

RESEARCH ARTICLE

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Grape seed proanthocyanidin extract inhibits glutamate-induced cell death through inhibition of calcium signals and nitric oxide formation in cultured rat hippocampal neurons

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Abstract

Background: Proanthocyanidin is a polyphenolic bioflavonoid with known antioxidant activity. Some flavonoids have a modulatory effect on $[Ca^{2+}]_i$. Although proanthocyanidin extract from blueberries reportedly affects Ca^{2+} buffering capacity, there are no reports on the effects of proanthocyanidin on glutamate-induced $[Ca^{2+}]_i$ or cell death. In the present study, the effects of grape seed proanthocyanidin extract (GSPE) on glutamate-induced excitotoxicity was investigated through calcium signals and nitric oxide (NO) in cultured rat hippocampal neurons.

Results: Pretreatment with GSPE (0.3-10 μ g/ml) for 5 min inhibited the $[Ca^{2+}]_i$ increase normally induced by treatment with glutamate (100 μ M) for 1 min, in a concentration-dependent manner. Pretreatment with GSPE (6 μ g/ml) for 5 min significantly decreased the $[Ca^{2+}]_i$ increase normally induced by two ionotropic glutamate receptor agonists, N-methyl-D-aspartate and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). GSPE further decreased AMPA-induced response in the presence of 1 μ M nimodipine. However, GSPE did not affect the 50 mM K^+ -induced increase in $[Ca^{2+}]_i$. GSPE significantly decreased the metabotropic glutamate receptor agonist (RS)-3,5-Dihydroxyphenylglycine-induced increase in $[Ca^{2+}]_i$, but it did not affect caffeine-induced response. GSPE (0.3-6 μ g/ml) significantly inhibited synaptically induced $[Ca^{2+}]_i$ spikes by 0.1 mM $[Mg^{2+}]_o$. In addition, pretreatment with GSPE (6 μ g/ml) for 5 min inhibited 0.1 mM $[Mg^{2+}]_o$ - and glutamate-induced formation of NO. Treatment with GSPE (6 μ g/ml) significantly inhibited 0.1 mM $[Mg^{2+}]_o$ - and oxygen glucose deprivation-induced neuronal cell death.

Conclusions: All these data suggest that GSPE inhibits 0.1 mM $[Mg^{2+}]_o$ - and oxygen glucose deprivation-induced neurotoxicity through inhibition of calcium signals and NO formation in cultured rat hippocampal neurons.

Background

Proanthocyanidins are polymers of flavonoid molecules that are widely available in fruits, vegetables, nuts, seeds, flowers, and bark, and especially in grape seeds [1]. These compounds possess a broad spectrum of antioxidative properties that provide potent protection against free radical-induced diseases, such as ischemia and reperfusion injury [2-4], aging [5], and carcinogenesis [6]. Proanthocyanidins are also known to possess

antibacterial, antiviral, anti-inflammatory, anti-allergic, and vasodilator properties [1,7].

Glutamate is a major neurotransmitter in the central nervous system. Glutamate increases intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in neurons by activating ionotropic and metabotropic glutamate receptors. In pathological conditions, including epilepsy and ischemia, a massive glutamate release leads to glutamate neurotoxicity [8,9]. The neurotoxicity is mainly due to N-methyl-D-aspartate (NMDA) receptors, which cause excessive elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and subsequent neuronal cell death [10]. Elevation of $[Ca^{2+}]_i$ following NMDA receptor activation

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stimulates nitric oxide synthase (NOS), an enzyme that induces formation of nitric oxide (NO) in neurons [11]. NO reportedly also mediates glutamate neurotoxicity [12,13].

Some flavonoids have modulatory effects on $[Ca^{2+}]_i$. (-)-Epigallocatechin gallate (EGCG) increase $[Ca^{2+}]_i$ in U87 cells [14] and inhibit glutamate-induced $[Ca^{2+}]_i$ increase in PC12 cells [15] and cultured rat hippocampal neurons [16]. Quercetin has stimulatory effects on voltage-dependent L-type Ca^{2+} channels in GH3 cells and inhibitory effects on L-type Ca^{2+} channels in NG108-15 cells [17]. In addition, EGCG [15], apigenin [18], and wogonin [19] have a neuroprotective effect in glutamate neurotoxicity. Proanthocyanidin extract from blueberries has reportedly reversed dopamine, $A\beta_{42}$, and lipopolysaccharide-induced dysregulation of Ca^{2+} buffering capacity [20]. However, there are no reports on the effect of proanthocyanidin on glutamate-induced $[Ca^{2+}]_i$ or cell death in cultured rat hippocampal neurons.

The present study determined whether grape seed proanthocyanidin extract (GSPE) affected glutamate-induced Ca^{2+} signalling and NO formation in cultured rat hippocampal neurons. It further examined whether GSPE protects neurons against neurotoxicity induced by low extracellular Mg^{2+} concentration ($[Mg^{2+}]_o$) and oxygen glucose deprivation.

Results

Effect of GSPE on glutamate-induced $[Ca^{2+}]_i$ increase

Since elevation of $[Ca^{2+}]_i$ is one of the major causes of glutamate excitotoxicity [10], the present study first examined the effect of GSPE on glutamate-induced $[Ca^{2+}]_i$ increase in cultured rat hippocampal neurons. Treatment with glutamate (100 μ M) for 1 min caused $[Ca^{2+}]_i$ increase. Reproducible response could be elicited by applying glutamate (100 μ M) for 1 min at 30-min intervals (peak 2/peak 1 = $97.6 \pm 2.4\%$, $n = 27$) (Figure 1A). Pretreatment with GSPE (0.3 μ g/ml) for 5 min did not affect the glutamate-induced $[Ca^{2+}]_i$ response (peak 2/peak 1 = $100.8 \pm 3.8\%$, $n = 15$) (Figure 1B). Pretreatment with higher concentrations of GSPE (1-6 μ g/ml) inhibited the glutamate-induced response in a concentration-dependent manner (peak 2/peak 1 = $92.0 \pm 2.1\%$ at 1 μ g/ml, $n = 17$; $86.5 \pm 3.5\%$ at 3 μ g/ml, $n = 16$; $71.9 \pm 2.3\%$ at 6 μ g/ml, $n = 21$). However, pretreatment with 10 μ g/ml GSPE did not further inhibit the glutamate-induced response (peak 2/peak 1 = $72.4 \pm 3.5\%$ at 10 μ g/ml, $n = 16$) (Figure 1C-G). Therefore, the present study used 6 μ g/ml of GSPE to quantify the inhibition of agonist-induced $[Ca^{2+}]_i$ increase. The 6 μ g/ml concentration of IHEA GSPE used in the present study was less than or equal to the serum levels of polyphenols after intake of grape seed proanthocyanidin extract in humans [21].

Effect of GSPE on ionotropic glutamate receptor agonist-induced $[Ca^{2+}]_i$ increase

To determine how GSPE inhibits glutamate receptor-induced $[Ca^{2+}]_i$ increase, the present study used two ionotropic glutamate receptor agonists, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA. Application of AMPA increased $[Ca^{2+}]_i$ by activating AMPA/kainate channels and then voltage-gated Ca^{2+} channels in neurons. Reproducible increase in $[Ca^{2+}]_i$ was induced by treatment with (S)-AMPA (10 μ M) for 1 min at 10-min intervals (peak 2/peak 1 = $93.7 \pm 5.4\%$, $n = 20$). Pretreatment with GSPE (6 μ g/ml) for 5 min significantly inhibited the AMPA-induced $[Ca^{2+}]_i$ response (peak 2/peak 1 = $80.8 \pm 2.0\%$, $n = 23$, $P < 0.05$) (Figure 2A1-A3).

