Neuroprotective and Neurotrophic Effects of Isorosmanol

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The neurotoxicity induced by beta-amyloid (Aβ), which is one of the major causes of Alzheimer's disease (AD), leads to synaptic loss and subsequent neuronal death. Therefore, modulation of Aβ-induced neurotoxicity, as well as regeneration of damaged synapses could be important therapeutic approaches to control AD. In this study, we found that isorosmanol, an abietane-type diterpene, protected PC12 cells against Aβ-induced toxicity. Furthermore, isorosmanol promoted the generation of neurites. The neurotrophic effect of isorosmanol was enhanced by co-treatment with nerve growth factor (NGF). In addition, the neurite outgrowth induced by isorosmanol was accompanied by F-actin redistribution and increased expression of neurofilaments. Taken together, these results suggest that isorosmanol possesses both neuroprotective and neurotrophic effects, that might be beneficial for controlling AD.

Key words: Isorosmanol, Neurotrophic Effect, Beta-amyloid

Introduction

Beta-amyloid (Aβ), which is a product of the proteolytic cleavage of amyloid precursor protein (APP) by β- and γ-secretases, is one of the major contributors to cognitive decline associated with Alzheimer's disease (AD) (Mattson *et al.*, 2004). Accumulating evidence suggests that neurotoxicity induced by aggregates of Aβ (oligomers) leads to synaptic loss and subsequent neuronal death (Cummings *et al.*, 1998). Further, the degradation of nerve growth factor (NGF), a neurotrophic factor that promotes nerve growth, is increased in AD, eventually leading to neuronal degeneration and synapse loss (Cuello and Bruno, 2007). Therefore, the modulation of Aβ-induced neurotoxicity and of neurotrophic factors has been recognized as an important therapeutic approach for controlling the onset of AD.

In the present study, isorosmanol (Fig. 1A), a diterpene isolated from rosemary by activity-guided fractionation, exhibited a neuroprotective effect against Aβ-induced toxicity in PC12 cells. Interestingly, the neuroprotective effect of isorosmanol was accompanied by a neurotrophic effect in PC12 cells. We found that the neurotrophic effect of isorosmanol was due to the redistribution of F-actin (filamentous actin) and the increase of neurofilaments.

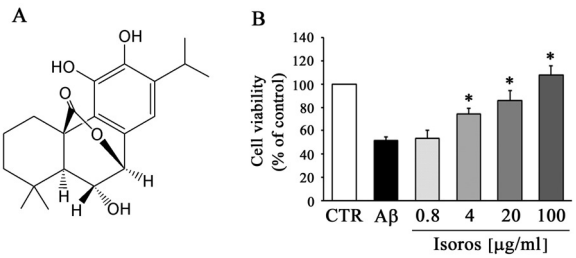

Fig. 1. Isorosmanol protects PC12 cells against Aβ-induced toxicity. (A) Chemical structure of isorosmanol. (B) PC12 cells were incubated with different concentrations of isorosmanol (Isoros; 0.8, 4, 20, and 100 μg/ml) in the presence of Aβ for 24 h; then the cell viability was estimated by the MTT assay. All data shown represent means ± SD. The values were obtained from three independent experiments. * $p < 0.05$, different from Aβ-treated groups.

Results and Discussion

Aβ (25–35) has been reported as an active toxic fragment of Aβ (1–42) (Pike *et al.*, 1991). Furthermore, Aβ (25–35) and Aβ (1–42) have been found to induce similar effects on neuritic atrophy and cell death (Tohda *et al.*, 2006). Therefore, in the present study, Aβ (25–35) was employed as a neurotoxicant, and the neuroprotective effect of isorosmanol against Aβ-induced toxicity was determined by measuring the viability of PC12 cells

