Protective Effect of ($\pm$) Isoborneol Against 6-OHDA-Induced Apoptosis in SH-SY5Y Cells

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Key Words

Parkinson's disease • Reactive oxygen species • 6-Hydroxydopamine • Mitogen-activated protein (MAP) kinase • Apoptosis

Abstract

Oxidative stress caused by dopamine (DA) may play an important role in the pathogenesis of Parkinson's disease (PD). ($\pm$) Isoborneol is a monoterpenoid alcohol present in the essential oils of numerous medicinal plants and is a known antioxidant. In this study, we investigated the neuroprotective effect of isoborneol against 6-hydroxydopamine (6-OHDA)-induced cell death in human neuroblastoma SH-SY5Y cells. Pretreatment of SH-SY5Y cells with isoborneol significantly reduced 6-OHDA-induced generation of reactive oxygen species (ROS) and 6-OHDA-induced increases in intracellular calcium. Furthermore, apoptosis induced by 6-OHDA was reversed by isoborneol treatment. Isoborneol protected against 6-OHDA-induced increases in caspase-3 activity and

cytochrome C translocation into the cytosol from mitochondria. Isoborneol prevented 6-OHDA from decreasing the Bax/Bcl-2 ratio. We also observed that isoborneol decreased the activation of c-Jun N-terminal kinase and induced activation of protein kinase C (PKC) which had been suppressed by 6-OHDA. Our results indicate that the protective function of isoborneol is dependent upon its antioxidant potential and strongly suggest that isoborneol may be an effective treatment for neurodegenerative diseases associated with oxidative stress.

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Introduction

The pathology of Parkinson's disease (PD) is caused by selective degeneration of nigral dopamine (DA) neurons [1]. While neurodegeneration also occurs in other regions of the brain in certain PD cases, the loss of DA neurons in the substantia nigra pars compacta (SNpc) is

the most pervasive and appears to contribute most significantly to PD symptoms [2, 3]. At present, only symptomatic treatments of PD are of proven efficacy. For example, levodopa (l-dopa) is used to restore dopamine content, but its use leads to long-term pro-oxidant damage. This is problematic, because PD progression is caused by oxidative stress and the generation of free radicals, from both mitochondrial impairment and DA metabolism [4-7]. Oxidative stress may be responsible for dopaminergic neurodegeneration by activating apoptotic cascades [8]. Therefore, regulation of intracellular reactive oxygen species (ROS) and modification of the apoptotic cascade may prevent apoptosis, and may represent a new approach for the prevention and treatment of PD.

For these reasons, therapies to treat PD recommend intake of antioxidants in combination with traditional medications [9, 10]. Because neurodegenerative disorders are mainly caused by oxidative damage, many plants have been tested successfully in both in vivo and in vitro models of PD. Antioxidants derived from plants, such as Ginkgo biloba, Huperzine A, salvanic acid A, and tea catechins, have been shown to have neuroprotective actions [11-15].

Isoborneol is a monoterpene present in the essential oils of numerous medicinal plants, including valerian (*Valeriana officinalis*), chamomile (*Matricaria chamomilla*) and lavender (*Lavandula officinalis*). It is used for analgesia and anaesthesia in traditional Chinese and Japanese medicine. Traditionally, extracts of these plants have been used to treat nervous system symptoms, such as anxiety, restlessness and insomnia [16-19].

The hallmark of PD is a loss of dopaminergic neurons in the substantia nigra, which leads to clinical and pharmacological abnormalities characterizing the disease. 6-OHDA is a selective catecholaminergic neurotoxin that is widely used to study mechanisms of cell death in dopaminergic neurons as well as other types of neurons [20-22]. The human dopaminergic neuronal cell line SH-SY5Y possesses many qualities of substantia nigra neurons [23] and is therefore widely used to study the death of dopaminergic neurons. In this study, we investigated whether the anti-oxidant properties of isoborneol would prevent the neurotoxic effects of 6-OHDA in SH-SY5Y cells. We examined the effects of isoborneol and 6-OHDA on cell viability and apoptosis. The possible mechanisms of isoborneol protection were investigated by measuring intracellular ROS, mitochondrial membrane potential, intracellular calcium concentration and the scavenging effect of isoborneol on the autoxidation of 6-OHDA.

