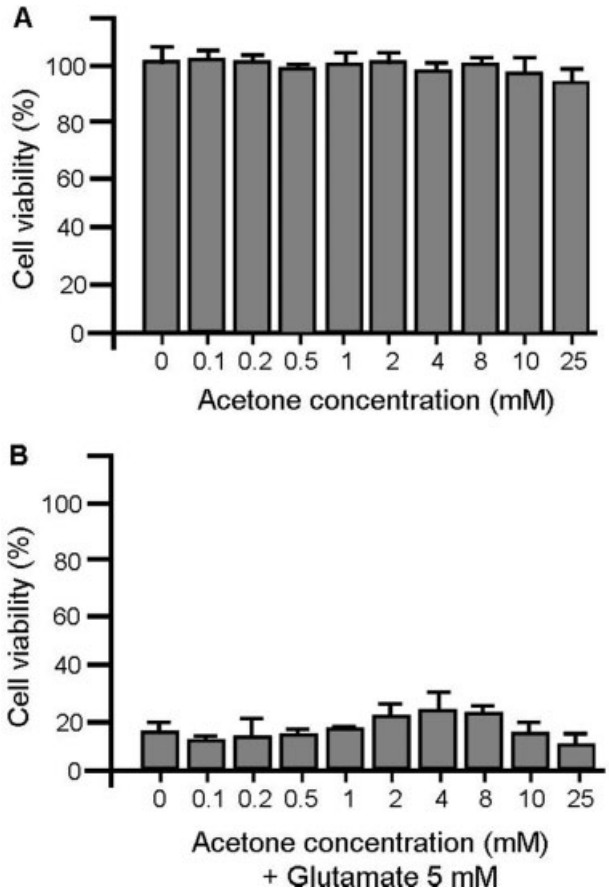

Fig. 4. Effects of acetone on HT22 cell viability. Cell survival was expressed as percentage neuroprotection and compared with controls set at 100%. **A:** Various concentration of acetone itself did not affect cell viability (each n = 24). **B:** Acetone did not protect significantly against glutamate-induced cell death in HT22 cell. Cells were treated with 5 mM glutamate for 12 hr following acetone treatment for 24 hr (mean ± SEM; each n = 32).

AA Decreases ROS Production in HT22 Cells

To elucidate further the underlying mechanism of AA-mediated neuroprotection, we measured ROS levels. As shown in Figure 6A, confocal images showing DCF fluorescence did not differ between normal (Fig. 6Aa) and AA-alone-pretreated cells (Fig. 6Ab). After glutamate treatment (2 hr), ROS levels gradually increased (Fig. 6Ac). At the 8-hr time point, DCF fluorescence appeared stronger than at the 2-hr time point (Fig. 6Ac,e). Interestingly, pretreatment with AA prevented glutamate-induced ROS production markedly at all experimental time points (Fig. 6Ad,f). We quantified ROS production at all experimental time points after glutamate treatment by using flow cytometry and found that AA significantly decreased glutamate-induced ROS production compared

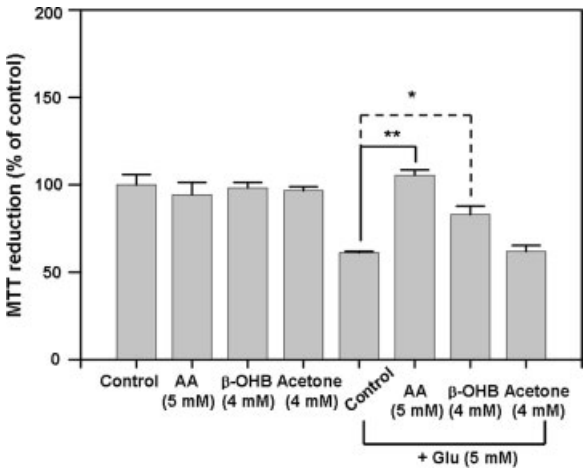

Fig. 5. Acetoacetate (AA) and β-hydroxybutylate (β-OHB) protect against glutamate (Glu)-induced cell death in primary hippocampal neurons, but acetone does not significantly protect against Glu-induced cell death. Cellular viability was assessed by MTT assay. Cell survival was expressed as percentage neuroprotection compared with controls set at 100%. These data represent a combination of oxidative Glu-induced toxicity and Glu receptor-induced excitotoxicity. Asterisks denote significant differences from untreated controls (mean ± SEM; each n = 32). *$P < 0.005$, **$P < 0.0005$, one-way ANOVA, followed by a Tukey's multiple-comparison test.

