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Original scientific paper

Lithium Effect on Glutamate Induced Damage in Glioblastoma Cells

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A B S T R A C T

Lithium, besides mood stabilization, might be involved in neuroprotection. Previously we have found that the treatment with lithium increased the levels of p21$^{WAF/Cip1}$ and survivin in human glioblastoma A1235 cells. The aim of the present study was to examine the cytotoxic effect of glutamate on these cells, and to determine whether lithium can protect A1235 cells against toxic effects of glutamate. Cytotoxicity of glutamate was examined by spectrophotometric MTT assay, while the expression of apoptosis related genes was examined by Western blot method. Glutamate was excessively cytotoxic for A1235 cells only in concentrations higher than 100 mM. It did not induce apoptosis, but rather suppressed survivin expression and increased the level of p21$^{WAF/Cip1}$. Pretreatment with lithium (2 mM) partially reverted change in survivin expression induced by glutamate, suggesting that lithium may have beneficial effect on glutamate induced cell damage in glioblastoma cells.

Key words: *glioblastoma cells, glutamate, lithium, survivin, p21$^{WAF/Cip1}$*

Introduction

Lithium has been used as an effective mood-stabilizing drug for the treatment of manic episodes and depression for 50 years[1,2]. However, both, the biochemical mechanisms underlying the pathogenesis of these disorders and the mechanisms by which lithium exerts its therapeutic effects are not yet completely understood. Experiments support multiple targets of lithium regulating the expression of numerous molecules and enzymes[2,3]**.** Recently, lithium has been found to protect neurons from the death induced by a wide array of neurotoxic insults such as glutamate, low extracellular potassium levels, oubain, valinomycin, aluminum, phenytoin, carbamazepine, C2-ceramide etc [2,4–13]. This effect was partially based on the altered level of apoptosis related genes[8,9,12,13]. Not only that lithium suppressed the induction of apoptosis, but it also exerted neurotrophic effects[2,14,15].

Glutamate is an excitatory neurotransmitter in the central nervous system, that operates as a modulator of synaptic transmission[16]. Activation of glutamate receptors can trigger cell death of neurons and some types of glial cells, particularly when the cells are coincidentally subjected to adverse conditions such as reduced levels of oxygen or glucose, increased levels of oxidative stress, exposure to toxic or other pathogenic agents, or a disease-causing genetic mutations. Excitotoxic cell death involves excessive calcium influx and release from internal organelles, oxyradical production, and engagement of programmed cell death cascade. It has been implicated in neuronal death in a variety of neuropsychiatric diseases[16,17].

In our previous study we have shown that the treatment with lithium increased the levels of p21$^{WAF/Cip1}$ and survivin in human glioblastoma cells[18], suggesting that increased expression of p21$^{WAF/Cip}$ (a protein with anti-apoptotic function), and survivin (a protein that supports the growth of cells by suppression of apoptosis and pro-

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motion of cell proliferation), can be the early events in the long-term cell response to lithium, that may be involved in the beneficial effects of this drug. The aim of the present study was to examine the cytotoxic effect of glutamate in glioblastoma cells, and to determine whether lithium can protect A1235 cells against toxic effects of glutamate.

Material and Methods

Cell culture

In this study, we used human glioblastoma cells A1235. They were grown in plastic Petri dishes as a monolayer culture in Dulbecco's medium (DMEM) (GIBCO BRL) with 10% fetal bovine serum (FBS) (GIBCO BRL) and antibiotics (50 g/mL streptomycin and 100 IU/mL penicillin) at 37 °C with 5% CO_2.

Lithium and glutamate

Lithium chloride and glutamate (Sigma, Steinheim, Germany) were dissolved in water at concentration of 20 M (lithium) or 6 M (glutamate). These stock solutions were sterilized by filtration (Millipore Corporation, Bedford, MA, 01730, USA) and stored at –20 °C. They were diluted with the growth medium immediately before use (glutamate in the final concentrations 10–200 mM, and lithium in the therapeutic doses 0.5 mM and 2 mM).

Cytotoxicity assay

The sensitivity of human glioblastoma cells to glutamate was determined using a colorimetric MTT assay[19], modified as described[20]. Briefly, 4.5×10^3 cells were seeded in 0.18 mL of medium per well in 96-well microtitre tissue culture plates. On the following day, different concentrations of glutamate were added (0.02 mL) to each well; each concentration was tested in quadruplicate. The cells were continuously treated for 72 h at 37 °C. Thereafter, the medium was aspirated and 20 μg/mL of MTT dye/0.04 mL medium was added to each well. Following 4 h of incubation at 37 °C, formazan crystals were dissolved in dimethyl sulfoxide (0.17 mL/well). The plates were mechanically agitated for 5 min and optical density at 540 nm was determined on a microtiter plate reader. If the influence of lithium pretreatment was studied, the cells were seeded as described above, the next day lithium was added (0.5 or 2 mM) and incubated for 48 h. Thereafter glutamate was added (80 –180 mM) and incubated for the next 24 h. The cytotoxicity was determined as described above. Each experiment was repeated three times.