Ca^{2+} -permeable AMPA receptors are expressed in hippocampal neurons early in development [22]. The present study tested whether proanthocyanin inhibits Ca^{2+} -permeable AMPA-receptor-mediated Ca^{2+} influx. Pretreatment with nimodipine (1 μ M) for 5 min inhibited the AMPA-induced $[Ca^{2+}]_i$ response (peak 2/peak 1 = $40.2 \pm 4.8\%$, $n = 11$, $P < 0.01$). Pretreatment with GSPE (6 μ g/ml) for 5 min further inhibited AMPA-induced response in the presence of nimodipine (1 μ M) (peak 3/peak 1 = $27.3 \pm 6.7\%$, $n = 9$, $P < 0.01$) (Figure 2B1-B3).

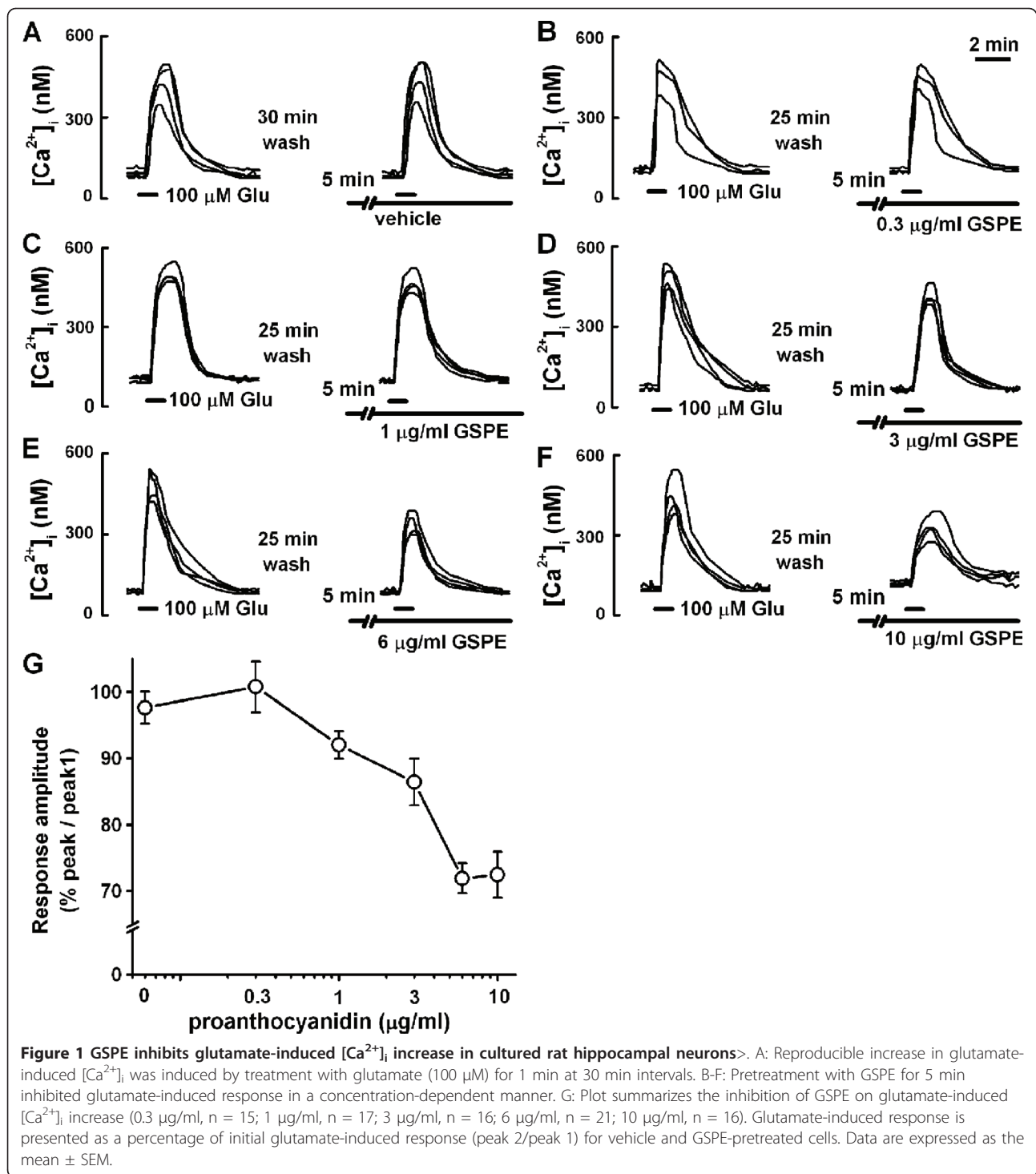
In addition, reproducible NMDA-induced $[Ca^{2+}]_i$ increase was induced by treatment with NMDA (100 μ M) for 1 min at 20-min intervals (peak 2/peak 1 = $94.9 \pm 7.6\%$, $n = 11$). Pretreatment with GSPE (6 μ g/ml) for 5 min also significantly inhibited NMDA-induced $[Ca^{2+}]_i$ response (peak 2/peak 1 = $71.1 \pm 2.6\%$, $n = 15$, $P < 0.05$) (Figure 3).

Effect of GSPE on high K^+ -induced $[Ca^{2+}]_i$ increase

Binding glutamate to its AMPA receptors induced an influx of Na^+ (partly Ca^{2+}) into neurons and depolarized the neurons. This depolarization induced secondary activation of voltage-gated Ca^{2+} channels [23]. To determine the effect of GSPE on glutamate-induced secondary activation of Ca^{2+} channels, the present study observed whether GSPE affects the depolarization-induced $[Ca^{2+}]_i$ increase by 50 mM K^+ HEPES-HBSS (Figure 4). Reproducible $[Ca^{2+}]_i$ increase was induced by treatment for 1 min with 50 mM K^+ HEPES-HBSS at 30-min intervals (peak 2/peak 1 = $91.2 \pm 2.7\%$, $n = 22$). Treatment with GSPE (6 μ g/ml) for 5 min did not affect high K^+ -induced $[Ca^{2+}]_i$ response (peak 2/peak 1 = $93.2 \pm 1.7\%$, $n = 27$, $P > 0.05$) (Figure 4).

