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$MgSO_4$ and lazaroid (U-83836E) partially protects glioma cells against glutamate toxicity *in vitro*

Selda Kabadere[1], Pınar Öztopçu[2], Seval Korkmaz[3], Kevser Erol[4] and **Ruhi Uyar[1]**

[1]Department of Physiology, Faculty of Medicine, Osmangazi University, [2]Department of Biology, Faculty of Art and Sciences; [3]Department of Pharmacology, Faculty of Pharmacy, Anadolu University; [4]Department of Pharmacology, Faculty of Medicine, Osmangazi University, Eskişehir, Turkey

Abstract. In this study, the possible effects of $MgSO_4$ and lazaroid (U-83836E) on glutamate toxicity on glial cells were investigated. C6 and human glioblastoma multiforme cells derived from two patients were grown in an incubator. First, determined IC_{50} dose of L-glutamate (L-glu) was given for 24 hours and removed, and then respective $MgSO_4$ or U-83836E doses were added to the culture medium. After 24 hours 3-(4,5-Dimethylthyazol--2-yl)-2,5-diphenyltetrazolium bromide, thiazolyl blue (MTT) test was applied. When compared to the L-glu-treated group, $MgSO_4$ at the dose of 0.01 mM induced C6 and human glioma cell growth by 17%, 15% and 5%, respectively. At the dose of 1 μM U-83836E also increased C6 and human glioma cell growth by 12%, 13% and 5%, respectively. In conclusion, although $MgSO_4$ and U-83836E do not strongly block glutamate-induced cell death, it is suggested that reduction of Mg^{2+} ions and free radical production may have a role in glutamate toxicity on glial cells.

The correspondence should be addressed to R. Uyar, Email: ruyar@ogu.edu.tr

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INTRODUCTION

The role of astrocytes in promoting neuronal survival and recovery following cerebral insult is becoming increasingly appreciated. Although much of the recent work on neuroprotection has focused on improving the survival of neurons, a prominent concomitant effect of ischemia is the death of glia, in particular astrocytes. Once damaged, the resulting reductions in astrocyte function may further contribute to central nervous system (CNS) losses (Chen et al. 2000). Improving astrocyte survival would lead to neuroprotection. Therefore, identification of the mechanisms responsible for astrocyte death after cerebral insult is highly desirable and should lead to the development of strategies aimed specifically at enhancing astrocyte survival.

Elevated levels of extracellular L-glutamate (L-glu) are responsible for neuronal damage and degeneration in brain disorders, including stroke, epilepsy and Parkinson's disease (Coyle and Puttfarcken 1993). Recent experimental data support the view that astroglia and neurons are intimate partners in L-glu synaptic transmission (Araque et al. 1999). In addition to being a neurotoxin, L-glu also acts as a gliotoxin. The toxic action of L-glu on cultured astrocytes can be summarized as follows. After L-glu treatment, cell swelling is initiated early (4-6 h) as indicated by microscopic observation. Later, morphological or biochemical signs of cell death are initiated (16-18 h) and almost all cells die after 24-30 h of L-glu exposure. However, the effect of L-glu is reversible because when extracellular L-glu is completely taken up, affected astrocytes recover and return to normal morphology (Chen et al. 2000). Following a traumatic event, a number of factors are released to initiate further injury processes. These factors include excitatory and inhibitory neurotransmitters and neuromodulators. We focused especially on released glutamate.

Astroglia express functional ionotropic and metabotropic Glu receptors (iGluRs and mGluRs) (Steinhauser and Gallo 1996). Once released, glutamate can interact with a number of membrane receptors including the ionotropic N-methyl-D-aspartate (NMDA) receptor and the phospholipase C-linked metabotropic receptor, among others. The NMDA receptor and its role in ischemic brain damage has been well characterized. These studies have demonstrated that opening of the channel is both magnesium and membrane potential dependent (Coyle and Puttfarcken 1993). In the alternative metabotropic pathway, glutamate interacts directly with a phospholipase C-linked receptor that initiates the inositol triphosphate second-messenger pathway. The increased inositol triphosphate concentration leads to a release of calcium from intracellular stores and activation of the calcium cascade. In the nervous system, L-glu induced calcium influx can produce nitric oxide by activation of nitric oxide synthase and arachidonic acid *via* phospholipase A_2, which facilitate generation of free radicals and membrane breakdown. Membrane breakdown causes a decrease in magnesium concentration (Hansson et al. 2000). This post-traumatic free magnesium decline is shown in cerebral edema, and neurologic and cognitive failure (Heath and Vink 1998). Magnesium ions have also been shown to limit neuronal injury in both *in vivo* and *in vitro* experiments (Izumi et al. 1991, Kass et al. 1988). In addition, $MgSO_4$ is a readily available and inexpensive NMDA receptor antagonist with a well-established clinical profile (McLean 1994).