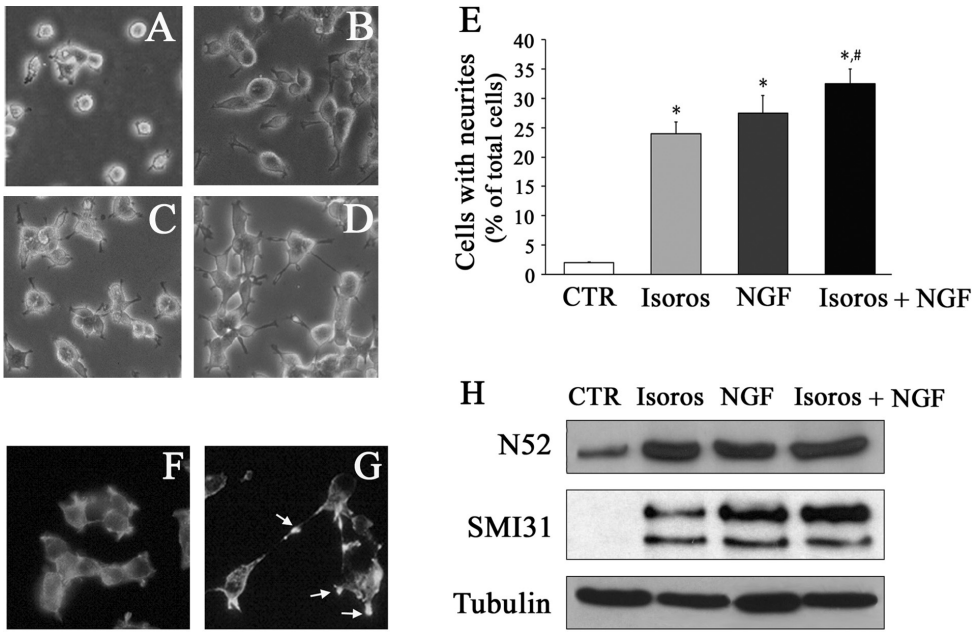

Fig. 2. Isorosmanol-induced neurite outgrowth in PC12 cells. (A–D) PC12 cells treated with (A) DMSO, (B) isorosmanol (Isoros), (C) NGF, or (D) isorosmanol (Isoros) + NGF for 24 h were fixed and examined under an inverted microscope. (E) The number of cells possessing neurites longer than the length of the cell body was counted. All data shown represent means ± SD. The values were obtained from three independent experiments. $^{*}p<0.05$, different from control (CTR) groups; $^{\#}p<0.05$, different from isorosmanol-treated groups. (F, G) The distribution of F-actin in PC12 cells treated with (G) or without isorosmanol (Isoros) (F) was examined under a fluorescence microscope. The arrows indicate the accumulation of F-actin in the growth-cone. (H) The expression of neurofilaments in PC12 cells treated with or without isorosmanol (Isoros) in the presence or absence of NGF was examined by Western blot analysis. All experiments were repeated at least three times.

incubated with Aβ in the absence or presence of isorosmanol using an MTT assay. Treatment with 10 μM Aβ (25–35) induced approximately 50% cell death (Fig. 1B). Treatment with isorosmanol alone did not decrease the cell viability when compared to DMSO-treated controls (data not shown). Conversely, pre-treatment with 4, 20 or 100 μg/ml of isorosmanol significantly inhibited the Aβ-induced toxicity in PC12 cells. Particularly, treatment of PC12 cells with 100 μg/ml isorosmanol completely blocked the Aβ-induced toxicity. These results suggest that isorosmanol can protect PC12 cells from Aβ-induced toxicity in a dose-dependent manner.

In addition to the neuroprotective effect against Aβ-induced toxicity, isorosmanol induced the neurite outgrowth in PC12 cells. PC12 cells in DMSO-treated control groups seldom possessed neurites (Fig. 2A), whereas there was a significant increase in neurite generation in cells treated with isorosmanol (10 μg/ml), NGF (100 ng/ml), or isorosmanol (10 μg/ml) + NGF (100 ng/ml) (Figs. 2B, C, and D, respectively) compared to the controls. As shown in Fig. 2E, the number of cells possessing neurites longer than the length of the cell body was significantly increased in cells treated with isorosmanol, NGF or isorosmanol + NGF compared to DMSO-treated controls. Furthermore, the cells treated with isorosmanol in the presence of NGF possessed significantly more neurites than cells treated with isorosmanol alone. These results suggest that isorosmanol possesses a neurotrophic effect in PC12 cells that is enhanced by co-treatment with NGF.

The neurotrophic effect of isorosmanol was confirmed by evaluating the effect of isorosmanol on F-actin redistribution with phalloidin-FITC conjugates. In DMSO-treated control cells, the nuclei were weakly immunostained with phalloidin-FITC conjugates, whereas fluorescent staining

was more evenly distributed in the cytoplasm and the cell membrane (Fig. 2F). However, treatment with isorosmanol induced concentrated fluorescent staining at the growth-cone, especially to the neurites (Fig. 2G). F-actin is one of the principal components of the cytoskeleton in eukaryotic species. In nerve cells, F-actin concentrates in the growth-cone of the axon. NGF induces F-actin redistribution from the cytoplasm to the growth-cone where it induces neurite growth (Sano and Iwanaga, 1992). Consistent with NGF, isorosmanol induced the F-actin redistribution from the cytoplasm and the cell membrane to the growth-cone or the neurites. These results indicate that isorosmanol can induce neurites in PC12 cells.

