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Nerve growth factor, ganglioside and vitamin E reverse glutamate cytotoxicity in hippocampal cells

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Abstract

The present work showed that glutamate decreased hippocampal cell viability in a dose-dependent manner. While no significant effect was observed after cell exposure to 0.1 mM glutamate, cell incubation for 0.5 h caused a progressive decrease of cell viability, which at 5 mM concentration reached 68% as compared to controls. No further effect was observed in the presence of 10 mM glutamate. While nerve growth factor (NGF) at the dose of 0.5 ng/ml presented no effect, it significantly reduced glutamate cytotoxicity at a higher dose (1 ng/ml) increasing the cell viability to 66%. Similarly, cell viabilities in the presence of the ganglioside GM_1 (5 and 10 ng/ml) after glutamate exposure were 19 and 73%, respectively. A dose–response relationship was observed after cell incubation with vitamin E (0.5 and 1 mM) which resulted in cell viability of the order of 34 and 70%, respectively. Surprisingly, a potentiation of the effect was observed after the association of NGF (0.5 ng/ml) plus ganglioside GM_1 (5 ng/ml) or vitamin E (0.5 mM) plus ganglioside GM_1 (5 ng/ml), after pre-incubation with glutamate. In these conditions, significantly higher viabilities were demonstrated (66 and 71% for the two associations, respectively) as compared to each one of the compounds alone (NGF 0.5 ng/ml—29.5%; ganglioside GM_1 5 ng/ml—19.4%). However, no potentiation was seen after the association of NGF plus vitamin E on glutamate pre-exposed cells. These results showed a cytoprotective effect of ganglioside GM_1, NGF and vitamin E on the glutamate-induced cytotoxicity in rat hippocampal cells.

Keywords: NGF (nerve growth factor); Ganglioside GM_1; Vitamin E; Glutamate; Hippocampal cell

1. Introduction

Several cell surface receptors are involved in excitotoxicity, including not only glutamate receptors, but also receptors for other neurotransmitters and neurotrophic factors (Mattson and Mark, 1996), and emphasis is placed on the roles of Ca^{2+} and free radicals as mediators of cellular damage. Other data (Deupree et al., 1996) support the hypothesis that activation of the NMDA receptor and subsequent influx of Ca^{2+} through this receptor play a critical role in excitatory amino acid induced neurotoxicity. Overactivation of receptors for the excitatory amino acid transmitter glutamate is believed to be a critical event in the sequence leading to neuronal cell death. Glutamate induces oxidative stress in neurons as the result of calcium influx through NMDA receptors and voltage-dependent channels. Ca^{2+} promotes generation of superoxide anion radicals, hydrogen peroxide, hydroxyl radicals, nitric oxide and peroxynitrite.

Activation of the NMDA receptor is subject to modification by several factors. Glycine potentiates NMDA receptor activation by glutamate and may actually be required for activation (Johnson and Ascher, 1987). Hydrogen ion concentration (pH) and redox state (Levy et al., 1990; Sucher et al., 1990) have strong influences on NMDA receptor activation. Acidic pH inhibits NMDA receptor activation, while reducing agents enhance NMDA receptor activation and Ca^{2+} influx.

Neurotrophic factors constitute an important class of endogenous modulators of excitotoxicity, and many reports of excitoprotective activities of neurotrophic factors have appeared recently (Mattson et al., 1993; Mattson and

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Scheff, 1994). Nerve growth factor (NGF) protected cultured rat cortical neurons against glutamate-induced neurotoxicity (Shimolama et al., 1993). In vivo studies have also been performed, and many have shown beneficial effects of neurotrophic factors, including NGF, in protecting neurons from several brain regions against excitotoxic/ischemic injury in vivo (Shigeno et al., 1991; Frim et al., 1993; Prehn et al., 1993).