Materials and Methods

Chemicals and Reagents

Dulbecco's modified Eagle's Medium (DMEM), fetal bovine serum (FBS), [4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), penicillin, streptomycin were obtained from Gibco corporation (Grand Island, NY). 6-Hydroxydopamine (6-OHDA), N-acetylcysteine (NAC), D- α -tocopherol (vitamin E), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), Fluo-4 acetoxymethyl ester (Fluo-4-AM), Hoechst 33258, Chelerythrine chloride, phorbol 12-myristate 13-acetate (PMA), isoborneol were purchased from Sigma-Aldrich (St. Louis). Rhodamine 123 (Rho 123), Acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin (Ac-DEVD-AMC) were bought from Calbiochem (San Diego, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis), unless stated otherwise. Elisa kits of Annexin-V-FITC/PI were obtained from BioSea Biotech Co. (Beijing, China).

Cell culture and treatment

The human neuroblastoma cell line SH-SY5Y (obtained from ATCC) was maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% FBS, penicillin/streptomycin (100 U/ml; 100 μ g/ml), and 2 mM L-glutamine, at 37°C in a humidified atmosphere containing 5% CO₂/95% air. For MTT assays, cells were seeded into 96-well culture plates at a density of 4×10^4 cells. For other assays, cells were plated onto 12-well plates at a density of 6×10^5 cells per well.

Cells were treated with different concentrations of 6-OHDA (25-300 μ M) for 24 h to investigate the neurotoxicity of 6-OHDA. Isoborneol (2.5, 5, 10 μ M) was added 1 h before treatment and then incubated with 100 μ M 6-OHDA for 24 h. In order to avoid the reaction of Isoborneol with 6-OHDA present in the medium, the cells were treated with Isoborneol for 1 h, washed three times, and then incubated with 6-OHDA for an additional 24 h in another group of experiments.

Assessment of cell viability

Cell viability was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. SH-SY5Y cells in 96-well plates were incubated with 6-OHDA for 24 h, and then 500 μ g/ml MTT (final concentration) was added to each well and the cells were incubated at 37°C for 2 h. MTT was removed, and the cells were lysed with dimethyl sulfoxide (DMSO). The absorbance was measured using a VictorTM 1420 Multilabel Counter (Wallac, Turku, Finland) at a wavelength of 570 nm. Control cells were treated in the same way with the exclusion of 6-OHDA, and the values of different absorbances were expressed as a percentage of control.

Staining of Nuclear DNA in Apoptotic Cells with Hoechst 33258

Cells exhibiting reduced nuclear size, chromatin condensation, intense fluorescence, and nuclear fragmentation were considered as apoptotic. The above apoptotic structure changes were visualized with the DNA-binding fluorescent dye, Hoechst 33258. After being treated with 6-OHDA and/or

Isoborneol for 24 h, the cells were fixed with 4% paraformaldehyde solution and stained with Hoechst 33258 (2 µg/ml) for 30 min, and then observed using Olympus (IX71) fluorescence microscope and affiliated Image-Pro Express software (Olympus, Japan).

Annexin-V and Propidium Iodide Staining

Quantitative detection of early apoptosis and late apoptosis/necrosis, induced by 6-OHDA, was assessed in SH-SY5Y cells following 24 h exposure to various concentrations of test agents diluted in complete DMEM. Following treatment, cells were stained using the Annexin-V-FITC kit according to the manufacturer's instructions. Briefly, cells were harvested by trypsinization and washed twice ice-cold phosphate-buffered saline (PBS) with a cell concentration of 5×10^5 – 1×10^6 /mL, centrifuged at 120 g, 5 min twice, and resuspended in 200 µL binding buffer. Then 10 µL of 20 µg/ml Annexin-V-FITC and 5 µL propidium iodide (PI) (final concentration 3.5 µM) were added for 15 min in the dark and finally 300 µL binding buffer was added. Samples were then immediately analyzed using FACSCalibur (Becton Dickinson, San Jose, USA) with CELLQuest software (Becton Dickinson). PI was added to samples to distinguish necrotic and late apoptotic events (annexin V–, PI+; annexin V+, PI+) from early apoptotic events (annexin V+, PI–).

Assessment of intracellular ROS production

Intracellular ROS were measured with the ROS-sensitive fluorophore 2',7'-dichlorofluorescein diacetate (DCFH-DA) as previously described [24]. Thirty minutes before the end of the incubation time, cells were treated with 10 µM DCFH-DA (final concentration). After PBS washing, cells were lysed in 1 ml of RIPA buffer and analyzed immediately by fluorescence spectrophotometric analysis at 510 nm. Data were normalized to total protein content.