The effects of isorosmanol on neurofilaments were examined by Western blot analysis using antibodies for phosphorylated neurofilament H (NF-H) and neurofilament M (NF-M) (SMI31) and total NF-H and NF-M (N52) (Fig. 2H). DMSO-treated control cells showed weak immunoreactivities, whereas treatment with isorosmanol, NGF or isorosmanol + NGF significantly increased the immunoreactivities of phosphorylated NF-H and NF-M, and total NF-H. The immunoreactivity using SMI31 was the highest in cells treated with isorosmanol in the presence of NGF. Previous reports indicated that NGF generates thin dendritic neurites and concomitantly dramatically increases the protein levels of neurofilaments (NFs) in PC12 cells (Yao *et al.*, 2005). In addition, NF-L and NF-M are detectable during initial neurite outgrowth, whereas NF-H is significantly expressed later in differentiation (Lee and Cleveland, 1996). Taken together, these results suggest that isorosmanol induces the generation of neurites by increasing the expression of NFs. However, mechanistic details of neurite outgrowth induced by isorosmanol should be studied further.

In conclusion, our study demonstrates that isorosmanol can protect PC12 cells against Aβ-induced cytotoxicity. In addition, isorosmanol induces neurotrophic outgrowth in PC12 cells by redistributing F-actin from the cytoplasm to the growth-cone and by increasing the levels of NF proteins. The neurotrophic effect of isorosmanol is further enhanced by co-treatment with NGF. Our results suggest that the neurotrophic and neuroprotective effects of isorosmanol could be beneficial for the treatment and control of neurodegenerative diseases such as AD.

Material and Methods

Chemicals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), and *N*,*N*-dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Aβ (25–35) was purchased from Bachem California Inc. (Torrance, CA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and horse serum were obtained from Gibco (Logan, UT, USA). Activity-guided fractionation of methanol extracts from rosemary resulted in the isolation of isorosmanol. Isorosmanol was dissolved in DMSO at 1 mg/ml concentration, and stored at −70 °C until use. Aβ (25–35) used for this study was pre-aggregated prior to use (Park *et al.*, 2008).

Assessing neuroprotective effects of isorosmanol against Aβ in PC12 cells

The ability of isorosmanol to rescue PC12 cells from Aβ (25–35)-induced toxicity was determined by measuring the potential of the cells to reduce MTT to MTT formazan (Park *et al.*, 2008). Briefly, exponentially growing PC12 rat pheochromocytoma cells ($4 \cdot 10^4$ cells per well) were pre-treated with different concentrations of isorosmanol (0.8, 4, 20, and 100 μg/ml) for 1 h, followed by treatment with Aβ (25–35) (10 μM) for an additional 24 h. Then, MTT solution (10 μl per well, 5 mg/ml stock solution in PBS) was added for 3 h at 37 °C. The cells were then lysed in the presence of 100 μl of lysis buffer (10% w/v of SDS in 0.01 M HCl) overnight at 37 °C. Next, the optical density of the resulting solutions was colorimetrically determined at 570 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Determination of neurite outgrowth

PC12 cells plated on collagen-coated cover glasses were incubated with isorosmanol (10 μg/ml), NGF (100 ng/ml), or isorosmanol (10 μg/ml) + NGF (100 ng/ml) for 24 h, after which the cells were fixed with 4% paraformaldehyde (pH 7.4). The number of cells possessing neurites longer than the length of the cell body was counted under a Nikon inverted microscope.

Determination of F-actin redistribution

PC12 cells cultured on collagen-coated cover glasses were incubated with or without isorosmanol (10 μg/ml) for 24 h. Then, the cells were fixed with 4% paraformaldehyde (pH 7.4) and permeabilized with 0.1% Triton X-100. After blocking with 10% bovine serum albumin, the cells were incubated with fluorescent phalloidin-FITC conjugates (1:2000; Sigma) for 2 h at room temperature. After several washes in phosphate buffered saline (PBS), the cells were mounted on glass slides. Pictures were taken under a Nikon fluorescence microscope.

Protein determination, electrophoresis, and immunoblotting

PC12 cells treated with isorosmanol (10 μg/ml) in the presence or absence of NGF (100 ng/ml) for 24 h were scraped into sample buffer and homogenized in a boiling water bath for 10 min. After determination of the protein concentrations by Lowry's method, proteins were separated on SDS-PAGE gels and transferred to Immobilon membranes (Millipore, Billerica, MA, USA). Immunodetection was performed according to Towbin *et al.* (1979). The following antibodies were used: phosphorylated neurofilament-H (NF-H) and neurofilament-M (NF-M) (clone SMI31; 1:2000; Sternberger Monoclonals, Baltimore, MD, USA), phosphorylated and non-phosphorylated NF-H (clone N52; 1:1000; Sigma) and tubulin (1:10,000; Sigma). Secondary anti-mouse IgG antibodies (1:1000; Santa Cruz, Santa Cruz, CA, USA) followed by enhanced chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ, USA) were used for the detection of proteins. Densitometric values were normalized using tubulin as an internal control. Scanning of the Western blots confirmed that the curve was linear in the range used for each antibody.

Data analysis

Data in text and figures are expressed as means ± SD. Two-group comparisons were evaluated with Student's *t*-tests. Differences were considered statistically significant at $p < 0.05$.

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