

REVIEW

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Protection of taurine and granulocyte colony-stimulating factor against excitotoxicity induced by glutamate in primary cortical neurons

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Abstracts

Background: Both taurine, an inhibitory neurotransmitter and granulocyte colony-stimulating factor (G-CSF), a growth factor, possess neuroprotective and neurotrophic properties in vitro. However, the mechanisms of their underlying neuroprotective effects are not fully understood.

Methods: In the present study, we investigated the potential protective benefits of taurine, G-CSF and the combination of taurine and G-CSF against excitotoxicity induced by glutamate in primary cortical neuronal cultures.

Results: 25 mM taurine, 25 ng/ml G-CSF and the combination of 25 mM taurine and 25 ng/ml G-CSF showed a protective effect reaching 75%, 75% and 88%, respectively. Furthermore, taurine exerted its protective effect through down-regulation of expression of GRP 78, CHOP, Bim and caspase 12.

Conclusion: The results showed that all of these treatments, taurine, G-CSF and the combination of taurine and G-CSF, protected primary cortical neurons against excitotoxicity induced by glutamate. ER stress is suppressed by taurine after glutamate toxicity.

Background

Taurine (2-aminoethanesulfonic acid), an inhibitory neurotransmitter, is present at high concentrations in many invertebrate and vertebrate systems [1-3]. Taurine has received much attention in the field of neuroprotection since the original experiments of Curtis and Watkins on the synaptic effects of inhibitory and excitatory amino acids [4,5]. Taurine is at a high level in the immature brain, serving as a trophic factor [6]. It has been thought to induce hyperpolarization, to inhibit firing of central neurons and to act as a modulator of synaptic activity in the brain [7-9]. The maintenance of the integrity of membranes, transmembrane Cl⁻ flux and intracellular calcium homeostasis are also important functions of taurine in the brain [10-13]. Taurine also acts as an osmoregulator and plays an antioxidant role [14-16]. In

addition, it has been related to neuroprotection against multiple neurological diseases including Alzheimer's disease, Huntington's disease and brain ischemia [17-19]. Moreover, taurine was found in neuronal systems to exert a protective function against toxicity induced by glutamate [20,21].

G-CSF is one of the few growth factors currently approved for clinical use for routine treatment of neutropenia [22]. It primarily stimulates proliferation, differentiation and maturation of cells committed to the neutrophilic granulocyte lineage through binding to the specific G-CSF receptor [23]. G-CSF also has been shown to have trophic effects on neuronal cells in vitro [24]. Moreover, G-CSF is an effective neuroprotectant in the treatment of a number of neurological diseases including stroke, Parkinson's disease and Alzheimer's disease [25-28]. In addition, apart from its protective role in neurons, G-CSF also dampens systemic inflammatory reactions, which may be of additional benefit in neurodegenerative conditions [29].

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Although it is established that taurine and G-CSF have many beneficial effects under a variety of conditions of cell damage, the protective mechanisms are still unclear. We have recently demonstrated that taurine protects PC12 cells against ER stress induced by oxidative stress [30]. Here, we studied the protective effect of taurine, G-CSF and the combination of taurine and G-CSF against excitotoxicity induced by glutamate in rat primary neuronal cultures. We demonstrated that ER stress is also involved in the excitotoxicity induced by glutamate. Moreover, taurine protects primary neurons by suppressing ER stress induced by glutamate.

Methods

Materials

Basal medium-Eagle, fetal bovine serum, poly-D-lysine, taurine, Penicillin-Streptomycin and other chemicals were purchased from Sigma (St. Louis, MO, USA). Mouse anti-actin, rabbit anti-GRP78, rabbit anti-CHOP/GADD153, rabbit anti-caspase-12 antibodies and secondary mouse and rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-Bim antibody was purchased from Assay Designs (Ann Arbor, Michigan, USA). Adenosine 5'-triphosphate (ATP) Bioluminescent assay kit was purchased from Promega (Madison, WI, USA). RIPA buffer was purchased from Thermo Scientific (Rockford, IL, USA). Pregnant Sprague Dawley rats were purchased from Harlan (Indianapolis, IN) and housed in the animal care facility at Florida Atlantic University. The procedures for the care and use of rats, in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, were approved by the Institutional Animal Care and Use Committee of Florida Atlantic University.