Measurement of mitochondrial membrane potential (MMP)

Mitochondrial membrane potential was measured by using rhodamine 123, a cationic fluorescent dye, as previously described [25]. After a 24-h incubation in normal medium with or without treatment, the cells were changed to serum-free medium containing 10 µM rhodamine 123 and incubated for 15 min at 37°C. The cells were then collected and the fluorescence intensity was analyzed within 15 min by a spectrofluorimeter (Hitachi F-4500, Japan, 490-nm excitation and 515-nm emission).

Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$)

In order to detect the concentration of intracellular Ca^{2+} , Fluo-4 AM was used as described previously [26]. Cells were collected after 6-OHDA or isoborneol incubation, washed, and resuspended in a standard medium (containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 1.5 mM $CaCl_2$, and 20 mM Hepes (final pH 7.4)), and then Fluo-4 AM (3 mM) was added, which was subsequently incubated for 30 min at 37°C. After washing three times, cells were resuspended in the

standard medium and transferred to a fluorometer cuvette. The fluorescence intensity of Fluo-4 was quantified by a fluorescence spectrophotometer (Hitachi F-4500) with an excitation wavelength of 490 nm and an emission wavelength of 526 nm. $[Ca^{2+}]_i$ was calculated from the Fluo-4 fluorescence intensity using the equation:

$$[Ca^{2+}]_i = K_d (F_0 - F_{min}) / (F_{max} - F_0) \text{ (nmol} \cdot \text{L}^{-1}\text{)}$$

For the purpose of calculation of $[Ca^{2+}]_i$, the K_d was assumed to remain constant between 10 and 25°C, and increased linearly up to 42°C and $K_d = 400 \text{ nmol L}^{-1}$ at 25°C.

The maximal Fluo-4 fluorescence intensity (F_{max}) was determined by adding 0.1% Triton X-100 and the minimal fluorescence (F_{min}) was determined by quenching Fluo-4 fluorescence with 5 mM EGTA. F_0 is the fluorescence measured without adding Triton X-100 or EGTA.

Measurement of Caspase-3 activity assay

Caspase-3 activity was measured with Acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin (Ac-DEVD-AMC) as the substrate. The cell pellets were lysed on ice in 10 mM Tris, 130 mM NaCl, 1% Triton X-100, 10 mM NaH_2PO_4 and 10 mM Na_2HPO_4 , pH 7.5 in the presence of a protease inhibitor cocktail (Calbiochem) for 20 min. Lysates were centrifuged for 30 min 13,600 g at 4°C and the supernatants were removed and assayed for protein contents. Aliquots of cell extracts containing 50 µg of protein were added to the assay buffer (20 mM HEPES at pH 7.5, 10% glycerol and 2 mM DDT) containing 20 µM Ac-DEVD-AMC, as the substrate and incubated in the dark at 37°C for 2 h. AMC liberation was measured using a Victor™ 1420 Multilabel Counter (Wallac, Turku, Finland) with excitation and emission wavelengths 360 nm and 460 nm. The increase in fluorescence was standardized using graded concentrations of free AMC. The results were calculated as release of AMC in pmol/min/mg protein.

Western blot

For detection of cytochrome c, the cell pellets were lysed for 30 min on ice in buffer (20 mM HEPES, pH 7.6, 20% glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 1.7 µg/ml aprotinin, 1.5 µM antipain, 1 µM pepstatin, 0.5 µM phenylmethylsulfonyl fluoride, 1 mM DTT and 0.5% SDS). Lysates were centrifuged for 30 min 13,600 g at 4°C. The supernatants were removed and the pellets were resuspended in buffer and sonicated for 10 s. Both supernatant (cytosol) and pellet were stored at -70°C until protein determination was performed. For detection of other proteins, cells were lysed in 50 mmol/l Tris-HCl, 150 mmol/l NaCl, pH 7.5, buffer containing 1% Nonidet P40, 0.5% sodium deoxycholate, 100 mmol/l NaF, 2 mmol/l Na_3VO_4 , 10 mmol/l phenylmethylsulfonyl fluoride, 500 µmol/l 4-(2-aminoethyl)-benzenesulfonyl fluoride, 150 nmol/l aprotinin, and 1 µmol/l leupeptin. Protein concentrations were measured with the Bradford Protein Assay. Equivalent amounts of protein were electrophoresed in 8% sodium dodecyl sulfate denaturing polyacrylamide slab gels. After transfer to an Immobilon-P Transfer Membrane (Millipore, Bedford, MA) at room temperature, membranes were blocked in Tris-buffered saline with 0.05% Tween 20 (TTBS) containing 5% nonfat dry milk, and incubated for 2 h at room temperature with primary