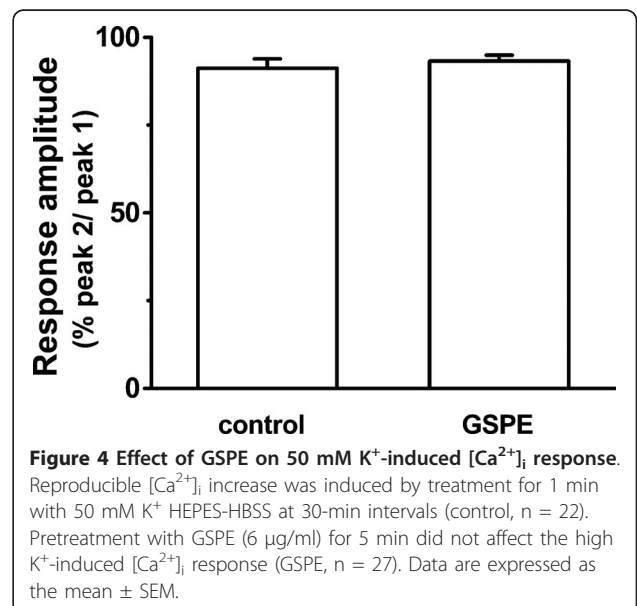
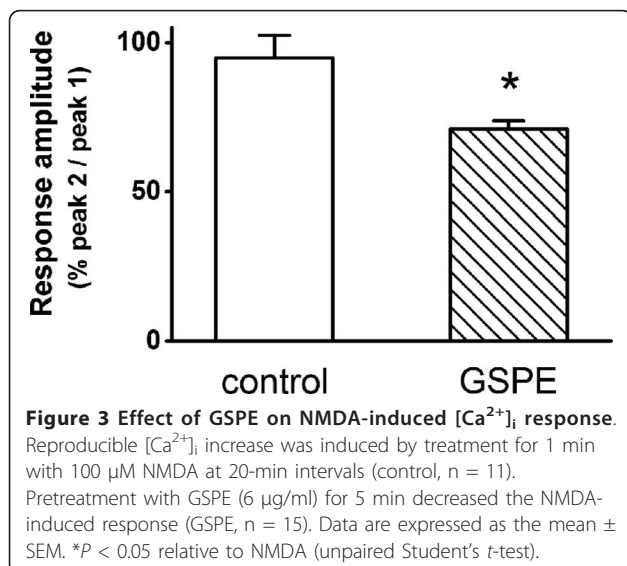
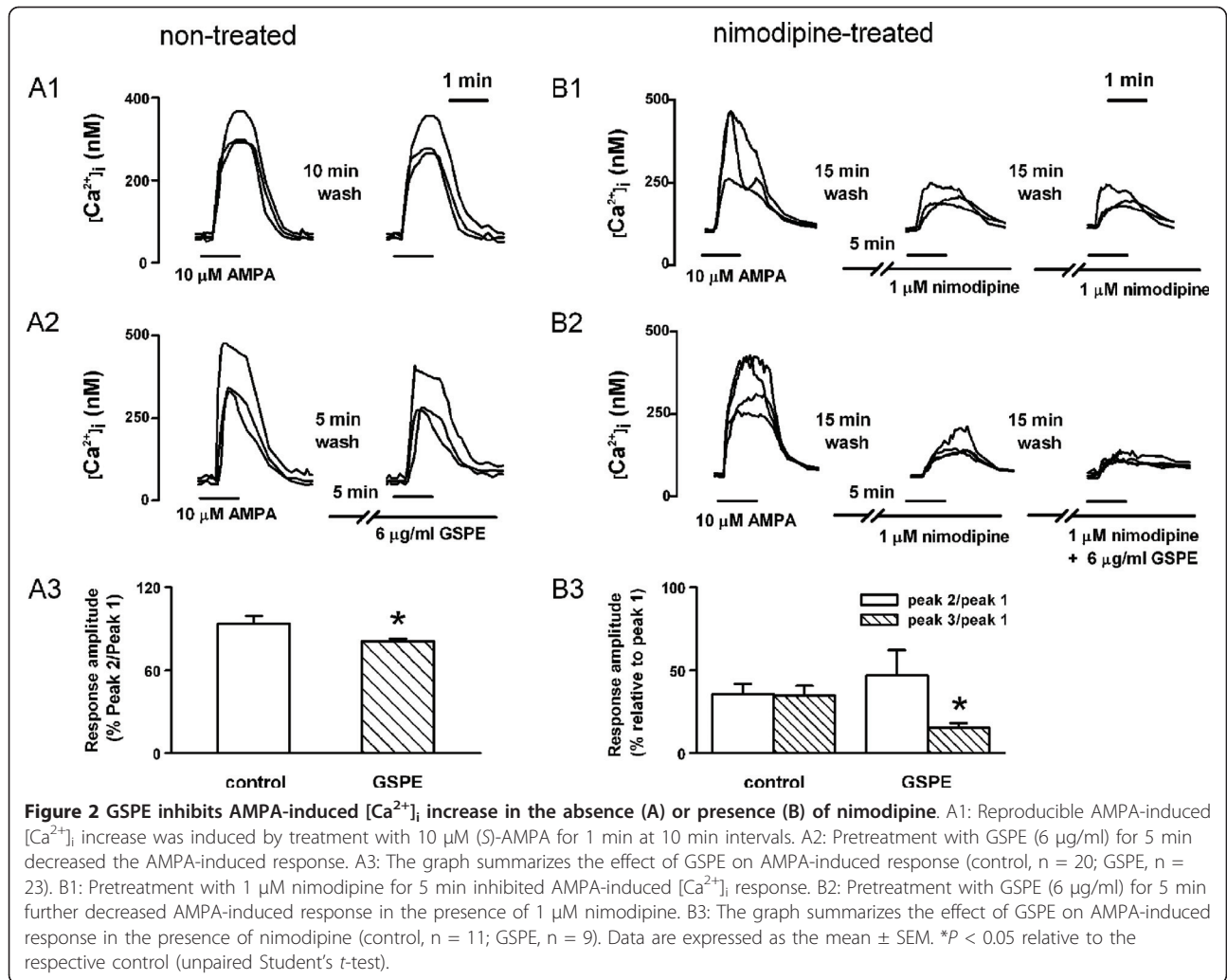
Effect of GSPE on metabotropic glutamate receptor agonist or caffeine-induced $[Ca^{2+}]_i$ increase

Group I metabotropic glutamate receptors, composed of mGluR1 and mGluR5, are exclusively expressed at



postsynaptic sites in the hippocampus [24]. They are linked to phosphatidylinositol metabolism and the formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. Binding of IP_3 to the IP_3 receptors initiates release of Ca^{2+} from intracellular stores [25]. The present study examined whether GSPE affects the

metabotropic glutamate receptor agonist response to DHPG-induced $[Ca^{2+}]_i$ increase. Reproducible $[Ca^{2+}]_i$ increase was induced by treatment with DHPG ($100 \mu\text{M}$) for 1 min at 30-min intervals (peak 2/peak 1 = $105.9 \pm 1.3\%$, $n = 31$). Pretreatment with GSPE ($6 \mu\text{g/ml}$) for 5 min significantly inhibited DHPG-induced



$[Ca^{2+}]_i$ response (peak 2/peak 1 = $68.9 \pm 1.8\%$, $n = 29$, $P < 0.05$) (Figure 5).

In addition to IP_3 receptors, ryanodine receptors can mobilize intracellular Ca^{2+} stores [26]. Reproducible $[Ca^{2+}]_i$ increase was induced by treatment with caffeine (10 mM) for 2 min at 10-min intervals (peak 2/peak 1 = $98.8 \pm 1.0\%$ of control response, $n = 14$). Pretreatment with GSPE (6 μ g/ml) for 5 min did not significantly affect caffeine-induced $[Ca^{2+}]_i$ response ($92.5 \pm 2.4\%$ of control response, $n = 9$, $P > 0.05$) (Figure 5).