Primary cortical neuronal cell culture

Primary cortical neuronal cell cultures were prepared using a previously described protocol [13]. Briefly, rat embryos at 17-18 days were removed and brains were isolated from the fetuses and kept in basal media Eagle (BME) supplemented with 2 mM glutamine, 26.8 mM glucose, and 20% heat-inactivated fetal bovine serum. This medium is referred to as growth medium-eagle (GME). The cortices then were dissociated by passing the tissue through a 14-G cannula. Cells were centrifuged at 200 g/min for 5 min at 25°C. The resulting pellet was resuspended in GME and plated on appropriate tissue culture plates precoated with 5 µg/ml of poly-D-lysine. Cells were maintained for 1 hour in a humidified incubator (37°C, 99% humidity and 5% CO₂) before the incubation medium was replaced with serum-free neurobasalmedium (GIBCO) supplemented with B27

and 500 µM glutamine. The cultures were maintained in an incubator for 14 -18 days.

Measurement of cell viability

Cells were measured by ATP assay. Neurons at 14 days in vitro were preincubated with 25 mM taurine for 1 hour. Then the neurons were treated with 100 µM glutamate for 4 hours. ATP solution was added to each well and cells were incubated for 10 minutes, after which levels of ATP were quantified in a luciferase reaction. The luminescent intensity was measured using a luminometer (SpectraMax, Molecular Devices) after transferring the lysate to a standard opaque walled multi-well plate.

Western blot analysis

Primary cortical neuron cultures were lysed in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing 1% (v/v) mammalian protease inhibitor cocktail from Sigma and separated on a SDS-PAGE. After proteins were transferred to a nitrocellulose membrane, the membrane was then blocked in blocking buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, 5% milk) for 1.5 hours at room temperature. After blocking, the corresponding primary antibody was incubated for one hour, followed by one hour incubation with the corresponding HRP-conjugated secondary antibody at room temperature. Extensive washes with blocking buffer were performed between each step. The protein immuno-complex was visualized using ECL detection reagents.

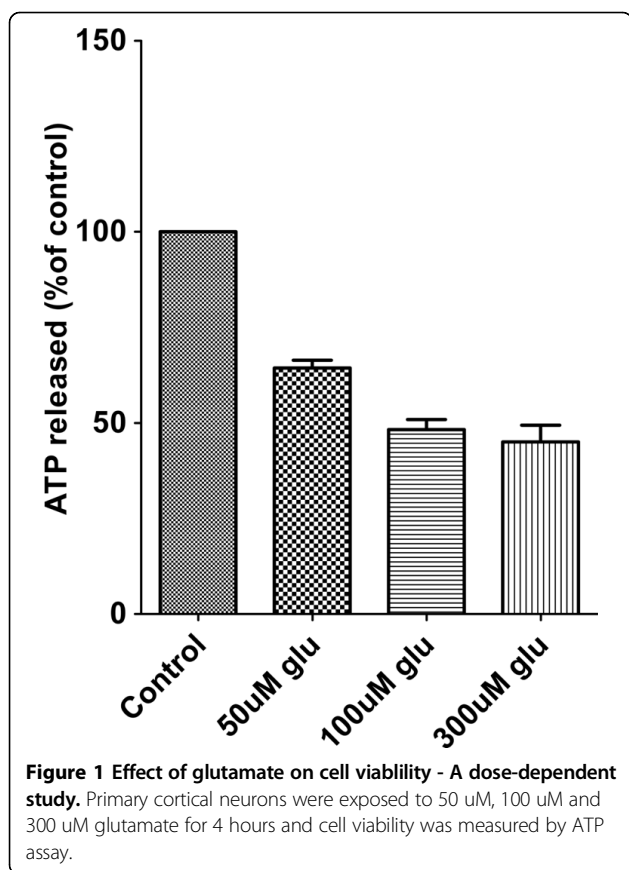
Statistical analysis

All data shown were expressed as the mean ± SEM. The Student's t-test or one-way ANOVA was used to compare means between groups. Differences of P<0.05 were considered statistically significant.

Results

Dose-dependent glutamate toxicity in primary neuron cultures

Excessive levels of the neurotransmitter glutamate trigger excitotoxic processes in neurons that result in cell death [31]. To identify the excitotoxic dose range of glutamate, rat cortical neurons were treated for 4 hours with 50, 100, 200 or 300 µM glutamate respectively. The results are shown in Fig. 1. We found that glutamate treatment caused a dose-dependent increase in neuronal apoptotic processes. There was approximately 50% survival of cortical neurons with 100 µM glutamate treatment for 4 hours (Fig. 1, lane 3). 100 µM glutamate was chosen as an optimal concentration to induce the excitotoxicity.