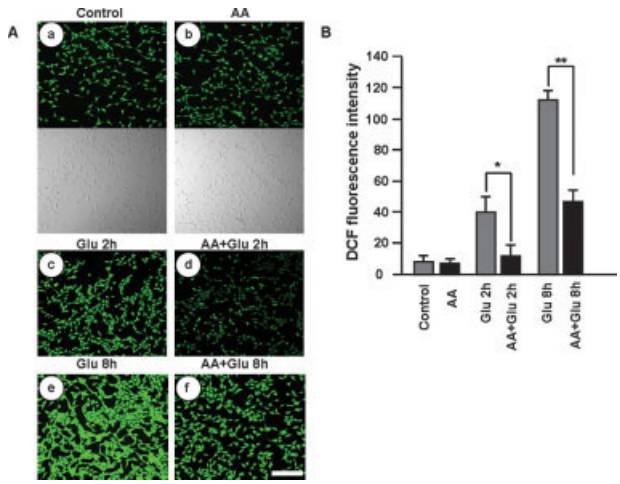

Fig. 6. Effect of acetoacetate (AA) on ROS production. **A:** Cells were incubated in the presence (b,d,f) or absence (a,c,e) of 5 mM AA for 24 hr. After this period, glutamate (5 mM) was added to individual culture dishes (c–f), and cells were then loaded with 2,7-dichlorofluorescencein (DCF) to measure ROS production at various time points after glutamate treatment (a: control; b: acetoacetate-alone treated cells; c,d: after 2 hr, e,f: after 8 hr). **B:** Quantification of ROS production. Values are the mean ± SEM from three independent cultures, performed in quadruplicate, compared with glutamate-alone treatment groups. Data were collected from 20,000 live cells for each plot. *$P < 0.005$, **$P < 0.0005$, Student's *t*-test.

with that in HT22 cells treated with glutamate alone (Fig. 6B).

Flow Cytometric Detection Of Apoptotic Cells by Dual Staining With Annexin V and PI

Cells were analyzed for apoptosis by staining with annexin V and PI (Fig. 7). Control (Fig. 7A) and AA-only-treated cells (Fig. 7D) clustered in the lower left (LL) quadrant of the dot-plot. After glutamate treatment (8 hr), the fraction of cells in the lower right (LR) quadrant of the dot-plot increased (Fig. 7B), which corresponds to early-phase apoptotic cells that have not yet lost outer membrane integrity but have lost phosphatidylserine asymmetry (Sewell et al., 2005). After glutamate treatment (12 hr), the fraction of cells in the upper right (UR) as well as LR quadrants of the dot-plot increased (Fig. 7C). UR represents the late-phase apoptotic cells that were stained with both annexin V and PI. These results suggest that fewer cells live 12 hr after glutamate treatment. However, pretreatment with AA caused a significant decrease in the percentage of glutamate-induced single-positive cells for annexin V (LR) or of double-positive cells for annexin V and PI (UR; Fig. 7E,F).

DISCUSSION

The present study was designed to investigate the potential capacity of ketone bodies to protect against the toxic effects of high concentrations of glutamate. After a brief exposure to low concentrations of glutamate, cell death is initiated by N-methyl-D-aspartate (NMDA) receptor activation and receptor-dependent phase (Choi, 1988; Tan et al., 1998). However, when there is a high concentration of extracellular glutamate (>200 μM), cystine uptake into cells is prevented, followed by the depletion of intracellular cysteine and glutathione, resulting in severe oxidative stress (Murphy et al., 1989; Schubert and Piasecki, 2001). In addition, Schubert and Piasecki (2001) recently reported that approximately 50% of the total cell death in primary mouse cortical neurons resulted from oxidative glutamate toxicity (300 μM). Therefore, we tested the potential role of ketone bodies in protecting against oxidative glutamate toxicity with the HT22 cell line and primary hippocampal neurons.

The results present here show that AA and β-OHB significantly reduce glutamate-induced neuronal cell death in HT22 cells and primary hippocampal neurons. Pretreatment with 5 mM AA provides the most effective protection against glutamate-induced neuronal cell death in HT22 cells (86% protection) and primary hippocampal neurons (completely protected). In the case of β-OHB, 4 mM β-OHB show maximal protection, 52% in HT22 cells and 80% in primary hippocampal neurons. However, pretreatment with acetone provide no effective protection against glutamate-induced neuronal cell death in HT22 cells or primary hippocampal neurons. In Figure 1A we can see that the higher concentration of AA (>10 mM) itself led to cell death. This is caused by a high lithium load, which is toxic to cells. In Figure 3A we can see that the higher concentration of β-OHB (>4 mM) itself led to cell death. HT22 cells were cultured in DMEM, whose Na concentration is 155 mM; addition of sodium