Effect of GSPE on 0.1 mM $[Mg^{2+}]_o$ -induced $[Ca^{2+}]_i$ spikes

The next study determined whether GSPE affects synaptically mediated $[Ca^{2+}]_i$ spikes. Previous studies have shown that reducing $[Mg^{2+}]_o$ to 0.1 mM can elicit intense $[Ca^{2+}]_i$ spikes (Figure 6A) which depend on synaptic transmission [27]. $[Ca^{2+}]_i$ spikes were induced by 0.1 mM $[Mg^{2+}]_o$ in the cultured rat hippocampal neurons 13 days after plating. The low $[Mg^{2+}]_o$ -induced $[Ca^{2+}]_i$ spikes gradually disappeared after treatment with GSPE (6 μ g/ml). At 10 min after exposure to GSPE, the frequency of $[Ca^{2+}]_i$ spikes was $12.8 \pm 8.7\%$ of the initial frequency (Figure 6B & 6C).

Effect of GSPE on 0.1 mM $[Mg^{2+}]_o$ -and glutamate-induced NO formation

NO is important for glutamate-induced neurotoxicity [12]. The present study determined whether GSPE affects low $[Mg^{2+}]_o$ -induced NO formation using the

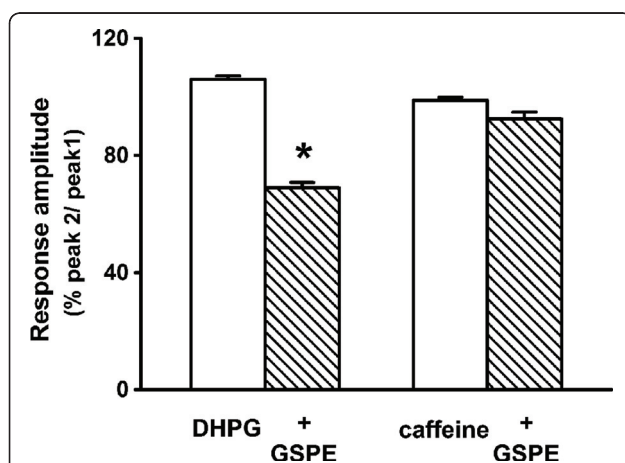


Figure 5 Effects of GSPE on DHPG- or caffeine-induced $[Ca^{2+}]_i$ response. Reproducible $[Ca^{2+}]_i$ increase was induced by treatment for 1 min with DHPG (100 μ M) at 30-min intervals (DHPG, $n = 31$). Pretreatment with GSPE (6 μ g/ml) for 5 min decreased the DHPG-induced response (+ GSPE, $n = 29$). Reproducible caffeine-induced $[Ca^{2+}]_i$ increase was induced by treatment for 2 min with DHPG (100 μ M) at 10-min intervals (caffeine, $n = 14$). However, GSPE did not affect caffeine-induced $[Ca^{2+}]_i$ increase (+ GSPE, $n = 9$). Data are expressed as the mean \pm SEM. * $P < 0.05$ relative to DHPG (unpaired Student's t -test).

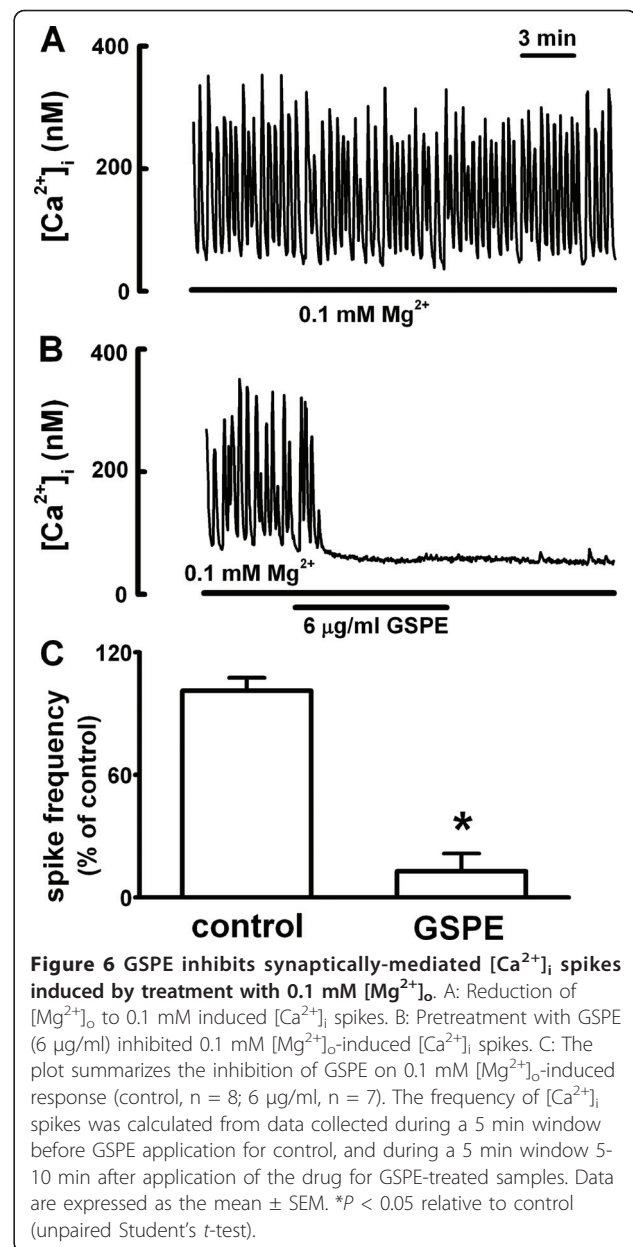
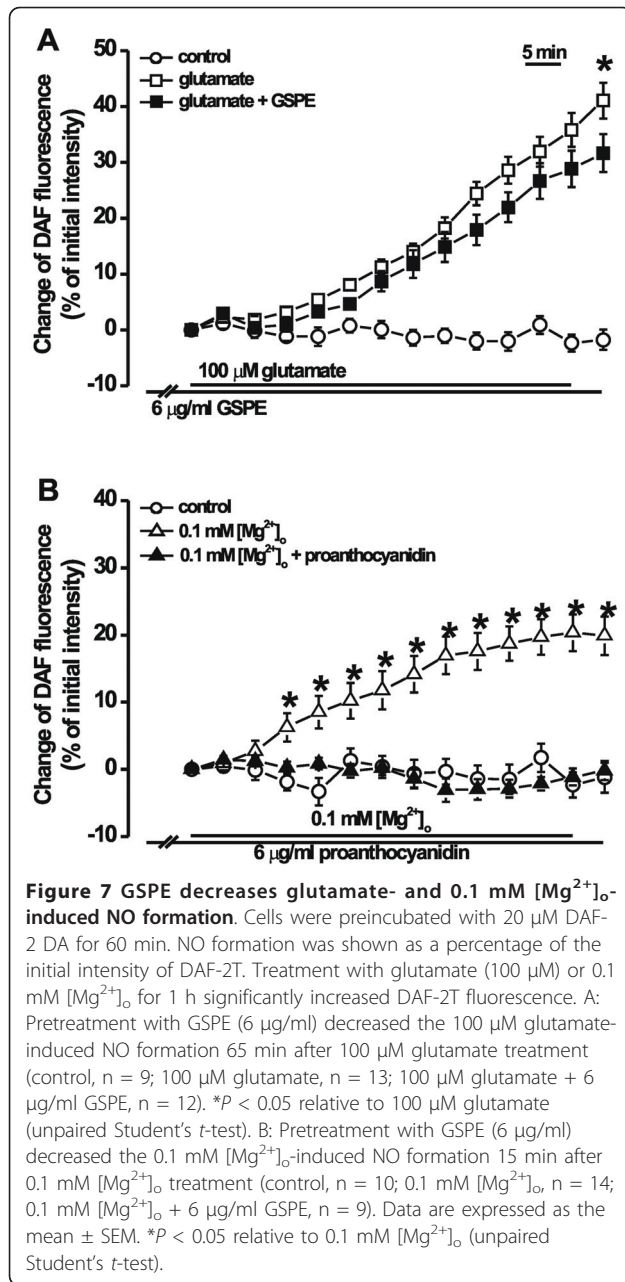