Western blot analysis

Protein extracts of cells were prepared by lysing cells in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris HCl, pH 8) with 10 mM EDTA and 1 mM PMSF for 30 min at 4 °C. The samples were then centrifuged for 15 min at 15 000 g. Protein concentration in supernatant was determined according to the Lowry method[21]. For each sample, 60 μg of protein were loaded on 12.5% SDS-polyacrylamide gel, electrophoresed, and transferred to nitrocellulose membrane (0.22 μm, Protran, Schleicher & Schuell). The membrane was blocked for 1 hour at room temperature with the blocking buffer (TBS containing 0.1% Tween 20 (w/v) (Sigma) and 5% milk (w/v) (Sirela, Croatia)). Primary antibodies (applied for 1 hour at room temperature, or overnight at 4 °C) were: anti-PARP (mouse monoclonal C2-10, Pharmingen, USA), anti-caspase-3 (mouse monoclonal E-8, Santa Cruz), anti $p21^{WAF/Cip1}$ (WAF1) (mouse monoclonal Ab-1, Oncogene), anti-survivin (rabbit polyclonal FL-142, Santa Cruz) and anti-ERK2 (rabbit polyclonal C-14, Santa Cruz). Anti-caspase-3 and anti-survivin were diluted 1:1000, anti-PARP 1:4000, anti-ERK2 1:3000, and anti-p21 1:300. Thereafter, membranes were incubated for 1 h with HRP-labeled secondary antibodies (Amersham Pharmacia Biotech, Sweden): sheep anti-mouse NA 931 (diluted 1:2500), and donkey anti-rabbit NA 934 (diluted 1:5000), and then developed by an ECL system according to manufacturer's instructions (Amersham). The experiments were repeated three times.

Results

Cytotoxic effect of glutamate on human glioblastoma cells is shown in Figure 1. Reduction in cell survival was detected for the doses higher than 30 mM, but for a high decrease in cell survival elevated doses (over 100 mM) of glutamate were needed. According to the survival curve, for the investigation of cell death following doses were selected: 80, 100, 150 and 180 mM. As shown in Figure 2, 24 h the treatment with glutamate did not induce the cleavage of PARP, or reduced the level of procaspase 3 (the hallmarks of apoptosis), but it reduced the level of survivin. Following the 72 h treatment, again, no cleav-

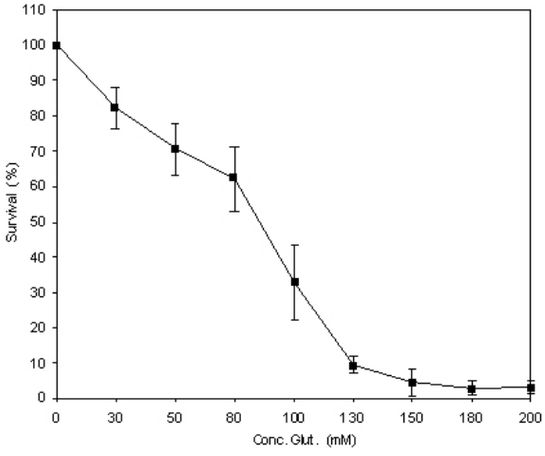

Fig. 1. Cytotoxic effect of 72 h treatment with glutamate (Glut) on survival of human glioblastoma A1235 cells assayed by a modified MTT method. Result in each point represents mean values from 3 experiments (X± SD).

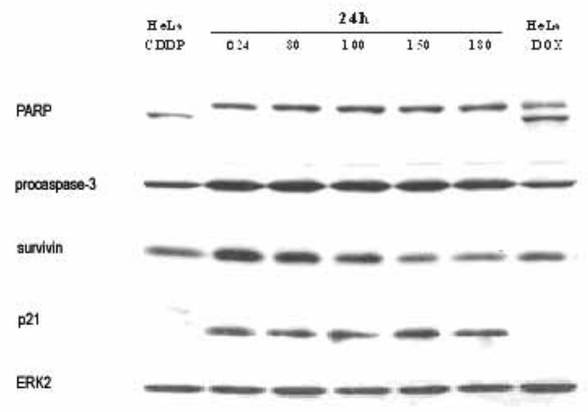

Fig. 2. Western blot analysis of PARP, procaspase-3, survivin and p21$^{WAF/Cip1}$ protein expression in human glioblastoma A1235 cells after 24 h treatment with 80, 100, 150 and 180 mM of glutamate. C24 are control cells. ERK-2 was used as equal loading control. HeLa cells treated with cisplatin (CDDP) or doxorubicin (DOX) were used as positive controls for PARP and caspase cleavage. (CDDP: cells were treated for 1 h with 80 μM cisplatin and collected 24 h after the treatment; DOX: cells were treated for 24 h with 2.6 μM DOX and collected 24 h later). Shown are representative blots.