There is now a substantial evidence that ganglioside GM_1 is effective in partially correcting the consequences of neuroinjury in a number of experimental models. Although the molecular mechanism for this neurotrophic activity is not fully understood, several works suggest that ganglioside GM_1 exerts a neuroprotective effect on various neurotransmitter systems (Hadjiconstantinou and Neff, 1998). Thus, it has been demonstrated (Fusco et al., 1993) that ganglioside GM_1 treatment to aged rats potentiates the NGF-induced increase of cholinoacetyltransferase in the striatum ipsilateral to the NGF infusion.

In addition, exogenously added gangliosides are known to promote neurite outgrow in a variety of cell types. Ganglioside GM_1 enhanced NGF-stimulated neuritogenesis and prevented apoptotic death of PC12 cells possibly by enhancing the dimerization and activation of the NGF TrKA receptor (Farooqui et al., 1997; Li et al., 1998). Also, ganglioside GM_1 treatment partially restored NGF and NGF mRNA in frontal cortex and hippocampus in the aged rat brain but not in the young one (Duchemin et al., 1997).

The prolonged elevations of $[Ca^{2+}]_i$ caused by glutamate will damage neurons through the calcium activation of proteases that degrade cytoskeletal and other proteins (Mattson and Mark, 1996). Convincing evidence supports a role for free radicals in excitotoxic and ischemic injury processes (Jesberger and Richardson, 1991; Richter and Kass, 1991). The evidence comes from studies showing that antioxidants can attenuate glutamate neurotoxicity, and that glutamate can induce free radical production in neurons. Free radicals promote elevation of $[Ca^{2+}]_i$, and elevation of $[Ca^{2+}]_i$ promotes free radicals production (Orrenius et al., 1989).

In vitro studies have shown that glutamate and metabolic insults can induce free radicals production in neurons (Mattson et al., 1995). Cell culture studies have shown that antioxidants can protect neurons against glutamate toxicity (Monyer et al., 1990; Schubert et al., 1992; Favit et al., 1992). Antioxidants including vitamin E and 21-aminosteroids were also reported effective in protecting neurons against excitotoxicity insults in vivo (Hall et al., 1989).

The objectives of the present work were to study the effects of the ganglioside GM_1, NGF, and vitamin E, alone and associated, on the glutamate-induced cytotoxicity in rat hippocampal cells in order to detect a possible antagonism or potentiation after combination of these drugs, and further clarify the mechanism of action of the glutamate neurotoxicity.

2. Materials and methods

2.1. Drugs

L-Glutamic acid and nerve growth factor (NGF) from *Vipera lebetina* venom were purchased from Sigma, St. Louis, MO, USA, monosialoganglioside (ganglioside GM_1) (Sygen) was a gift from TRB Pharma, SP, Brazil and vitamin E from Vit Gold-Vitagold, Washington, DC, USA. These drugs were dissolved in phosphate-buffered saline (PBS) before use.

2.2. Cell culture

Primary cultures of hippocampus cells were prepared from 1-day old Wistar rats as described by Akaike et al. (1991) with minor modifications. Briefly, cells were plated (5.0×10^6 cells per 35 mm) on dishes previously coated with rat tail collagen. Culture dishes were incubated in Eagle's minimal essential salt medium (Eagle's MEM) supplemented with 10% heat-inactivated fetal bovine serum, 5% heat-inactivated horse serum, streptomycin (100 mg/ml), penicillin (100 U/ml), amphotericin B (5 mg/ml), glutamine (2 mM), glucose (11 mM), $NaHCO_3$ (24 mM) and HEPES (10 mM). Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. After 7 days of plating, nonneuronal cells were removed by adding 10^{-5} M cytosine arabinoside for 24 h. Only mature (9–10 days in vitro) cultures were studied.

Table 1
Percentage of viability of hippocampal cultures after exposure to glutamate

Glutamate (mM)	Time of exposure (0.5 h)	Control (%)	Time of exposure (2 h)	Control (%)
0.1	88.75 ± 3.11 (4)	96.8	86.00 ± 2.98 (4)	93.9
1.0	75.85 ± 4.83 (8)	82.8	80.16 ± 1.69 (8)	87.5
5.0	29.24 ± 2.97 (17)[a]	31.9	33.00 ± 6.72 (3)[a]	36.0
10.0	26.87 ± 11.13 (4)[a]	29.3	16.50 ± 3.92 (5)[a]	18.0

Values are means ± S.E.M. The number of experiments is in parentheses. Cells viability (%) in the control group was 91.6 ± 1.0 ($N = 27$). [a] $P < 0.05$ vs. control (ANOVA and Tukey's test).