Figure 6 GSPE inhibits synaptically-mediated $[Ca^{2+}]_i$ spikes induced by treatment with 0.1 mM $[Mg^{2+}]_o$. A: Reduction of $[Mg^{2+}]_o$ to 0.1 mM induced $[Ca^{2+}]_i$ spikes. B: Pretreatment with GSPE (6 μ g/ml) inhibited 0.1 mM $[Mg^{2+}]_o$ -induced $[Ca^{2+}]_i$ spikes. C: The plot summarizes the inhibition of GSPE on 0.1 mM $[Mg^{2+}]_o$ -induced response (control, $n = 8$; 6 μ g/ml, $n = 7$). The frequency of $[Ca^{2+}]_i$ spikes was calculated from data collected during a 5 min window before GSPE application for control, and during a 5 min window 5-10 min after application of the drug for GSPE-treated samples. Data are expressed as the mean \pm SEM. * $P < 0.05$ relative to control (unpaired Student's t -test).

NO indicator DAF-2DA. Treatment with glutamate (100 μ M) or low $[Mg^{2+}]_o$ for 1 h significantly increased DAF-2T fluorescence. While the 0.1 mM $[Mg^{2+}]_o$ -induced increase in NO formation was markedly inhibited by pretreatment with GSPE (6 μ g/ml) for 5 min, and the glutamate-induced NO formation was slowly inhibited at a later phase (Figure 7A & 7B).

GSPE protects neuronal cells against 0.1 mM $[Mg^{2+}]_o$ - and oxygen glucose deprivation-induced cell death

Reduction of $[Mg^{2+}]_o$ in the solution used to bathe cultured CNS neurons elicits an intense pattern of excitatory activity and $[Ca^{2+}]_i$ spikes and causes neuronal cell



death [28-30]. The present study was an examination of whether GSPE protects cells against cell death induced by low $[Mg^{2+}]_o$. Cell viability was determined by counting the number of viable neurons before and 20-24 h after treatment (Figure 8 & Figure 9A). In the control cells, cell survival was decreased slightly (Figure 8, data shown in Methods section). Reduction of $[Mg^{2+}]_o$ markedly decreased neuronal cell survival ($52.6 \pm 0.9\%$ of the control) (Figure 8 & Figure 9A). A similar proportion of the GSPE-treated cells died relative to the control cells (Figure 8 & Figure 9A). However, a 0.1 mM $[Mg^{2+}]_o$ -induced decrease in cell survival was markedly inhibited

by 6 $\mu g/ml$ GSPE ($78.6 \pm 9.9\%$ of control) (Figure 8 & Figure 9A). The effect of GSPE on oxygen glucose deprivation-induced cell death was examined further (Figure 9B). The cells in glucose-free BSS, with and without GSPE (6 $\mu g/ml$), were gassed with 85% N_2 , 10% H_2 , and 5% CO_2 for 90 min, and then were regrown in DMEM supplemented with 10% horse serum and penicillin/streptomycin in a CO_2 incubator for 24 h. Oxygen glucose deprivation decreased neuronal cell survival to $57.8 \pm 2.2\%$ of the control. However, treatment with GSPE (6 $\mu g/ml$) increased cell survival to $81.3 \pm 7.3\%$ of the control.

Discussion

The present study used an *in vitro* rat hippocampal culture model to determine the inhibitory mechanisms of GSPE in low $[Mg^{2+}]_o$ or oxygen glucose deprivation-induced neuronal cell death. GSPE reduced the glutamate-induced $[Ca^{2+}]_i$ increase by inhibiting the AMPA, NMDA, and DHPG-induced $[Ca^{2+}]_i$ increase in hippocampal neurons. Synaptically mediated low $[Mg^{2+}]_o$ -induced $[Ca^{2+}]_i$ spikes were also inhibited by GSPE. GSPE inhibited low $[Mg^{2+}]_o$ or oxygen glucose deprivation-induced neuronal cell death by inhibition of both $[Ca^{2+}]_i$ increase and Ca^{2+} -dependent NO formation.

Glutamate depolarizes membranes by an influx of Na^+ (partly Ca^{2+}) through non-NMDA receptors, which secondarily activate voltage-gated Ca^{2+} channels and induce Ca^{2+} influx [23]. Glutamate also induces Ca^{2+} influx directly through NMDA receptor channels and Ca^{2+} -permeable non-NMDA AMPA receptor channels. In the present study, GSPE inhibited glutamate, AMPA, and NMDA-induced $[Ca^{2+}]_i$ increase, but it did not affect the depolarization-induced $[Ca^{2+}]_i$ increase from 50 mM K^+ HEPES-HBSS, suggesting that GSPE inhibits AMPA-induced $[Ca^{2+}]_i$ increase by inhibiting Ca^{2+} influx directly through Ca^{2+} -permeable AMPA receptors. In fact, Ca^{2+} -permeable AMPA receptors are strongly expressed in hippocampal neurons, especially early in development [22]. All these data suggested that GSPE inhibited Ca^{2+} influx through Ca^{2+} -permeable AMPA channels and NMDA channels. This data are indirectly supported by other reports that flavonoids such as baicalin, baicalein, and EGCG, decreased glutamate or NMDA-induced $[Ca^{2+}]_i$ increase [15,31].