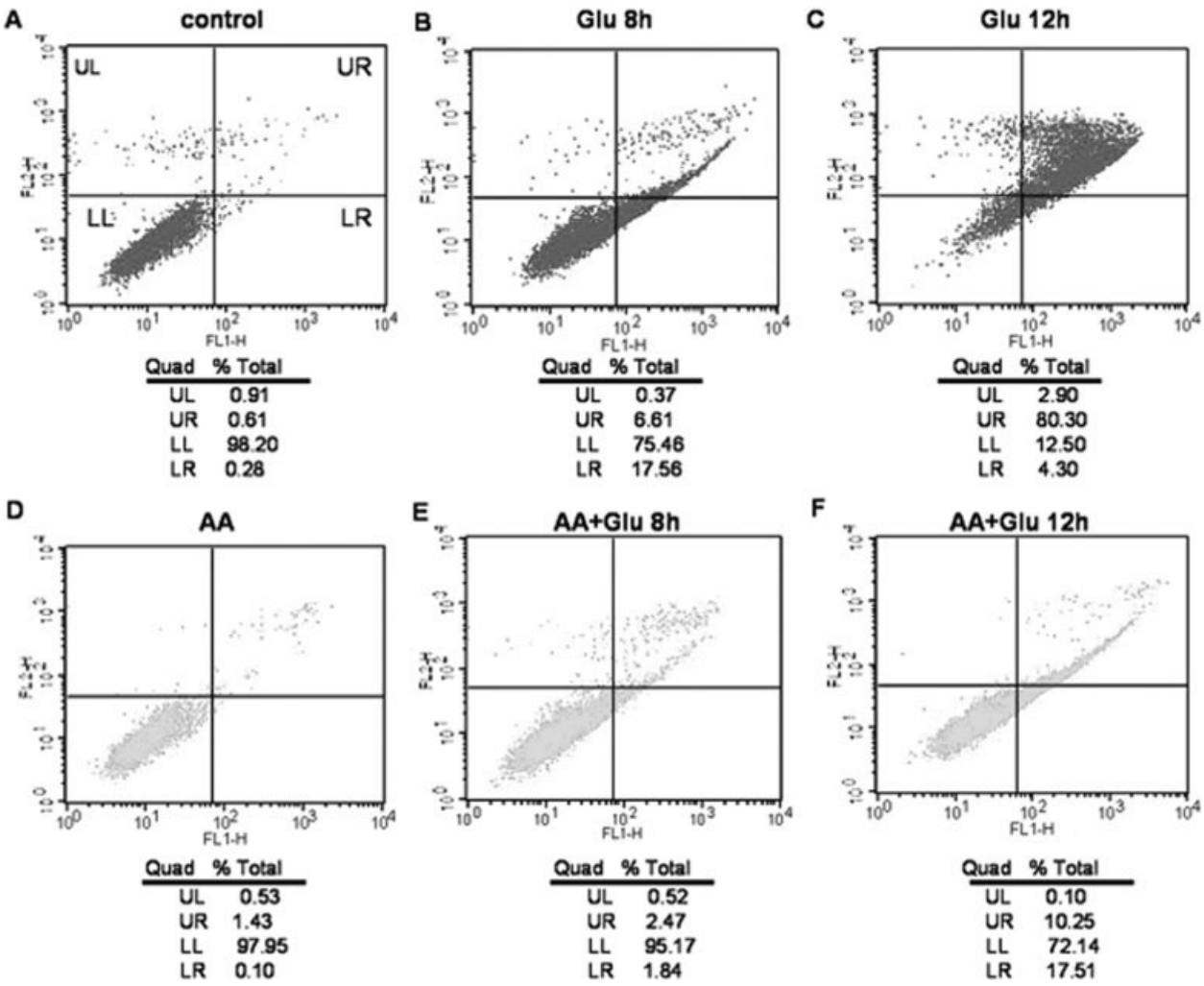

Fig. 7. Flow cytometric analysis. **A–F:** Representative cytograms of annexin V (FLI-H) vs. PI (FL2-H) fluorescence intensities as determined by flow cytometric analysis in HT22 cells. Quadrants designated lower left (LL) are primarily annexin V- and PI-negative; upper left (UL) are PI-positive, indicating necrosis; lower right (LR) are annexin V-positive, indicating early apoptosis; upper right (UR) are annexin V- and PI-positive, indicating late apoptosis. **G:** Graph summarizes cell phase as determined by flow cytometric analysis. Asterisks denote significant differences from untreated controls (mean ± SEM). $*P < 0.05$, one-way ANOVA, followed by a Tukey's multiple-comparison test.