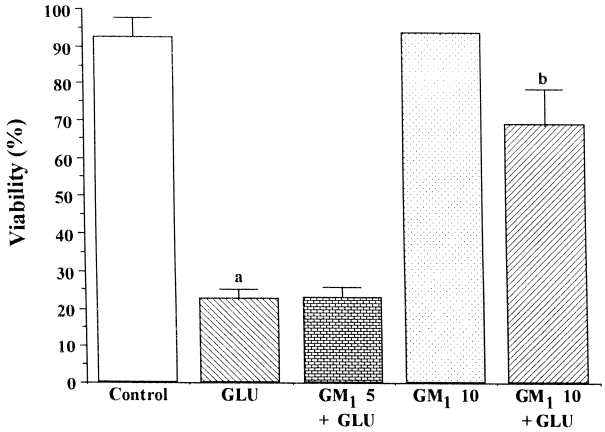

Fig. 1. Effect of ganglioside GM_1 (GM_1) on glutamate-induced cytotoxicity in rat hippocampal cells. After cells exposure to glutamate (Glu) (5 mM), cultures were then incubated with glutamate-free medium containing GM_1 (5 or 10 ng/ml) for 24 h. The ordinate indicates the viability of cultures calculated as described in Section 2. (a) vs. control; (b) vs. Glu at $P < 0.05$ (ANOVA and Scheffe's test).

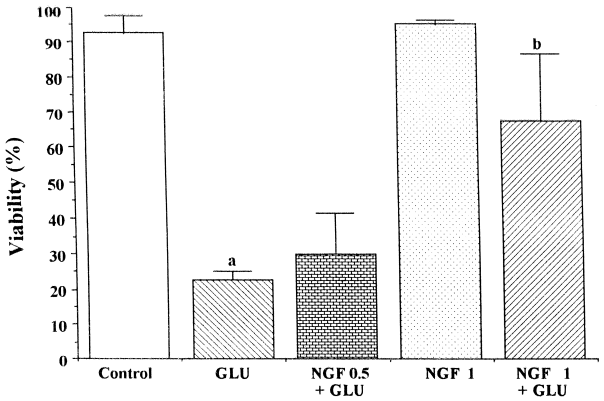

Fig. 2. Effect of NGF on glutamate-induced cytotoxicity in rat hippocampal cells. After cells exposure to glutamate (Glu) (5 mM), cultures were then incubated with glutamate-free medium containing NGF (0.5 or 1 ng/ml) for 24 h. The ordinate indicates the viability of cultures calculated as described in Section 2. (a) vs. control; (b) vs. Glu at $P < 0.05$ (ANOVA and Scheffe's test).

2.3. Evaluation of drug-induced protection against glutamate cytotoxicity

For studying the glutamate-mediated cytotoxicity, the culture-conditioned medium was collected, and cells were incubated with Locke's solution (in mM: 154 NaCl, 5.6 $NaHCO_3$, 2.3 $CaCl_2$, 5.6 glucose, 10 μM glycine, 5 HEPES) at pH 7.4, with or without glutamate (1, 2.5, 5, 10 mM) for 30 min. After this period, cells were washed with phosphate-buffered saline (PBS) and placed into fresh medium in the absence and presence of ganglioside GM_1 (5 and 10 ng/ml), NGF (0.5 and 1 ng/ml), and vitamin E (0.5 and 1 mM) alone or in association. Cell viability was conducted 24 h later. Three experiments in triplicate each were done.