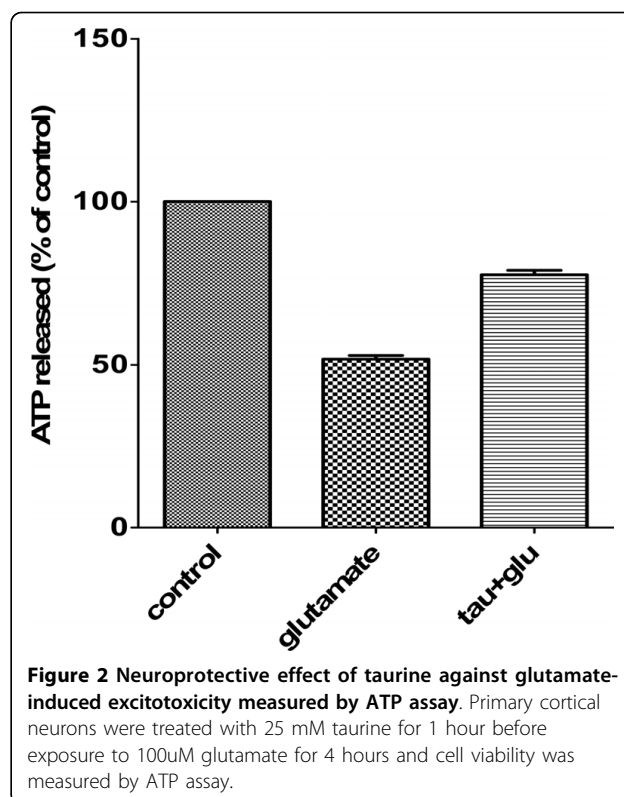


Protective effects of taurine against glutamate toxicity in primary neuron cultures

Previously, we found that 25 mM taurine resulted in the optimal neuroprotection against glutamate induced excitotoxicity [32]. For this reason, we selected the 25 mM taurine concentration for testing cell viability using the ATP assay. For testing the protective effect of 25 mM taurine against glutamate in cortical neurons, cells were seeded in 96-well plates and treated with or without 25 mM taurine for 1 hour followed by 100 uM glutamate exposure for 4 hours. The cell survival results are shown in Fig. 2. The treatment of 25 mM taurine increased the cell survival by 75% compared to the condition with 100 uM glutamate treatment.

Protection of G-CSF against glutamate toxicity in primary neuronal cultures

G-CSF has been widely investigated in terms of protection of neurons in stroke, as shown in numerous papers [25,26,33-36]. Glutamate has been shown to play a key role in the pathogenesis of stroke [37]. However, there has been little research on the protective function of G-CSF in glutamate induced excitotoxicity in vitro. G-CSF was previously shown to exhibit a protective effect in cerebellar granule cells exposed to glutamate toxicity