age of PARP, or alteration in the level of procaspase 3 was detected. While the level of survivin was similar to the one found in the control, the expression of p21$^{WAF/Cip}$ significantly increased (Figure 3).

The pretreatment with lithium (0.5 and 2.0 mM) did not alter the survival of cells treated with glutamate (the data obtained with 2 mM lithium are depicted on Figure 4). The analysis of apoptosis related proteins showed no

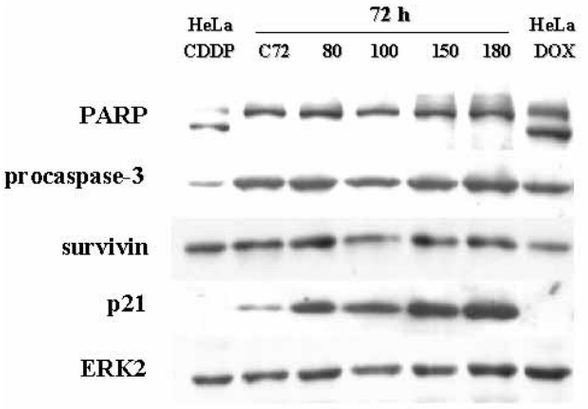

Fig. 3. Western blot analysis of PARP, procaspase-3, survivin and Magnetic Resonance after 72 h treatment with 80, 100, 150 and 180 mM of glutamate. C72 are control cells. ERK-2 was used as the control for equal loading. HeLa cells treated with cisplatin (CDDP) or doxorubicin (DOX) were used as positive controls for PARP and caspase cleavage. (CDDP: cells were treated for 1 h with 80 μM cisplatin and collected 24 h after the treatment; DOX: cells were treated for 24 h with 2.6 μM DOX and collected 24 h later). Shown are representative blots.

cleavage of PARP, or alteration in the level of procaspase 3, but partially reverted levels of survivin expression induced by glutamate if the cells were pretreated with 2 mM dose of lithium (Figure 5).

Discussion

Lithium has been used clinically for more than 50 years as a mood-stabilizing drug in the treatment of bipolar disorders and other mood disorders[1,2]. Neuroprotective effects of this drug have been evidenced by preclinical and clinical studies as well[2,4–13]. Glutamate is an

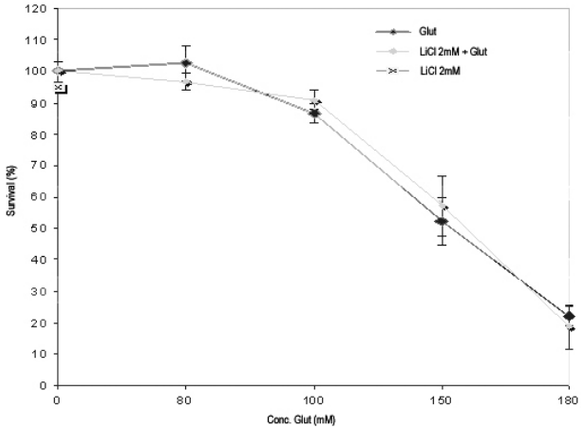

Fig. 4. Survival curves of glioblastoma A1235 cells following 24 h incubation with glutamate (Glut) with or without 48 h pretreatment with 2 mM lithium (LiCl). Pooled data from three experiments (X±SD).

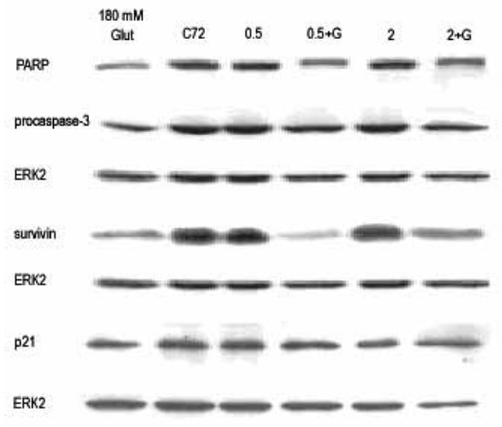

Fig. 5. Western blot analysis of PARP, procaspase-3, survivin and p21$^{WAF/Cip1}$ protein expression in human glioblastoma A1235 cells following: 24 h treatment with glutamate (Glut, 180 mM), 48 h treatment with 0.5 mM lithium (0.5), 48 h treatment with 2 mM lithium (2), 48 h treatment with 0.5 mM lithium and then incubation for 24 h with 180 mM glutamate (0.5 + G), 48 h treatment with 2 mM lithium and then incubation for 24 h with 180 mM glutamate (2 + G). C72 are control cells. Shown are representative blots.