The group I metabotropic glutamate receptor agonist, DHPG, induces a release of Ca^{2+} from IP_3 -sensitive stores by activating PLC [25,32]. In the present study, GSPE inhibited DHPG-induced $[Ca^{2+}]_i$ increase. Although the working mechanism of GSPE is not obvious, GSPE may inhibit DHPG-induced Ca^{2+} release from IP_3 -sensitive stores or DHPG-induced activation of PLC. Therefore, further research is needed to determine whether proanthocyanidin inhibits release of Ca^{2+} from

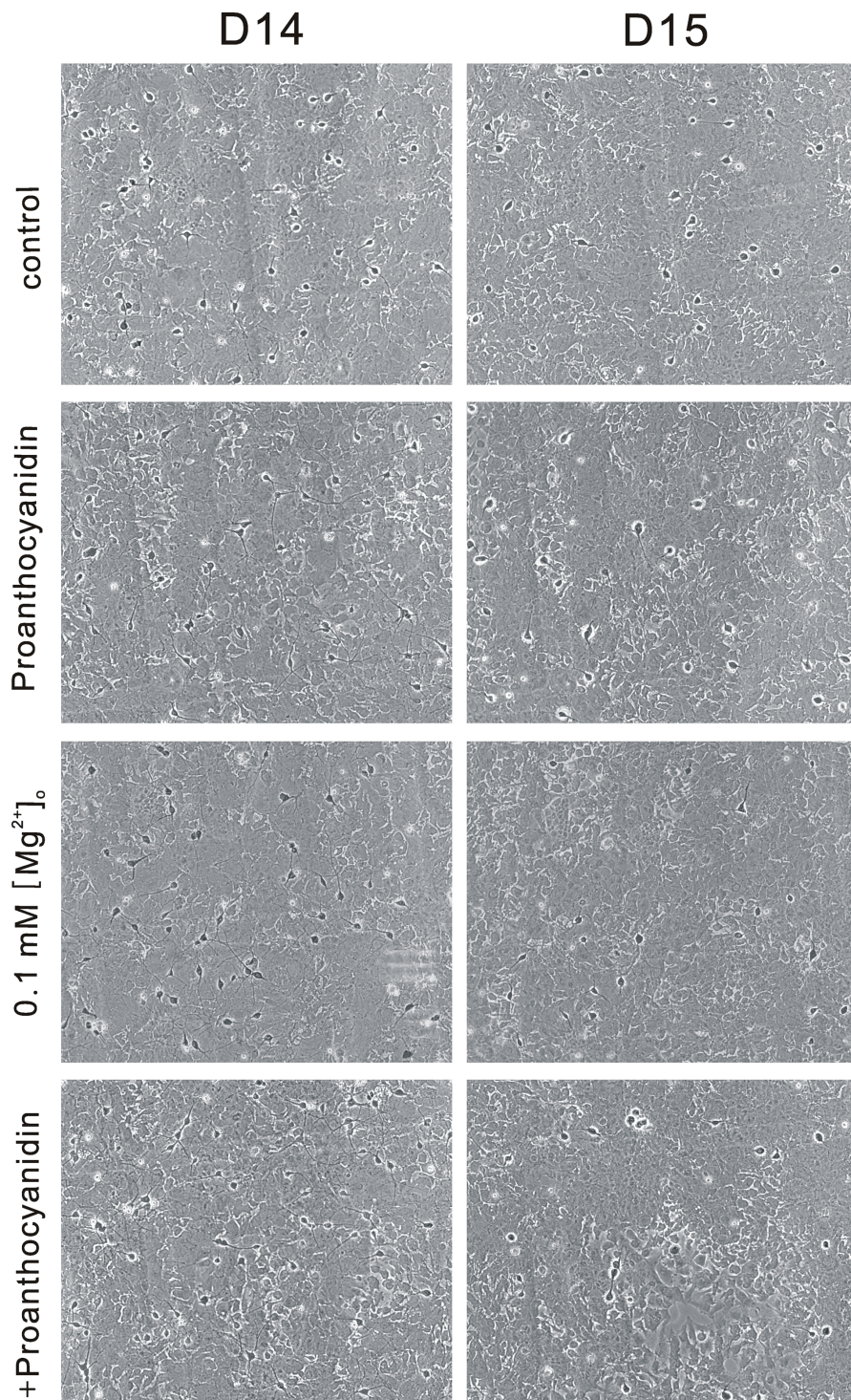
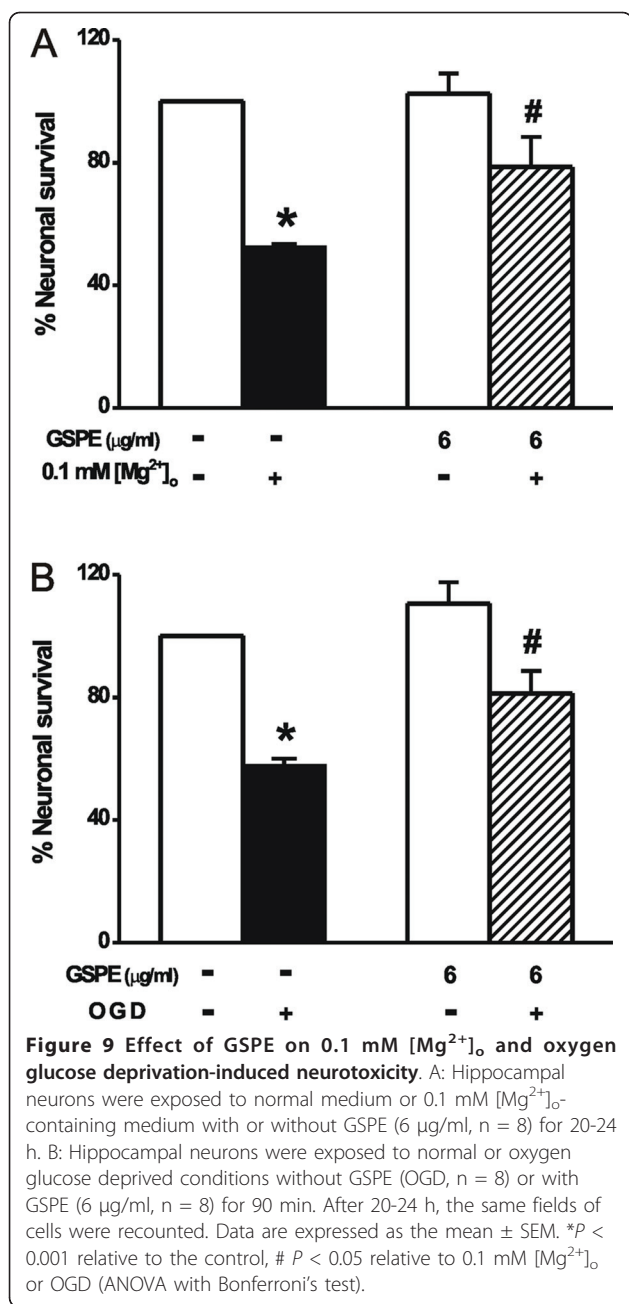


Figure 8 GSPE protects cells against 0.1 mM [Mg²⁺]_o-induced neurotoxicity. Phase-contrast photomicrographs showed the same field of cultured rat hippocampal neurons before treatment (left, D14) and 20-24 h after treatment (right, D15). The cells were treated with normal medium (control), GSPE (6 µg/ml), 0.1 mM [Mg²⁺]_o and 0.1 mM [Mg²⁺]_o plus GSPE (+ GSPE)-containing medium at 14 days in culture. Hippocampal neurons (identified by a light halo around the soma and long fine processes) grew on a bed of non-neuronal cells that formed a mosaic beneath them.



IP₃-sensitive stores or metabotropic glutamate receptor-induced activation of PLC.

In the present study, GSPE inhibited glutamate-induced $[Ca^{2+}]_i$ increase by inhibiting AMPA, NMDA, and metabotropic glutamate receptor-induced $[Ca^{2+}]_i$ increase. Reduction of $[Mg^{2+}]_o$ in cultured central nervous system neurons to 0.1 mM elicited $[Ca^{2+}]_i$ spikes that depend on glutaminergic synaptic transmission [27,29,33]. In the present study, GSPE inhibited low $[Mg^{2+}]_o$ -induced $[Ca^{2+}]_i$ spikes. All these data suggest a possibility that proanthocyanidin can inhibit glutaminergic

synaptic transmission in hippocampal neurons. In the present study, GSPE did not affect the depolarization-induced $[Ca^{2+}]_i$ increase induced by high K^+ , which is involved in neurotransmitter release in the synaptic terminal. Thus, it is not clear whether proanthocyanidin inhibited synaptic transmission by decreasing glutamate release in presynaptic sites.