is harmful to cell culture. In HT22 cells, glutamate induces a form of programmed cell death with characteristics of both apoptosis and necrosis, termed *oxytosis* (Tan et al., 1998). In the present study, we can see that 24 hr of pretreatment with 5 mM of AA decreased the appearance of annexin V-positive HT22 cells, which are indicative of the early stage of apoptosis, and PI-positive HT22 cells, which are indicative of necrosis. In the case of simultaneous application of AA and glutamate, glutamate-induced neuronal cell death is protected compared with HT22 cells treated with glutamate alone. AA also appears to be effective even after initial insult. However, a 24-hr pretreatment with AA is more effective than cotreatment against glutamate-induced oxidative stress in the HT22 cell line. In our preliminary data, pretreatment with 5 mM AA for 12 hr did not lead to significantly greater effects against glutamate toxicity than a cotreatment with AA and glutamate (data not shown). These findings suggest that the neuroprotective effects of AA, at least in part, might be through a change in mitochondrial metabolism, which may render AA not only a preventive agent but also a putative therapeutic substance in cases of neuronal damage. Consistently with our data, previous reports suggested that AA and β-OHB protect neurons against various insults (Garcia and Massieu, 2003; Kashiwaya et al., 2000; Suzuki et al., 2001; Masuda et al., 2005). Among these studies, that of Garcia and Massieu (2003) showed that AA rescues neurons from elevated glutamate concentrations after glutamate uptake is inhibited by L-trans-pyrrolidine-2,4-dicarboxylate (PDC). Also, Kashiwaya et al. (2000) showed that β-OHB can protect cultured neurons from 1-methyl-4-phenylpyridinium (MPP^+) and a frag-

ment of amyloid protein, $A\beta_{1-42}$, through the prevention of defects in mitochondrial energy generation.

It is well known that glutamate decreases the mitochondrial membrane potential ($\Delta\psi$) and increases ROS production (Pereira and De Oliveira, 2000). Most of the ROS in oxidative glutamate toxicity are generated from the mitochondrial electron transport chain. In addition, Tan et al. (1998) reported that ROS gradually increase during the first 6 hr of glutamate treatment, followed by an explosive increase in the rate of production after 6 hr in HT22 cells. Therefore, we measured ROS levels via the peroxide-sensitive dye DCF, which has the advantage of providing insight into total cytosolic ROS levels (Pereira and De Oliveira, 2000). Consistently with previously published reports (Tan et al., 1998), our data show that an 8-hr glutamate treatment of HT22 is consistent with an increase in ROS levels. Furthermore, we found that pretreatment with AA significantly prevented glutamate-induced ROS production in HT22 cells. Therefore, the present results suggest that, in hippocampal neurons, the neuroprotective effect AA may be related to a decrease in mitochondrial ROS production. In contradiction to our data, it was reported that pretreatment with 5 mM AA caused ROS production and depleted GSH levels in primary rat hepatocytes (Abdelmegeed et al., 2004). This contradictory effect of AA could be due to a cell-specific ability for biosynthesis of ketone bodies. The enzymes for ketone body synthesis are located mainly in mitochondria of liver hepatocytes (Casazza et al., 1984). Although astrocytes can produce ketone bodies from fatty acids in the brain, neuronal cells do not have this capability. Recently, Sullivan et al. (2004) reported that ketosis, induced by a KD, significantly decreased ROS production in the hippocampus, presumably by increasing the expression of mitochondrial uncoupling protein activity. Additionally, Ziegler et al. (2003) reported that a KD increases antioxidant activity, accompanied by an increase in glutathione peroxidase. Marie et al. (1990) showed that brain edema and infarct size were reduced after fasting, and we showed that kainic acid-induced hippocampal cell death was protected in mice fed a KD (Noh et al., 2003). The studies described above indicate that ketone bodies might directly affect neuronal cell death induced by glutamate toxicity. In addition, it was reported that AA blocked seizures in the Frings' model (Rho et al., 2002) and that acetone suppressed pentylenetetrazole (PTZ)-induced seizure (Likhodii and Burnham, 2002). These results imply that AA and acetone act, at least in part, not only as neuroprotective but also as antiepileptic agents. On the other hand, ketoacidosis, especially diabetic ketoacidosis, produces many problems owing to the high levels of ketone bodies (Laffel, 1999). In diabetic ketoacidosis, ketone body concentration is raised to 25 mM (Nordli et al., 1992). Therefore, the major difference of ketoacidosis and mild ketosis from KD or fasting is the acidity caused by the concentration of blood ketone bodies that are acidic.

In conclusion, our results demonstrate, for the first time, that AA displays a protective effect against glutamate-induced oxidative stress on HT22 cells and primary hippocampal neuron by decreasing glutamate-induced ROS production. The results are clinically significant because the rate of cell death in seizure, ischemia, and CNS trauma is independent of glutamate receptors. In addition, discovery of alternative dietary sources for use in KD may provide benefits in the treatment of epilepsy without undesirable side effects.

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