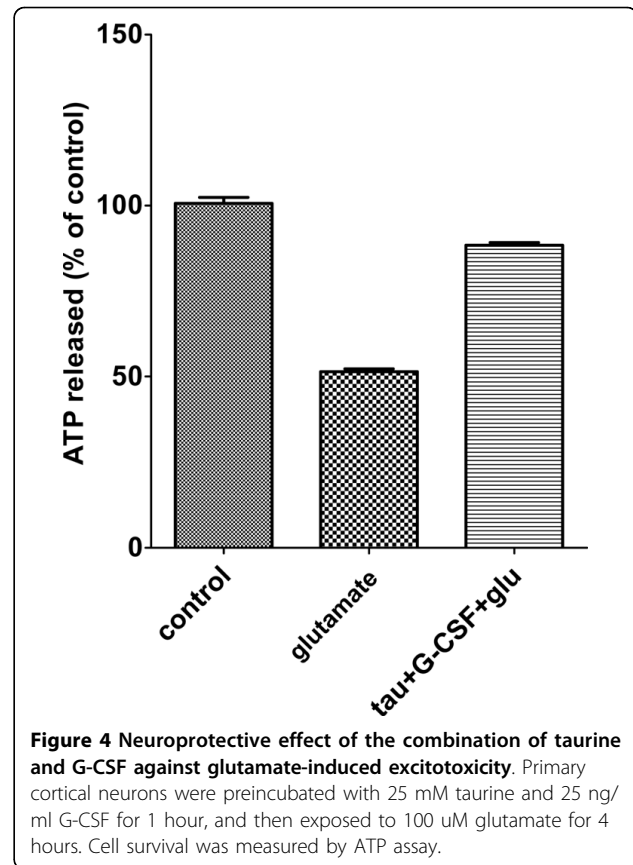
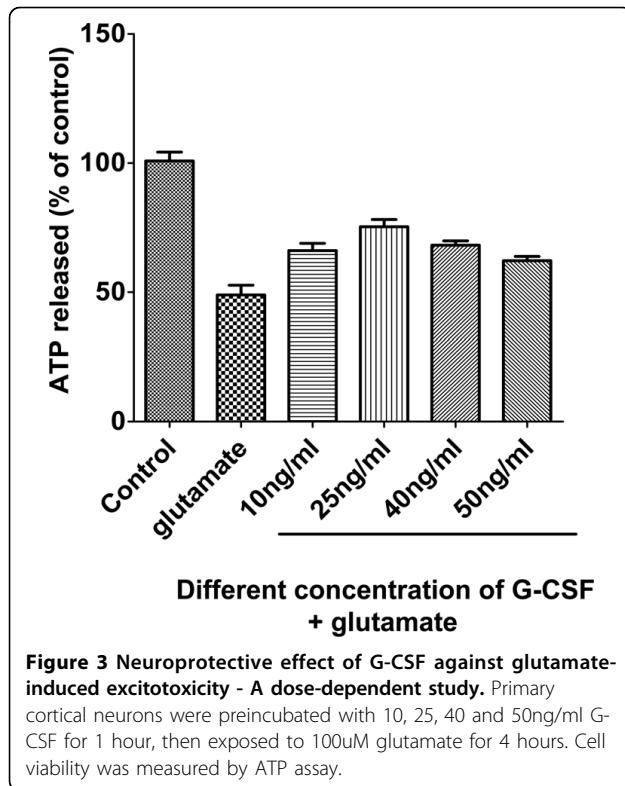
[25]. In the current study, we demonstrated the protective function of G-CSF at a range of concentrations from 10 to 40 ng/ml against excitotoxicity induced by glutamate in primary neuronal cultures (Fig. 3). G-CSF treatment resulted in an enhanced cell survival at several concentrations, with the highest protection of 75% occurring at 25 ng/ml.

The protective effect of the combination of taurine and G-CSF in primary neuronal cultures

To test whether the combination of taurine and G-CSF promotes protection against glutamate induced toxicity, we treated primary neurons with 25 mM taurine plus 25 ng/ml G-CSF for 1 hour, followed by glutamate treatment for 4 hours. The results are shown in Fig. 4. The combination of taurine and G-CSF increased the protective effect against glutamate toxicity to 88% cell survival compared to 75% cell survival from taurine or G-CSF treatment alone.

Taurine protects neurons against glutamate excitotoxicity by suppressing the expression of GRP78, CHOP, Caspase-12 and Bim

To investigate if ER stress can be induced by glutamate and then suppressed by taurine, specific ER stress effector proteins were analyzed by western blot. Glucose regulated protein-78 (GRP78) is an ER-associated

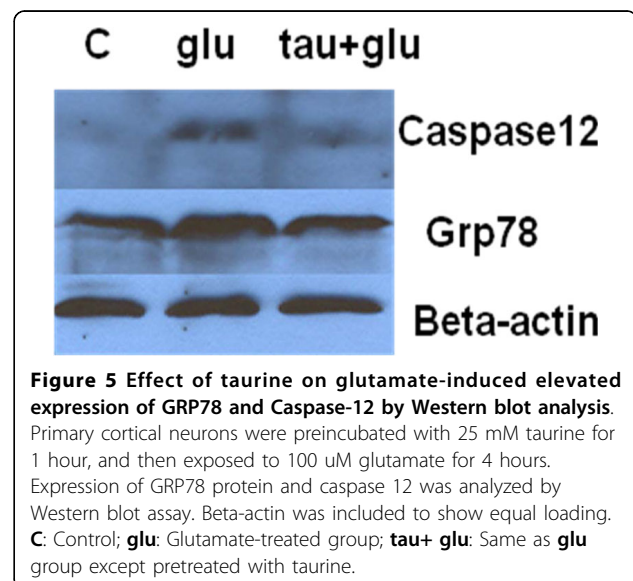


chaperone, which facilitates protein folding in ER [38]. The expression of GRP78 protein was up-regulated in primary neurons after treatment with 100 uM glutamate for 4 hours. However, taurine restored the level of GRP78 to control levels, as shown in Fig. 5. C/EBP homologous protein (CHOP), also known as growth arrest and DNA damage inducible protein 153 (GADD153), is an important ER stress marker [39]. Fig. 6 shows that the expression of CHOP was up-regulated by glutamate. Taurine treatment restored CHOP expression to the control level (Fig. 6). Both Caspase-12 and Bim play an essential role in the progression of programmed cell death during the proapoptotic phase of the ER stress response [40,41]. Taurine reversed the induction of Caspase-12 and Bim caused by glutamate in primary neurons, as shown in Fig. 5 and Fig. 6.