Fig. 1. Effect of Isoborneol and/or 6-OHDA on SH-SY5Y cell viability. (A) Cells were incubated with increasing concentrations of Isoborneol for 24 h, and cell viability was assessed by MTT assay. (B) Cells were incubated in drug-free medium or medium containing different concentration of 6-OHDA, and cell viability was assessed. (C) Cells were preincubated with increasing concentrations of Isoborneol for 1 h, and then 6-OHDA (100 μM) was added for an additional 24h (co-treatment, black bar) or the cells were preincubated with different concentrations of isoborneol for 1 h and then washed with medium for three times, and 6-OHDA (100 μM) was added for an additional 24 h (pretreatment, light gray bar). (D) SH-SY5Y cells were pre-incubated with different concentrations of vitamin E for 24 h and then concurrently incubated with vitamin E and 100 μM 6-OHDA for 24 h. Data from four independent experiments are presented as means±S.E.M.. P values were calculated by ANOVA test. *P < 0.05, **P < 0.01 vs control cells; #P < 0.05, ###P < 0.01 vs 6-OHDA -treated cells.

antibody against p-JNK, p-ERK, ERK1, cytochrome c, Bcl-2, nNOS, Bax (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Phospho-PKC (pan) (BII Ser660) (Cell Signaling Technology, Inc., Beverly, MA), and incubated for 1 h with secondary antibodies conjugated with peroxidase. The membrane was developed using the enhanced chemiluminescence reagents (Amersham Biosciences) and was exposed to XBT-1 film (Kodak, Shantou, China). Membranes were stripped to prepare them for a second round of probing with β-actin or total mitogen-activated protein (MAP) kinase antibodies. Quantification was performed using the computerized imaging program Quantity One (Bio-Rad).

Spectrophotometric studies of 6-OHDA autoxidation

Toxicity of 6-OHDA is directly correlated to its autoxidation rate [5, 27]. Thus, we monitored spectrophotometrically the autoxidation of 6-OHDA in phosphate buffer (pH 7.4). The autoxidation of 6-OHDA was followed spectrophotometrically by monitoring the formation of

p-quinone at 490 nm or the formation of the corresponding thiol conjugate at 350 nm [27, 28]. The deeper color demonstrates the stronger capacity to remove the H₂O₂ produced in the autoxidation of 6-OHDA. N-acetyl-CySH (NAC), as a positive control, was used in the measurement of the autoxidation of 6-OHDA [27]. A Pharmacia-LKB (Cambridge) spectrophotometer (model Ultrospec III) was used. The cuvette holder was thermostatically maintained at 37°C, and a magnetic stirrer was used for a continuous mixing of the sample. For each assay, 2.5ml of phosphate buffer (pH 7.4) was incubated in a quartz cuvette for 15 min to reach the given temperature. Then, the autoxidation was initiated with addition of 25 ml of stock solution of 6-OHDA at a final concentration of 0.5 mM. The monitoring of the corresponding kinetics was immediately initiated and maintained for the subsequent 3 min. Also, changes in the color of the assayed solutions were followed by direct visual inspection at preselected times (2, 8, 12, 24, 72, 96, 120, 168, 240, 288, and 360 h).

Fig. 2. Effect of Isoborneol on 6-OHDA-induced nucleic morphology changes. Apoptotic nuclei were visualized with 33258 staining. (A) untreated cells; (B) cells exposed to 100 μ M 6-OHDA; (C) 10 μ M Isoborneol for 24 h; or cells were concurrently incubated with 2.5 μ M (D), 5 μ M (E), 10 μ M (F) isoborneol and 100 μ M 6-OHDA. Arrows indicate the apoptotic cells. Scale bar: 50 μ m.