It has been indicated that the generation of free oxygen radicals and consequent lipid peroxidation of cell membranes are involved in pathogenesis of neuronal injury in cerebral ischemia and neurodegenerative disorders (Nakao et al. 1994). The lazaroids have been developed for acute treatment of CNS injury. They are 21-aminosteroids that lack glucocorticoid activity and are specifically designed to localize within cell membranes and inhibit lipid peroxidation. Lazaroids have been shown to ameliorate tissue damage in animal models of CNS trauma, ischemia and subarachnoid hemorrhage (Durmaz et al. 1999a, Hall et al. 1990). It is also indicated that lazaroid treatment prevents death of cultured rat embryonic mesencephalic neurons following glutathione depletion (Grasbon-Frodl et al. 1996). In addition to antiperoxidative effects, lazaroids have antiproliferative activity at higher doses (Arora et al. 1996, Durmaz et al. 1999b). U-83836E which we used for experiments combines the piperazinyl pyrimidine antioxidant portion of 21-aminosteroids with the antioxidant ring of α-tocopherol.

Since the possible effect of $MgSO_4$ and U-83836E on glutamate toxicity on glial cell survival has not been investigated before, the known cytoprotective properties of $MgSO_4$ and U-83836E led the authors to study their effects against glutamate toxicity induced on glioma cells *in vitro*.

METHODS

All chemicals were purchased from Sigma. Since rat glioma cell line (C6) is a useful model to study glial cell

properties and to investigate the mechanism of glutamate gliotoxicity *in vitro* (Kato et al. 1984), experiments were performed on C6 and human glioma cells obtained from two patients. Tumor specimens obtained from the patients at the time of surgery were examined histopathologically to confirm tumor diagnosis, and a part of each tumor tissue was prepared for cell culture as described previously (Kornblith 1978). After the tumor sample had been washed with phosphate buffered saline (PBS, without calcium and magnesium) in sterile petri-dishes and minced by scalpel, further dissociation of the cells was achieved enzymatically, according to the method of Chen and Mealey (Chen and Mealey 1970). Then the cell aggregates, treated with 0.25% trypsin-EDTA solution and incubated for 10-15 min at 37°C, were mechanically dispersed by using a 10 ml pipette. Trypsin activity was inhibited by adding growth medium and the cells were centrifuged at 1 000 rpm for 5 min at 4°C. Supernatant was removed and the pellet was seeded into 75 cm^2 flasks. Cells were cultivated in 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F-12 supplemented with 10% fetal calf serum and 1% penicillin-streptomycin solution (Unterman et al. 1991). The cultures were humidified at 37°C and 5% CO_2 atmosphere and fed 2-3 times a week until they approached confluency. Samples of monolayer culture grown on slide chambers were used for morphologic examination and glial fibrillary acidic protein (GFAP) staining. The glial origin of the cells obtained from the patients was demonstrated by immunoperoxidase staining with monoclonal antibody against GFAP, and found to be positive in more than 80% of the cells (Durmaz et al. 1999c). Since they become smaller and doubling time was shorter, after the fifth passage glioma cells of the patients were considered as transformed cells (Durmaz et al. 1999b). C6 was also cultivated in the same medium (see above). For drug treatments, monolayer cultures were washed with Hank's balanced salt solution (without calcium and magnesium) and treated with 0.25% trypsin--EDTA. After removal, the cells were centrifuged at 1 000 rpm for 5 min at 4°C and counted with a Coulter counter. Cell viability was assessed by trypan blue dye exclusion and found to be higher than 98%.