excitatory neurotransmitter in the central nervous system, that may cause cell death[16]. The aim of the present study was to evaluate possible neuroprotective effect of lithium on glutamate induced damage in human glioblastoma A1235 cells.

As evident from Figure 4, glioblastoma cells are resistant to high doses of glutamate. The possible explanation of this resistance could be that glioma cells from patients or established cell cultures release large amounts of glutamate[22]. These cells may be protected from glutamate excitotoxic effect by impaired glutamate uptake[22], or by specific genetic alterations that reduce their ability to undergo cell death. In the latter case high intracellular content of antioxidant gluthatione could prevent glutamate induced oxidative stress[23]. As shown in the present study, glutamate applied in high doses did not induce the cleavage of PARP, or reduced the level of procaspase 3 (the hallmarks of apoptosis) (Figures 2 and 3). The absence of apoptosis could be based on the observation that glutamate can induce different types of cell death varying from apoptosis, over apoptosis-like cell death to necrosis, suggesting the involvement of cell-type and cellular context specificity in this process[24–26]. This is not surprising, because the death patterns may overlap or integrate, providing a variety of cellular responses to various circumstances or stimuli[27].

To examine the possible protective effect of lithium on glutamate induced damage in glioblastoma cells, we focused our investigation on two proteins, survivin and $p21^{WAF/Cip}$, because our previous study indicated that lithium (applied in therapeutic doses of 0.5 and 2 mM) altered their expression[18]. Survivin is a member of the family of inhibitors of apoptosis (IAPs) proteins. However, recent studies have revealed its dual function[28,29]. The cell cycle-dependent expression of survivin (increased in the G2/M phase of cell cycle) and its antiapoptotic function led to the hypothesis that survivin connected cell cycle with apoptosis, thus providing a death switch for the termination of defective mitosis[28,29]. $p21^{WAF/Cip}$, a cell cycle regulatory molecule, is a member of cyclin–dependent kinase inhibitors[30]. Its overexpression can result in cell cycle arrest in either G1, S or G2 phase of the cell cycle. However, a number of recent studies have indicated that $p21^{WAF/Cip1}$ can assume both, anti- or pro-apoptotic functions in response to toxic agents depending on cell type and cellular context[30].

In conclusion, the result of the present study reveals that the treatment of glioblastoma cells with high doses of glutamate reduces the expression of survivin. In spite of the fact that lithium pretreatment did not alter the survival of glutamate treated cells (if measured following three days incubation), therapeutic dose of lithium (2 mM) induced very early alterations in survivin expression (it reverted glutamate induced suppression of survivin), suggesting that lithium may have beneficial effect on glutamate induced cell damage in glioblastoma cells.

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UČINAK LITIJA NA GLUTAMATOM IZAZAVANA OŠTEĆENJA U STANICAMA GLIOBLASTOMA

S A Ž E T A K

Osim stabilizacije raspoloženja, litij može imati i neuroprotektivni učinak. Prije smo pokazali da tretranje ljudskih stanica glioblastoma A1235 litijem povećava ekspresiju proteina $p21^{WAF/Cip1}$ i survivina. Cilj ovog istraživanja bio je ispitati citotoksični učinak glutamata na istim stanicama, i odrediti da li litij može zaštititi A1235 stanice od toksičnog učinka glutamata. Citotoksičnost glutamata određivana je spektrofotometrijskom MTT metodom, a ekspresija apoptotskih gena Western blot metodom. Glutamat je bio izrazito toksičan za A1235 stanice samo u koncentracijama višim od 100 mM. Nije izazivao apoptozu, već smanjivao ekspresiju survivina i povećavao ekspresiju $p21^{WAF/Cip1}$. Predtretman sa litijem djelomično je dokinuo promjene u ekspresiji survivina inducirane glutamatom. U zaključku, visoke koncentracije glutamata toksične su za stanice glioblastoma. Kod tih koncentracija predtretman stanica sa litijem (2 mM) dokida supresiju survivina izazvanu glutamatom, što upućuje na zaključak da bi litij mogao imati pozitivan učinak na glutamatom izazvana oštećenja u stanicama glioblastoma.