Statistical analysis

Data analysis was completed using the OriginPro 7.5 software (Originlab, Northampton, MA). Data are expressed as mean $\pm$ standard errors of mean (S.E.M.). Differences were analyzed with one-way analysis of variance (ANOVA) followed by post hoc analysis using Bonferroni, and p value <0.05 (*) was considered statistically significant. All experiments were performed a minimum of three times.

Results

Isoborneol dose-dependently protects SH-SY5Y cells from 6-OHDA-induced cell death

6-OHDA induced cell death in a dose-dependent manner (Fig. 1B). The survival rate of SH-SY5Y cells was about 30% following treatment with 100 μ M 6-OHDA for 24 h. Cells treated with 2.5–40 μ M isoborneol for

24 h showed an increased survival rate, whereas higher doses of isoborneol were toxic (Fig. 1A), suggesting that isoborneol has a positive effect on cell viability at low doses. Cells were also pretreated with isoborneol for 1 h, washed, and then treated with 6-OHDA for an additional 24 h. The results (Fig. 1C) showed that isoborneol attenuated the 6-OHDA –induced decrease of cell viability, although the pretreatment effects were reduced compared with the coinubation of isoborneol and 6-OHDA, and this difference in protection between pre-treatment and cotreatment group is not statistically significant, indicating that the antioxidant effect of isoborneol is an effect mainly occurring in the cells. As shown in Fig. 1D, vitamin E, a well-known anti-oxidant [29, 30] dose-dependently protects SH-SY5Y from 6-OHDA-induced decreases in cell viability.

Isoborneol rescues 6-OHDA-induced changes in nuclear morphology

Exposure of SH-SY5Y cells to 100 μ M 6-OHDA for 24 h led to apoptotic nuclei formation. Apoptotic nuclear characteristics were decreased significantly when cells were incubated with 0.1–10 μ M isoborneol and 100 μ M 6-OHDA concurrently (Fig. 2). Isoborneol alone had no significant effect.

Protective effect of isoborneol against 6-OHDA-induced apoptosis of SH-SY5Y cells

To identify morphological changes, we examined the cells using the annexin V and PI double-stain system. The annexin V[−]/PI[−] population were normal healthy cells. Meanwhile annexin V⁺/PI[−] cells were in early apoptosis, and annexin V⁺/PI⁺ cells were in necrosis/late apoptosis. Typical examples are shown in Fig. 3 (A–F). After 24 h of incubation with 6-OHDA, the percentage of early apoptotic cells increased dramatically (Fig. 3H). Isoborneol showed a dose-dependent protective effect, while isoborneol alone had no effect (Fig. 3C). The protective effect of 10 μ M isoborneol is equivalent to that of 100 μ M vitamin E (Fig. 3G).

Isoborneol inhibits the 6-OHDA-induced elevation in intracellular ROS levels

Intracellular ROS levels were determined with DCFH-DA. As shown in Fig. 4A, exposure of SH-SY5Y cells to 100 μ M 6-OHDA for 24 h led to a significant increase in DCF signal compared with the control group. Isoborneol pretreatment inhibited the increase in DCF fluorescence in a concentration-dependent manner in the 2.5–10 μ M range.

Inhibition of 6-OHDA autoxidation by isoborneol

As shown in Fig. 4B, there was a rapid increase in absorbance at 490 nm that followed rectangular hyperbolic kinetics. The initial rate for this process was calculated to be $48.2 \pm 4.7 \times 10^{-3} \Delta A/\text{min}$. The presence of NAC (10 mM) resulted in a relatively slow linear increase in the absorbance at 350 nm ($19.7 \times 10^{-3} \pm 3.26 \times 10^{-4} \Delta A/\text{min}$), and the solution remained colorless for 13 days. The autoxidation of 6-OHDA in the presence of isoborneol (1, 10 or 100 μ M) also proceeded with a slow linear increase in absorbance at 490 nm, with slopes of $20.4 \times 10^{-3} \pm 1.82 \times 10^{-4}$, $17.1 \times 10^{-3} \pm 2.47 \times 10^{-4}$ and $13.8 \times 10^{-3} \pm 3.79 \times 10^{-4} \Delta A/\text{min}$, respectively. The solution with 10 μ M isoborneol developed a bright yellow color after 72 h, which after 8 d changed to a black pigment. In general, this high capacity shown by isoborneol to