L-glu was dissolved in PBS at 10 mM concentration and stock solution was used for desired doses (Han et al. 1997). $MgSO_4$ was dissolved and diluted in F-12. U-83836E was dissolved in dimethylsulfoxide (DMSO) at 20 mM concentration and then diluted to 2.5 mM stock solution in fatty acid free bovine serum albumin in PBS (30 mg/ml). By using the stock solution, serial dilutions were made at a ratio of 1:10 in fatty acid free bovine serum albumin to reach desired final concentrations of 0.1μM and 1 μM lazaroid when added to 250 μl well medium (Hall et al. 1991). All solutions were prepared freshly before each experiment.

For experiment protocol, the cells at exponential growth phase were seeded in 96 well microtiter plates (2×10^4 cells/well) and incubated for 24 hours in 10 wells for control and 10 wells for each tested drug dose.

Experimental groups were (i) control – included growth medium only; (ii) L-glu – concentrations from 15 mM to 25 mM were used to determine 50% inhibitory concentration (IC_{50}); (iii) L-glu + $MgSO_4$ – first treated with IC_{50} dose of L-glu for 24 hours, after washout of L-glu, different $MgSO_4$ concentration (0.01 mM, 0.1 mM or 1 mM) were applied and cells were incubated for the next 24 hours (Zhou et al. 1999); (iv) L-glu + U-83836E – first treated with IC_{50} dose of L-glu for 24 hours and subsequently, after L-glu washout, treated with the concentrations of 0.1 μM and 1 μM U-83836E for 24 hours (Durmaz et al. 1999b).

Drug cytotoxicity screening was performed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl blue (MTT) colorimetric assay which was used originally by Mossmann and modified by Alley et al. (Alley et al. 1988, Mossmann 1983). Since the formation of formazan as a product of MTT has been found to correlate with the number of living cells (Alley et al. 1988), MTT assay was performed before density limitation of cell growth occurred in control wells. The optical density read from the drug-treated wells was converted to a percentage of living cells against the control using the following formula: absorbance of treated cells in each well × 100/the mean absorbance of control cells. The dose response curves were calculated for each drug at the above-mentioned concentrations and expressed as the mean percent fraction of control ± standard error of mean (SEM). Statistical significance was ascertained by one-way analysis of variance, followed by Tukey's multiple comparison test. The results are the means of three to five independent assays and a *P* value less than 0.05 was considered to be significant.

RESULTS

The response of cells from two different sources to L-glu toxicity was varied. The IC_{50} value of L-glu on C6

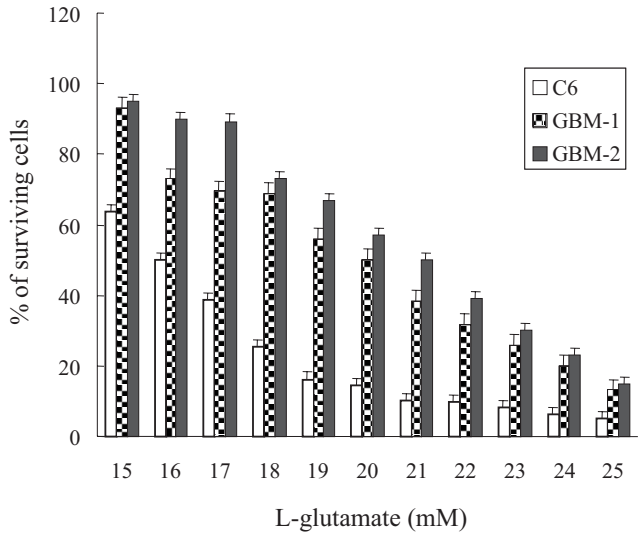

Fig. 1. Dose dependence of the antiproliferative effect of L-glu on C6 and two low passage human glioblastoma multiforme (GBM) cells. IC_{50}= 16 mM, 21 mM and 20 mM, respectively (*n*=24).