In the present study, GSPE completely inhibited low $[Mg^{2+}]_o$ -induced NO formation, and it slightly inhibited glutamate-induced formation. GSPE reportedly has potent inhibitory action on NO production presumably through of the inhibition of Ca^{2+} -dependent nitric oxide synthase [34]. In neuronal cells, NO was synthesized from Ca^{2+} -dependent enzymes, neuronal nitric oxide synthase [35,36]. Therefore, the inhibition of excessive Ca^{2+} influx or Ca^{2+} release from intracellular stores and formation of NO by glutamate in the present study suggest that proanthocyanidin inhibits NO formation by inhibiting glutamate or low $[Mg^{2+}]_o$ -induced $[Ca^{2+}]_i$ increase.

Previous investigations have reported that proanthocyanidin protects multiple target organs from drug- and chemical-induced toxicity. GSPE protects cells against acetaminophen-induced hepato- and nephrotoxicity, amiodarone-induced lung toxicity, doxorubicin-induced cardiotoxicity, and dimethylnitrosamine-induced splenotoxicity [37]. GSPE inhibited 12-O-tetradecanoylphorbol-13-acetate and O-ethyl-S,S-dipropyl phosphorodithioate-induced brain neurotoxicity [2,37]. Grape seed extract has also been reported to reduce brain ischemic injury in gerbils [4,38] and rats [39], suggesting that the neuroprotective effects of proanthocyanidin are mediated by its antioxidant effects and antiapoptotic effects, respectively. However, there have been no reports on the underlying roles of calcium signalling or NO formation in proanthocyanidin-induced neuroprotection. GSPE inhibited low $[Mg^{2+}]_o$ - and oxygen glucose deprivation-induced neuronal cell death as well as both $[Ca^{2+}]_i$ increase and Ca^{2+} -dependent NO formation. Ischemic insults have reportedly induced $[Ca^{2+}]_i$ increase and formation of NO in neurons [10,12,40,41]. In addition, proanthocyanidin blueberry extract is reported to have reversed dopamine, $A\beta_{42}$, and lipopolysaccharide-induced dysregulation of Ca^{2+} buffering capacity, thereby inducing neuroprotection in hippocampal neurons [20]. These results suggest that proanthocyanidin might inhibit ischemia-induced neuronal cell death by inhibiting glutamate-induced $[Ca^{2+}]_i$ signalling and NO formation as well as antioxidant effects and antiapoptotic effects.

The daily intake of proanthocyanidins may vary from tens to several hundred mg/day depending on diet [42]. Proanthocyanidins, especially oligomeric proanthocyanidins, are more easily absorbed and are present in blood

after oral intake [21,43]. Catechin and epicatechin are reportedly bioavailable to the brain after ingestion of oligomeric proanthocyanidin [43], which suggests that oligomeric proanthocyanidins can cross the blood-brain barrier and affect neuronal cells. In fact, the IH636 grape seed proanthocyanidin extract (GSPE) used in the present study was composed of more than 73% oligomeric polyphenolic compounds including monomeric, dimeric, trimeric, and tetrameric proanthocyanidin [44]. Although the biological efficacy of GSPE has been studied previously in humans [37,44], the bioavailability of GSPE used in the present study remains unknown. However, it should be noted that this particular concentration of grape seed proanthocyanidin extract (GSPE) was less than or equal to the serum concentration in humans following intake of 200 mg/kg proanthocyanidins or oligomeric proanthocyanidins [21]. These data suggest a possibility that IH636 grape seed proanthocyanidin extract (GSPE) can induce neuroprotection after intake of oligomeric proanthocyanidin in humans as well as animals.

Conclusions

The results of the present study showed that IH636 grape seed proanthocyanidin extract protected neuronal cells against the low $[Mg^{2+}]_o$ - and oxygen glucose deprivation-induced neurotoxicity in cultured rat hippocampal neurons. The neuroprotective effects of proanthocyanidin might have been mediated by inhibition of glutamate-induced calcium signalling and NO formation. These results demonstrated that proanthocyanidin, and especially oligomeric polyphenolic compounds, may have future utility as neuroprotective agents or as supplements against glutamate excitotoxicity-related neurologic disorders such as epilepsy, traumatic brain injury, and ischemia.

Methods

Materials

Materials were purchased from the following companies: IH636 grape seed proanthocyanidin extract (GSPE) from InterHealth Nutraceuticals (Benicia, CA, USA); Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) from Invitrogen (Carlsbad, CA, USA); fura-2 acetoxymethyl ester (AM) from Molecular Probes (Eugene, OR, USA); 4,5-diaminofluorescein diacetate (DAF-2DA) from A.G. Scientific (San Diego, CA, USA); N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), (S)-3,5-dihydroxyphenylglycine (DHPG) and all other reagents from Sigma (St. Louis, MO, USA).

Primary rat hippocampal cell culture

Rat hippocampal neurons were grown in primary culture as previously described [45] with minor

modifications. Adult maternal Sprague-Dawley rats (250-300 g) were used in the present study. All experimental procedures performed on the animals were conducted with the approval of the Catholic Ethics Committee of the Catholic University of Korea and were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (revised 1996). Fetuses were removed on embryonic day 17 from maternal rats anesthetized with urethane (1.3 g/kg b.w., i.p.). Hippocampi were dissected and placed in Ca^{2+} - and Mg^{2+} -free Hank's balanced salt solution, pH 7.4. Cells were dissociated by trituration through a 5-ml pipette and then a flame-narrowed Pasteur pipette. Cells were pelleted and resuspended in Dulbecco's modified Eagle's medium (DMEM) without glutamine and supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/ml and 100 μ g/ml, respectively). Dissociated cells were then plated at a density of 50,000 cells/well onto 25-mm-round cover glasses that were coated with poly-L-lysine (0.1 mg/ml) and washed with H_2O . The cells were grown in a humidified atmosphere of 10% CO_2 -90% air (pH 7.4) at 37°C. The medium was replaced 72-90 h after plating with DMEM supplemented with 10% horse serum and penicillin/streptomycin and fed every 7 days by exchange of 25% of the medium. The cells were cultured without mitotic inhibitors for a minimum of 12 days. The cells were used after 14-15 days in culture. During this period, neurons developed extensive neuritic networks, and formed functional synapses.