Fig. 3. Protective effect of Isoborneol against 6-OHDA-induced apoptosis of SH-SY5Y. SH-SY5Y cells were incubated in drug-free medium (A) or medium containing 100 μ M 6-OHDA (B) or 10 μ M isoborneol (C) for 24 h; or cells were preincubated with 2.5 μ M (D), 5 μ M (E), 10 μ M (F) isoborneol or 100 μ M Vitamin E (G) and then exposed to 100 μ M 6-OHDA for 24 h. The results shown in (H) are the means and SE for three different independent experiments.

remove H₂O₂ may act in biological systems by preventing the Fenton reaction between Fe²⁺ and H₂O₂ to form HO. Thus, the presence of isoborneol appears to represent a very significant protective agent to the oxidative stress derived from the production of H₂O₂ because the reactivity shown by this antioxidant against H₂O₂ is higher than that achieved with other well-known H₂O₂ scavenging substances such as NAC.

Fig. 4. Effect of isoborneol on 6-OHDA induced apoptosis-related events. (A) Isoborneol decreases 6-OHDA-induced elevation in intracellular ROS level. Cells were exposed to 6-OHDA with or without different concentration of Isoborneol for 24 h. ** $P < 0.01$ vs control cell; # $P < 0.05$, ### $P < 0.01$ vs 6-OHDA -treated cells. (B) Isoborneol inhibits the autooxidation of 6-OHDA. The autooxidation of 0.5 mM 6-OHDA was followed spectrophotometrically in the absence (control) and in the presence of 10 mM NAC and the increasing concentration of isoborneol. The wavelengths used were 490 nm for p-quinone and 350 nm for thiol conjugates. Data are expressed as mean $\pm$ S.E.M. for three independent determinations. (C) Isoborneol inhibits 6-OHDA-induced decrease in mitochondrial membrane potential. SH-SY5Y cells exposed to 100 μ M 6-OHDA without or with Isoborneol were incubated with rhodamine 123, and then the fluorescence intensity was recorded. * $P < 0.05$, ** $P < 0.01$ vs control cell; # $P < 0.05$, ### $P < 0.01$ vs 6-OHDA -treated cells. (D) Isoborneol prevents 6-OHDA-induced elevation in $[Ca^{2+}]_i$. Cells exposed to 100 μ M 6-OHDA with or without Isoborneol were incubated with Flu-4 AM, and $[Ca^{2+}]_i$ was measured. Data are expressed as percentage of the untreated control $\pm$ SEM. ** $P < 0.01$, vs control cell; ### $P < 0.01$ vs 6-OHDA -treated cells. (E) Isoborneol attenuates 6-OHDA-induced increase of caspase-3 activity. SH-SY5Y cells were pre-incubated with various concentrations of isoborneol for 1 h before 100 μ M 6-OHDA was added to the medium, and the cells were further incubated for 12 h and 24 h. Caspase-3 activity was fluorometrically measured from the cytosolic fraction using Ac-DEVD-AMC as the substrate. All of the data are expressed as means $\pm$ SEM from six dependent experiments. * $P < 0.05$, *** $P < 0.01$ vs control cell; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.01$ vs 6-OHDA -treated cells at each time points respectively.

Fig. 5. Involvement of PKC in the neuroprotection effect of Isoborneol. (A) SH-SY5Y cells were preincubated without or with 3 μM chelerythrine chloride (CC) for 30 min, and then 100 μM 6-OHDA, isoborneol (2.5, 5, 10 μM), or PMA (100 nM) were added for an additional 24h. Cell viability was measured by MTT assay. * $P < 0.01$, vs control cell; # $P < 0.05$, ## $P < 0.01$ vs 6-OHDA -treated cells. (B) SH-SY5Y cells were stimulated with 100 μM 6-OHDA for indicated times. (C) SH-SY5Y cells were treated with 100 μM 6-OHDA and/or 2.5, 5, 10 μM isoborneol concurrently for 2 h. Western blot analyses with an anti-phospho-PKC (pan) antibody are shown. (D) Results are shown as means±SEM of three independent experiments. * $P < 0.01$, vs control cell; # $P < 0.05$, ## $P < 0.01$ vs 6-OHDA -treated cells.

Isoborneol suppresses the 6-OHDA-induced decrease in mitochondrial membrane potential

Next we determined whether isoborneol could inhibit the 6-OHDA-induced decrease in mitochondrial membrane potential. Our results showed that isoborneol pretreatment inhibits the decrease in membrane potential at 2.5-10 μM in a dose-dependent manner (Fig. 4C). Isoborneol (10 μM) treatment alone elevated the mitochondrial membrane potential compared with control.