Discussion

In the present study, we have demonstrated the potent protection by taurine and by G-CSF in an *in vitro* model of primary cortical neuronal cell death induced by glutamate. Taurine and G-CSF protected primary cortical neurons against glutamate-induced neurotoxicity as determined by measuring cell viability using the ATP assay. On the other hand, we found that the combination of taurine and G-CSF gave a synergistic enhancement of protection against glutamate in primary cortical neurons. We have further shown that the suppression of

ER stress is an essential underlying mechanism for taurine-induced neuroprotection. Our investigation of the intracellular mechanisms downstream of ER stress demonstrated a reversal by taurine of glutamate-induced increases in GRP78, CHOP, Caspase-12 and Bim levels.



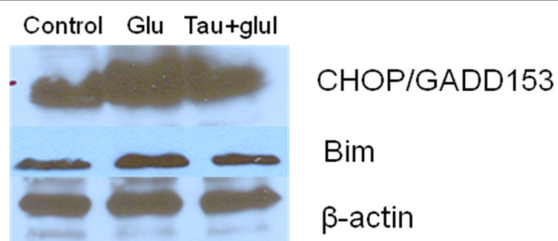


Figure 6 Effect of taurine on glutamate-induced elevated expression of CHOP and Bim by Western blot analysis. Primary cortical neurons were treated the same as described in Figure 5 except that the expression of CHOP and Bim was analyzed by Western blot assay.

A previous paper reported that taurine and basic fibroblast growth factor (bFGF) in combination gave an enhanced neuroprotection in granule neurons against glutamate induced excitotoxicity [42]. They showed that neuroprotection was obtained only through the combined action of taurine and bFGF in a cerebellar granule neuron rich culture, but not by these factors alone. Therefore, they believed that taurine can augment bFGF function under certain conditions. Here, we demonstrated that taurine or G-CSF administrated alone showed a neuroprotective effect. Furthermore, an enhanced protection against glutamate was also observed with a combination of taurine and G-CSF. The clinical application of taurine was investigated and found to be effective in studies as early as 1974 when it was applied to treatment for refractory epilepsy [43]. Both taurine and G-CSF have been shown to be potential drugs for ischemia or stroke in clinical applications [44,45]. Since the combination of taurine and G-CSF have synergistic neuroprotective effects against glutamate excitotoxicity, as demonstrated in this paper, this strongly suggests that the combination of taurine and G-CSF may be more effective than the individual agents in treatment of neurological diseases, such as stroke.

Many neurological disorders such as Alzheimer's disease, stroke and Parkinson's disease have been linked to the overactivation of glutamatergic transmission and excitotoxicity as a common pathway of neuronal injury [46-48]. Previous studies have also shown that ER stress is induced in neurons by glutamate toxicity [49,50]. Recently, kainic acid (KA), a non-NMDA glutamate receptor agonist, was found to cause the disintegration of the ER membrane in hippocampal neurons and to cause ER stress [51]. In this study, we demonstrated glutamate induced ER stress associated with the up-regulation of the proteins GRP78, CHOP, Bim and caspase-12.

Although taurine has been investigated and applied to treat many diseases, the protective mechanism is still not fully understood. We have already demonstrated

that ER stress induced by H₂O₂ in PC12 cells was prevented by taurine treatment [30]. In the present study, our results show that taurine reduces the ER stress induced by glutamate in primary neuronal cultures.

Conclusion

In the present study, we demonstrated that both taurine and G-CSF protect primary cortical neurons against glutamate-induced cell death. Interestingly, we found that the combination of taurine and G-CSF results in an enhanced protective effect. Because both taurine and G-CSF are neuroprotective agents that are approved for clinical use, the combined administration of these two factors may constitute a viable therapy with potentially enhanced therapeutic efficacy. Moreover, taurine suppressed the ER stress induced by glutamate. Further investigation will be performed to examine the specific pathway responsible for ER stress induced by glutamate and to identify molecular targets in the ER stress pathway that are specifically inhibited by taurine, G-CSF and their combination.

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Competing interests

The authors declare that they have no competing interests.

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