2.4. Cell viability

Glutamate neurotoxicity was quantified by examining cultures under inverted light microscopy. Cell viability was assessed by means of Trypan blue exclusion. After the experiment, cell cultures were immediately stained with 0.75% Trypan blue for 10 min at room temperature, fixed with isotonic formalin (pH 7.0 at 2–4°C), then rinsed with physiological saline. Cells stained with Trypan blue were regarded as nonviable. The viability of the cultures was calculated as the percentage of the ratio of the number of unstained cells (viable cells) to the total number of cells counted (viable cells plus nonviable cells). In each experiment over 200 cells on three dishes were counted at random to obtain means ± S.E.M. of the cell viability.

2.5. Statistical analysis

Single factor repeated analysis of variance (ANOVA), and Scheffe's and Tukey's tests were used to analyze data with $P < 0.05$ indicating significance. Data are expressed as means ± S.E.M.

3. Results

3.1. Effect of glutamate on cell viability

Table 1 shows a dose–response curve of glutamate at several concentrations when incubated with hippocampal cells for 0.5 h. No significant effect was observed after cell exposure to 0.1 mM glutamate. However, a progressive percentage decrease of cell viability was detected at higher concentrations, reaching a 68.1% decrease at 5 mM concentration. No further decrease was observed at 10 mM, and in this case the effect was similar to that observed with

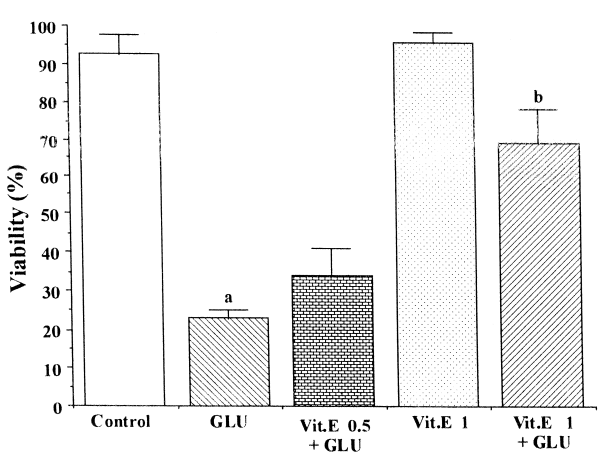

Fig. 3. Effect of vitamin E (Vit. E) on glutamate-induced cytotoxicity in rat hippocampal cells. After cells exposure to glutamate (Glu) (5 mM), cultures were then incubated with glutamate-free medium containing Vit. E (0.5 or 1 mM) for 24 h. The ordinate indicates the viability of cultures calculated as described in Section 2. (a) vs. control; (b) vs. Glu at $P < 0.05$ (ANOVA and Scheffe's test).

the 5 mM concentration. As far as the effect of time exposure to glutamate is concerned, no significant differences were shown in cell viability after 0.5 or 2 h with either glutamate concentration which lead us to decide that a 0.5 h cell exposure to glutamate was sufficient for the manifestation of glutamate excitotoxicity.

3.2. Effect of ganglioside GM_1, NGF and vitamin E alone or in association on glutamate-induced cytotoxicity

Fig. 1 shows the effect of ganglioside GM_1, 5 and 10 ng/ml on the glutamate-induced cytotoxicity in hippocampal cells. Glutamate (5 mM) decreased the cell viability to 25.4% (control cells showed a 93.1% viability). Cells exposed to glutamate in the presence of ganglioside GM_1 (5 and 10 ng/ml) showed viability of 19.4 and 72.5%, respectively. While NGF at the dose of 0.5 ng/ml presents no effect, it significantly reduced glutamate cytotoxicity at a higher dose (1 ng/ml), increasing the cell viability to 66.3% (Fig. 2).

A dose-dependent effect was observed after the hippocampal cells incubation with vitamin E (0.5 and 1 mM), with cell viability of the order of 34.0 and 69.6%, respectively (Fig. 3). Surprisingly, a potentiation of the effect was observed after the association of NGF (0.5 ng/ml) plus ganglioside GM_1 (5 ng/ml), and vitamin E (0.5 mM) plus ganglioside GM_1 (5 ng/ml) after exposure to glutamate, with percentages of cell viability of 66.1 and 71.0%, respectively, as compared to the percentage of cell viability observed with each compound alone (29.5 and 19.4%) (Fig. 4). On the other hand, only an additive effect was seen after the association of vitamin E (0.5 mM) plus NGF (0.5 ng/ml) after glutamate exposure (56.0% cell viability) (Fig. 4).