Isoborneol prevents the 6-OHDA-induced elevation in $[Ca^{2+}]_i$

To determine whether isoborneol could attenuate the 6-OHDA-induced increase in intracellular calcium

concentration, we used Fluo-4AM to measure intracellular calcium. Exposure of SH-SY5Y cells to 100 μM 6-OHDA for 24 h led to a 2.0 fold increase of $[Ca^{2+}]_i$. Isoborneol (10 μM) alone increased $[Ca^{2+}]_i$ slightly. Isoborneol pretreatment dose-dependently and significantly suppressed $[Ca^{2+}]_i$ elevation (Fig. 4D).

Isoborneol reduces the 6-OHDA-induced increase of caspase-3 activity

Caspase-3 is a key protein regulator of apoptosis [31]. We examined caspase-3 activity in SH-SY5Y cell extracts by monitoring the cleavage of the fluorogenic substrate Ac-DEVD-AMC. 6-OHDA-treated SH-SY5Y cells exhibited a 14-fold and 5.3-fold increase over control

Fig. 6. Effect of isoborneol on 6-OHDA-induced ERK and JNK phosphorylation in SH-SY5Y. (A), (C) SY5Y cells were treated with 100 μ M 6-OHDA for the indicated times. (B), (D) SY5Y cells were exposed to 100 μ M 6-OHDA and/or 2.5, 5, 10 μ M isoborneol for 2h. Cell lysates containing equal amounts of protein were analyzed by Western blot for p-ERK, ERK, p-JNK and JNK. (E)-(F) Results are shown as means $\pm$ SEM of three independent experiments for (B) and (D) respectively. * $P < 0.05$, vs control cell; # <0.05 vs 6-OHDA -treated cells.

at 12 h and 24 h, respectively (Fig. 4E). Isoborneol pretreatment significantly reduced elevated caspase-3 activity both at 12 h and at 24 h in a dose dependent manner. Isoborneol alone caused a minor decrease in caspase-3 activity compared to controls at the two time points.

Involvement of Protein Kinase C (PKC) in the neuroprotective effect of isoborneol

The magnitude of loss of PKC function has been correlated with the severity of oxidative stress damage [32, 33]. Therefore, we investigated whether PKC is involved in the neuroprotective effect of isoborneol against

6-OHDA-induced neurotoxicity. We examined the effect of a general PKC inhibitor, chelerythrine chloride, on the ability of isoborneol to protect SH-SY5Y cells against 6-OHDA-oxidative toxicity. As shown in Fig. 5A, prior exposure to chelerythrine chloride (3 μ M) completely abolished the protective effect, indicating PKC involvement in the neuroprotective action of isoborneol. Furthermore, 30 min pre-treatment with phorbol 12-myristate 13-acetate (PMA) (100 nM), a direct activator of PKC, significantly increased protection against 6-OHDA-induced cell toxicity compared to 6-OHDA treatment alone. This provides further evidence that PKC activation mediates the protective effect. Consistent with

Fig. 7. The effect of isoborneol on the apoptosis-related proteins in 6-OHDA treated SH-SY5Y cells. SH-SY5Y cells were incubated with 2.5, 5, 10 μ M isoborneol for 1 h before 100 μ M 6-OHDA was added to the medium, and the cells were further incubated for 12 h. Then, cells were harvested, and the apoptosis-related proteins were analyzed by western blot with antibody against: (A) cytochrome C (C = cytosol, P = pellet containing nucleus and mitochondria), (B) Bcl-2, (C) and Bax. The results are representative of three independent experiments. Quantification of the data presented in (D), (E), (F) and (G) respectively. Data is expressed as a percentage of untreated control, normalized by the corresponding β -actin level. All of the data are expressed as means $\pm$ SEM from at least three dependent experiments. * $P < 0.05$, ** $P < 0.01$ vs control cell; # <0.05 , ## <0.01 vs 6-OHDA -treated cells.

the above results, PKC phosphorylation, assessed by detection with anti-phospho-PKC (pan) antibody, decreased as early as 1 h after 6-OHDA administration, with a maximal decrease at 2 h (Fig. 5B). Pretreatment of isoborneol abolished the decrease in PKC phosphorylation caused by 6-OHDA, further supporting the involvement of PKC in isoborneol protective action (Fig. 5, C and D).