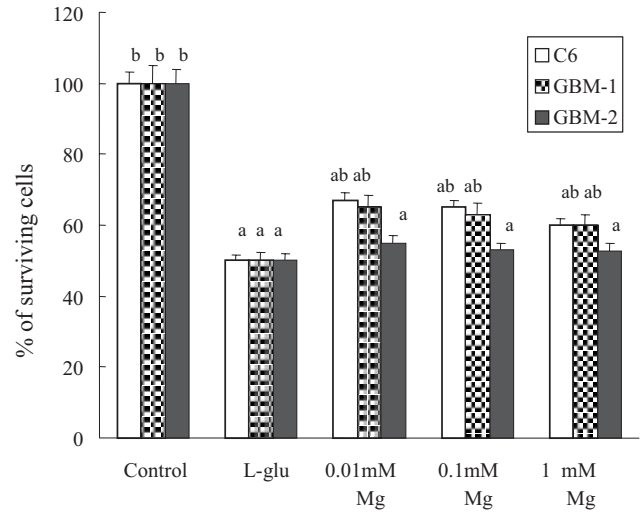

Fig. 2. Influence of $MgSO_4$ added after 24 h treatment with IC_{50} of L-glu on C6 and two human glioblastoma multiforme cells (*n*=24). (a) *P*<0.001 when all the groups compared with control; (b) *P*<0.001 when all the groups compared with the L-glu group.

was determined as 16 mM, whereas on low passage human glioma cells it was 20 mM and 21 mM, respectively *(P*<0.001) (Fig. 1).

Since concentrations of more than 1 mM $MgSO_4$ inhibited the cell proliferation (data not shown), they were not tested against glutamate toxicity. On C6 cells $MgSO_4$ at concentrations of 0.01 mM, 0.1 mM and 1 mM increased the glioma proliferation by 17%, 15% and 10%, respectively *(P*<0.001). When 0.01 mM and 0.1 mM $MgSO_4$ groups were compared to each other, there was not any significant difference (*P*>0.05), whereas 1 mM $MgSO_4$ group was statistically different from both 0.01 mM and 0.1 mM $MgSO_4$ groups *(P*<0.001) (Fig. 2).

On the first low passage human glioblastoma multiforme cells 0.01 mM, 0.1 mM and 1 mM of $MgSO_4$ raised the cell survival by 15%, 13% and 10%, respectively (*P*<0.001). When the three doses of $MgSO_4$ groups were compared to each other, there was not any significant difference (*P*>0.05) (Fig. 2).

On the second human glioma cells, $MgSO_4$ at the concentrations of 0.01 mM, 0.1 mM and 1 mM increased the glioma survival by 5%, 3% and 3%, respectively. The three $MgSO_4$ groups were statistically different from the control *(P*<0.001), but not from the only L-glu treated group or from each other (*P*>0.05) (Fig. 2).

At the concentrations of 0.1 μM and 1 μM U-83836E, which was added after treatment for 24 hours with IC_{50} dose of L-glu increased the C6 survival by 8% and 12%, respectively. On the first human glioma cells, U-83836E at the concentrations of 0.1 μM and 1 μM raised the cell survival by 11% and 13%, respectively *(P*<0.001). These two groups were not statistically different from each other *(P*<0.05) (Fig. 3).

The 0.1 μM and 1.0 μM U-83836E increased the glioma survival by 2% and 5%, respectively, on the sec-

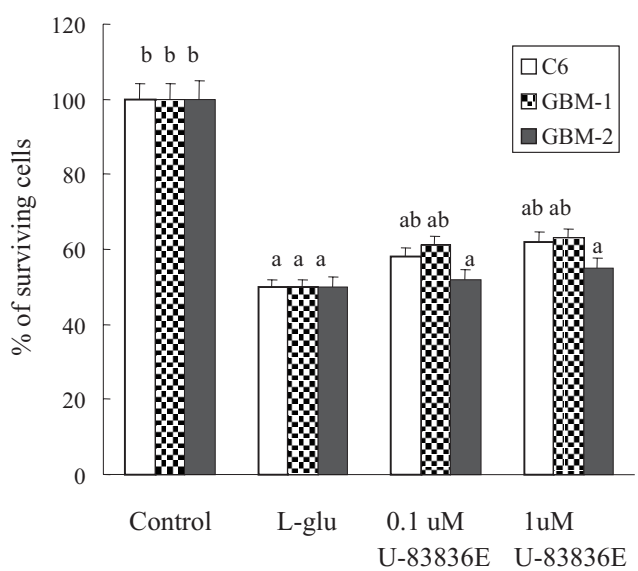

Fig. 3. Influence after 24 h treatment with IC_{50} of L-glu on C6 and two low passage human glioma cells (*n*=24).

ond low passage human glioblastoma multiform cells. Both doses of U-83836E exerted a statistically significant effect, in comparison with the control ($P<0.001$) but not with the only L-glu treated group ($P>0.05$) (Fig. 3).