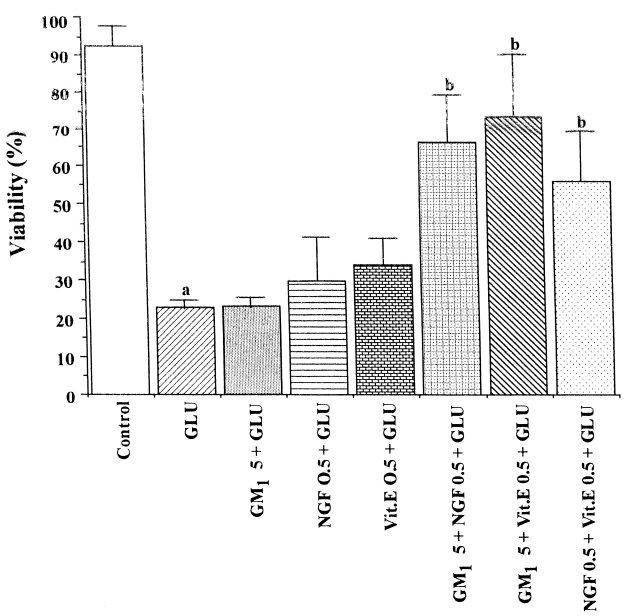

Fig. 4. Effect of ganglioside GM_1 (GM_1), NGF and vitamin E (Vit. E) on glutamate-induced cytotoxicity in rat hippocampal cells. After cells exposure to glutamate (Glu) (5 mM), cultures were incubated with glutamate-free medium containing GM_1 (5 ng/ml), NGF (0.5 ng/ml) or Vit. E (0.5 mM) alone or in association for 24 h. The ordinate indicates the viability of cultures calculated as described in Section 2. (a) vs. control; (b) vs. Glu at $P < 0.05$ (ANOVA and Scheffe's test).

4. Discussion

The present work showed that glutamate was able to cause excitotoxicity on hippocampal cells at 5 mM concentration, bringing cells viability to only 29% (controls presented a cell viability higher than 90%). The maximum effect was reached after 0.5 h exposure, with no further change after this period of time. Glutamate causes damage not only to neurons but also in several other cell lines and kills sensitive neurons through several steps downstream to receptor activation such as increased free Ca^{2+} levels, activation of various enzymes and accumulation of reactive oxygen species. Perhaps the strongest evidence that calcium is involved in the neuronal injury induced by glutamate came from cell culture studies. Thus, removal of extracellular calcium protected rat neocortical (Choi, 1987) and hippocampal (Mattson et al., 1989) cultured neurons against glutamate toxicity, demonstrating the requirement for calcium influx in the excitotoxic process.

Free radicals promote elevation of $[Ca^{2+}]_i$ and elevation of $[Ca^{2+}]_i$ promotes free radical production (Orrenius et al., 1989). Free radicals can damage proteins involved in regulation of $[Ca^{2+}]_i$ and may also damage neurons (Mattson and Mark, 1996). Cells possess mechanisms to protect themselves from free radicals that are produced during cellular metabolism or that arise from exogenous sources. The defense systems include antioxidant enzymes and free radical scavengers (Halliwell, 1987) such as vitamin E which blocks the autocatalytic peroxidation cascade in membranes.

Several studies have reported that neurotrophic factors can increase levels of antioxidant enzymes. For instance, NGF increases glutathione levels in cultured PC12 cells (Pan et al., 1994). Also, Nistico et al. (1992) reported an increase in the levels of superoxide dismutase and glutathione peroxidase in the brains of NGF-infused rats. Mattson et al. (1995) found that neurotrophic factors including NGF increased to various degrees superoxide dismutase and glutathione reductase activities in rat cortical cell cultures.