Digital $[Ca^{2+}]_i$ imaging

To measure $[Ca^{2+}]_i$, hippocampal cells were incubated in 4 μ M fura-2 AM in HEPES-buffered Hank's salt solution (HHSS: 20 mM HEPES, 137 mM NaCl, 1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, 0.4 mM KH_2PO_4 , 0.6 mM $Na_2H_2PO_4$, 3.0 mM $NaHCO_3$, and 5.6 mM glucose) containing 0.5% bovine serum albumin for 45 min at 37°C. The cover glass was then mounted in a flow-through chamber that was superfused at a rate of 1.5 ml/min. Digital calcium imaging was performed as described by Rhie et al. [46]. The chamber containing the fura-2-loaded cells was mounted on the stage of an inverted microscope (Nikon TE300, Tokyo, Japan), and alternately excited at 340 nm and 380 nm by rapidly switching optical filters (10 nm band pass) mounted on a computer-controlled wheel (Lambda 10-2, Sutter Instruments Inc., Novato, CA, USA) placed between a 100 W Xe arc lamp and the epifluorescence port of the microscope. Excitation light was reflected from a dichroic mirror (400 nm for fura-2) through a 20 \times objective (Nikon; N.A. 0.5). Digital fluorescence images (510 nm, 40 nm band-pass) were collected with a computer-controlled, cooled, charge-coupled device camera (1280 \times

1035 binned to 256×207 pixels, Quantix, Photometrics, Tucson, AZ, USA). Image pairs were collected every 2–20 s using an Axon Imaging Work Bench 2.2 (Axon Instruments, Inc., Foster City, CA, USA); exposure to excitation light was 120 ms per image. $[Ca^{2+}]_i$ was calculated from the ratio of the background-subtracted digital images. Cells were delimited by producing a mask that contained pixel values above a certain threshold applied to the 380 nm image. Background images were collected at the beginning of each experiment after removing cells from another area to the coverslip. Autofluorescence from cells not loaded with the dye was less than 5% and thus not corrected. Ratio values were converted to free $[Ca^{2+}]_i$ by the equation $[Ca^{2+}]_i = K_d\beta(R - R_{min})/(R_{max} - R)$, in which R was the 340/380 nm fluorescence emission ratio and $K_d = 224$ nM was the dissociation constant for fura-2. R_{min} , R_{max} , and β was determined in ionomycin-permeabilized cells in calcium-free and saturated solutions ($R_{min} = 0.325$, $R_{max} = 9.23$, $\beta = 7.61$).

$[Ca^{2+}]_i$ measurement using fura-2-based-photometry

$[Ca^{2+}]_i$ spikes were measured using fura-2-based-microfluorimetry [45]. The chamber containing the fura-2-loaded cells was mounted on an inverted microscope (Nikon S-100F, Nikon, Tokyo, Japan). For the excitation of fura-2, light from a 75 W Xe arc lamp (LPS-220, Photon Technology International, NJ, USA) was passed through band-pass filters (340/20 and 380/20 nm, respectively). Excitation light was reflected sequentially from a dichroic mirror (400 nm) through a $40\times$ phase contrast oil immersion objective (Nikon, Tokyo, Japan). Emitted light was reflected through a 510 nm filter to a photomultiplier tube (Model 710, Photon Technology International, NJ, USA) operating in photon-counting mode. Recordings were defined spatially with a rectangular diaphragm (D-104C, Photon Technology International, NJ, USA). $[Ca^{2+}]_i$ spikes were induced by HHSS containing 0.1 mM $MgCl_2$ and 10 μ M glycine. $[Ca^{2+}]_i$ was calibrated by the same method that was used for the digital $[Ca^{2+}]_i$ imaging. R_{min} , R_{max} , and β were 0.86, 14.89, and 7.42, respectively.

Measurement of nitric oxide (NO)

To measure the formation of NO, the cells were incubated in an NO indicator DAF-2DA (20 μ M) in HHSS without BSA for 60 min at 37°C. After DAF-2DA loading, the cells were rinsed with HHSS for 10 min and placed in a flow-through chamber. DAF-2T (the fluorescent triazolofluorescein produced by NO and DAF-2 reaction) images were obtained through excitation at 480 nm and emission at 535 nm/25 nm (DM 505 nm) [47] after treatment with or without GSPE.

Toxicity

For toxicity experiments, cells were plated on microetched coverslips (Belco Biotechnology, Vineland, NJ, USA) and at least 100 neurons were counted. In 0.1 mM Mg^{2+} medium-induced excitotoxicity experiments, coverslips were exposed for 24 h to the 0.1 mM Mg^{2+} medium with or without GSPE at 14 days in culture. After 20–24 h, the same fields of cells were recounted. In oxygen glucose deprivation-induced excitotoxicity experiments [48], cultures were washed 3 times with a balanced salt solution (BSS: 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.0 mM NaH_2PO_4 , 26.2 mM $NaHCO_3$, 1.8 mM $CaCl_2$, and 10 mg/L phenol red) lacking glucose and were aerated with an anaerobic gas mix (95% N_2 /5% CO_2) for 10 min to remove residual oxygen, then were transferred to an anaerobic chamber (1025/1029 Anaerobic System, ThermoForma, Ohio, USA) containing a gas mixture of 5% CO_2 , 10% H_2 , and 85% N_2 for 90 min. To terminate the oxygen glucose deprivation, cells were removed from the anaerobic chamber and then carefully washed with DMEM supplemented with 10% horse serum and penicillin/streptomycin. After 20–24 h, the same fields of cells were recounted.

Viable neurons were identified based on morphological criteria; they were phase-bright, had rounded somata, and extended long fine processes. Cell death was determined by comparing the number of viable neurons before and after treatment [30,49]. Viable neurons obtained were normalized and expressed as a percentage of sham-treated sister cultures (defined as 100%). Control experiments showed that the loss of viable neurons assessed in this manner was proportional to the number of neurons damaged. In control cells (medium exchange only), $28.4 \pm 1.5\%$ of the cells in the 0.1 mM Mg^{2+} experiment (Figure 8) and $27.3 \pm 1.2\%$ of the cells in the OGD experiment died.

Statistical analysis

Data are expressed as the mean \pm SEM. Significance was determined using a Student's *t*-test or one-way analysis of variance (ANOVA) followed by a Bonferroni test.

Lists of Abbreviations

AM: acetoxymethyl ester; AMPA: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; $[Ca^{2+}]_i$: intracellular free Ca^{2+} concentration; DAF-2DA: 4,5-diaminofluorescein diacetate; DHPG: (*RS*)-3,5-Dihydroxyphenylglycine; DMEM: Dulbecco's modified Eagle's medium; EGCG: (-)-epigallocatechine gallate; FBS: fetal bovine serum; GSPE: grape seed proanthocyanidin extract; HHSS: HEPES-buffered Hank's salt solution; IP3: inositol 1,4,5-trisphosphate; $[Mg^{2+}]_o$: extracellular Mg^{2+} concentration; NMDA: N-methyl-D-aspartate;

NO: nitric oxide; **NOS:** nitric oxide synthase; **OGD:** oxygen glucose deprivation; **PLC:** phospholipase C

Acknowledgements

This research was supported by the Biogreen 21 Program (Code #PJ007186), Rural Development Administration, Korea.

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Authors' contributions

SHY designed the study. S-HA, HJK, IJ, YJ H, M-JK, D-JR and SJH performed the experiments and analyses. S-HA, HJK, Y-HJ, and SHY wrote the paper. All authors read and approved the final manuscript.

Received: 12 April 2011 Accepted: 3 August 2011

Published: 3 August 2011

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doi:10.1186/1471-2202-12-78

Cite this article as: Ahn et al.: Grape seed proanthocyanidin extract inhibits glutamate-induced cell death through inhibition of calcium signals and nitric oxide formation in cultured rat hippocampal neurons. *BMC Neuroscience* 2011 **12**:78.

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