DISCUSSION

Astrocytes have been shown to protect neurons against glutamate toxicity (Rosenberg and Aizenman 1989). The protective effect of astrocytes is linked to their capacity to remove the compound responsible for the neuronal death from the extracellular space. As proposed by Harris and Rosenberg (1993) based on experiments performed with glutamate, the cellular organization should be of particular importance because astrocytes form a physical buffer allowing the interception of H_2O_2 before its interaction with neurons (Harris and Rosenberg 1993). Therefore, protection of astrocytes from glutamate toxicity is very important for further protection of neurons.

In this study the IC_{50} values of L-glu on C6 and two human glioma cells were determined as 16 mM, 20 mM and 21 mM, respectively. Our results indicate that 50% inhibitory concentration of L-glu might depend on different species. In addition to species, the toxic effect of L-glu varies with time and medium used. Furthermore, Han and coauthors found that in three different batches of C6 cells purchased from American Type Culture Collection on three different occasions, glutamate at 10 mM for 28 hours was cytotoxic to only one batch of cells. A higher concentration of glutamate (20 mM) was required to achieve a comparable cytotoxic response in another batch of cells (Han et al. 1997). Kato and coauthors showed that the ID_{50} for glutamate-induced damage to C6 for 48 hours was about 4 mM (Kato et al. 1984). Since we treated the C6 cells with L-glu for 24 hours and allowed them to recover for 24 hours, our result of IC_{50} of glutamate on C6 cells is different from other studies.

Following traumatic brain injury, intracellular free Mg^{2+} level is decreased approximately 60% in the brain (Heath and Vink 1998). We postulated that if we add Mg^{2+} to the culture medium, we might be able to protect the glial cells from death. In this study the most effective dose of $MgSO_4$ was determined to be 10 μM and increased the C6 and human glioma cells survival by 17%, 15% and 5%, respectively. C6 and the first human glioma survival rates were significantly different from that of the L-glu treated group, whereas the second glioma survival rate was not. Our results demonstrated that Mg^{2+} ions have a partial protective effect on the late stage of glutamate-induced toxicity of glial cells. Mg^{2+} is a natural antagonist of calcium channels and any decline in Mg^{2+} concentration is likely to lead to significant increases in calcium concentration and cell death. The hyperglisemic effect of Mg^{2+} can protect the cell survival rate. Furthermore, reductions in Mg^{2+} to less than 0.25 mM will significantly depress DNA transcription, RNA aggregation and protein synthesis (Ohnishi and Ohnishi 1995). Any significant alteration of free magnesium concentration from optimum might exert considerable metabolic disturbance. Included in this list of enzymes are those involved in glycolysis, Krebs cycle activity and ion homeostasis (Ohnishi and Ohnishi 1995). Furthermore, a decrease in magnesium concentration is known to destabilize the cell membrane with a resultant increase in membrane fluidity and permeability (Ohnishi and Ohnishi 1995). Mg^{2+} added to our culture medium may affect the biochemical and physiologic properties that may influence the development of irreversible glial cell injury after ischemic conditions.

Since our recent study demonstrated that lower doses of U-83836E slightly increased glial cell survival (Durmaz et al. 1999b), we chose 0.1 μM and 1 μM concentrations of U-83836E to test possible protective effect. We found that U-83836E at the concentration of 1 μM increased the C6 and two human glioma cell survival by 12%, 13% and 5%, respectively. Although experimental studies with lazaroids in CNS injury models were generally supportive of its use in assorted forms of CNS injury, the majority of the clinical trials involving stroke and trauma patients demonstrate a lack of efficiency of lazaroids (Marshall et al. 1998). In support of our results, Sureda and coauthors *in vitro* showed that U-83836E completely inhibited reactive oxygen species production induced by glutamate on rat cerebellar granule cells, but they concluded that this lazaroid offered only partial protection against excitotoxicity due to its antioxidant activity (Sureda et al. 1999).

CONCLUSION

Both Mg^{2+} and U-83836E have some protective effect on the late stages of glutamate-induced toxicity of glial cells. However, neither antioxidant therapy nor Mg^{2+} therapy would be sufficient to block the gliotoxic effects of glutamate.

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