Neurotrophic factors including NGF, constitute an important class of endogenous modulators of excitotoxicity, and are known to protect neurons against injury from several causes (Zhang et al., 1993; Mattson et al., 1993). It has been proposed that neurotrophic factors may protect neurons from excitotoxic/metabolic insults probably by the enhancement of calcium homeostatic mechanisms and suppression of reactive oxygen species accumulation (Mattson et al., 1993). Neurotrophic factors are known to

bind and activate receptor tyrosine kinases which initiate a cascade of kinase phosphorylations that ultimately result in activation of transcription (Schlessinger and Ulbrich, 1992). Data from Mattson et al. (1995) suggest two general mechanisms whereby neurotrophic factors suppress glutamate-induced accumulation of reactive oxygen species and protect neurons against excitotoxicity. One involves reducing the elevation of $[Ca^{2+}]_i$ (Mattson et al., 1993) which contributes to induction of reactive oxygen species (Lafon-Cazal et al., 1993) and the other involves induction of increased antioxidant enzyme activities.

MacGregor et al. (1996) showed that ascorbic acid was able to reduce kainate-induced damage of the rat hippocampus. According to these authors, the protective activity of ascorbate may be the result of its action as a free radical scavenger or as an antagonist of glutamate receptors. Exogenously added gangliosides are also known to promote neurite outgrowth in a variety of cell types (Hynds et al., 1997), including neuroblastoma cells (Mummert and Schenguind, 1997). Neuronal death after glutamate cells exposure can be decreased by the pretreatment with ganglioside (Favaron et al., 1988). This effect seems to result in the antagonism produced by gangliosides of the glutamate induced increase in the $[Ca^{2+}]_i$ together with the persistent phosphokinase C activation, consequence of the stimulation of the glutamatergic receptor. Manev et al. (1990) studying several synthetic gangliosides, showed that these compounds protect cells from glutamate-induced toxicity, possibly preventing the binding of glutamate to its receptor.

We have previously shown (Cunha et al., 1997) that glutamate after 24 h exposure induced cytotoxicity in PC12 cells in a dose-dependent manner and at concentrations as low as 1 mM. The effect was also time-dependent and not fully developed until somewhere between 6 and 24 h. Protection against glutamate cytotoxicity was observed after ganglioside GM_1 treatment, and this effect was time and dose-dependent. In the present work, glutamate-induced cytotoxicity was blocked not only by NGF but also by ganglioside GM_1, and vitamin E, and ganglioside GM_1 seems to potentiate the effects caused by both compounds. The mechanisms involved in the ganglioside GM_1–NGF interaction are not clearly defined. It is known that ganglioside GM_1 can be incorporated to neuronal membranes and might interfere with the membrane fluidity and alter proteins in the membrane (Li et al., 1986; Karpiak et al., 1991). Also, Skaper et al. (1991) showed that the death of cultured neurons induced by activation of NMDA receptors is reduced by gangliosides, agents which can be directed to act on the Ca^{2+} amplification events necessary for excitatory amino acid induction of cell death.

However, while NGF seems to act through specific receptors (Greene and Shooter, 1980), which involves signalling transducing cascade dependent from tyrosine kinase (Kaplan et al., 1991; Klein et al., 1991), the mechanism of action of ganglioside GM_1 is not completely clarified. An interesting hypothesis is that ganglioside GM_1 can indirectly act potentiating actions of neurotrophic factors. This fact was shown in vitro with PC12 cells where ganglioside GM_1 increased NGF-induced neurite growth (Ferrari et al., 1983). Ganglioside GM_1 affects receptor phosphorylation of the platelet-derived growth factor and epidermic growth factor (Brenner et al., 1986). Thus, it is possible that ganglioside GM_1 modulates NGF receptor phosphorylation leading to a signal amplification. In PC12 cells, ganglioside GM_1 potentiates neurite growth by NGF (Ferrari et al., 1983) and blockades the NGF inhibition by phosphokinase inhibitors (Ferrari et al., 1992). In addition, other second messenger systems are also affected by NGF and ganglioside GM_1 (Cuello, 1990; Levi and Alleman, 1991).

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