Effect of isoborneol on 6-OHDA-induced p-ERK regulation

We performed additional experiments to examine involvement of MAPK pathways, which are associated with oxidative stress-induced cell death and cell survival [34, 35] in the neuroprotective action of isoborneol.

6-OHDA induced a significant increase in the immuno-reactivity of phosphorylated JNK and a dramatic decrease in that of phosphorylated ERK at 2 h in SH-SY5Y cells (Fig. 6, A and C). Pretreatment with isoborneol prevented the reduction in ERK1/2 phosphorylation induced by 6-OHDA (Fig. 6B) in a dose-dependent manner. Treatment with 10 μ M isoborneol increased the levels of phosphorylated ERK1/2. Meanwhile, total levels of ERK1/2 (non- and phosphorylated) were not affected by any of the treatments. Interestingly, isoborneol also prevented elevation of phosphorylated JNK by 6-OHDA, suggesting that inhibition of JNK activity is involved in isoborneol-induced protection as shown in Fig. 6D. Isoborneol (10 μ M) alone could partly decrease JNK activity compared to the control.

Effect of isoborneol on apoptosis-related proteins: Bcl-2, Bax and cytochrome C in 6-OHDA-treated SH-SY5Y cells

Cytochrome C can activate the caspases responsible for apoptosis after it is released into the cytosol from mitochondria. As shown in Fig. 7, A, D and E, cytochrome C was released following treatment with 100 μ M 6-OHDA at 12 h. This release was dose-dependently inhibited by isoborneol pretreatment. We examined the role of Bcl-2 and Bax proteins, two major members of the Bcl-2 family of proteins. We showed that 6-OHDA (100 μ M) treatment caused a significant increase in the pro-apoptotic protein, Bax (Fig. 7, C and G), and a dramatic decrease in the levels of the anti-apoptotic protein, Bcl-2 (Fig. 7, B and F). Pretreatment with isoborneol prevented the decrease in Bcl-2 caused by 6-OHDA in a dose-dependent manner. Isoborneol pretreatment inhibited the elevation of Bax caused by 6-OHDA dose dependently in SH-SY5Y cells.

Discussion

PD is characterized by a profound loss of pigmented dopaminergic neurons in the substantia nigra. Even though the cause of PD remains unknown, several lines of evidence strongly suggest the involvement of oxidative stress and mitochondrial dysfunction. Moreover, the autoxidation and enzymatic oxidation of DA leads to the generation of ROS. Therefore, oxidative stress is suggested to play a major role in the neurodegenerative process of PD.

6-OHDA is a potent neurotoxin that causes degeneration of dopaminergic neurons, and several reports showed that 6-OHDA has been used as a selective catecholaminergic neurotoxin to produce cell and animal models of PD [36-39]. Although the precise molecular mechanism of 6-OHDA cytotoxicity remains uncertain, it has often been linked to the production of ROS. In view of the strong ability of 6-OHDA to autoxidize and produce ROS and inhibit complex I of the electron transport [40, 41], it might be expected that reductant reagents could prevent autoxidation and formation of ROS to diminish neurotoxicity of this compound. In this study, our results suggested that isoborneol reduced the damaging effect of 6-OHDA on SH-SY5Y cells. Isoborneol decreases 6-OHDA-induced cell death and ROS production and inhibited the autoxidation rate of 6-OHDA in SH-SY5Y cells. Furthermore, dopamine and 6-OHDA toxicity has been directly correlated to their

autoxidation rate [5, 27]. Based on these findings, we postulate that the anti-oxidative properties of isoborneol may contribute to the protection of SH-SY5Y cells from 6-OHDA-induced ROS.

ROS, induced by 6-OHDA, react with biological target molecules and damage the mitochondrial membrane. The damage eventually results in the loss of mitochondrial membrane potential, leading to release of apoptotic proteins into the cytoplasm and disruption of Ca^{2+} homeostasis, and which stimulates Ca^{2+} -dependent enzymes and triggers apoptosis of cells [42-44]. Consistent with other reports, cells incubated with 6-OHDA showed elevated intracellular Ca^{2+} . Isoborneol (2.5 -10 μ M) prevented 6-OHDA damage to the mitochondrial membrane and attenuated the increase in intracellular Ca^{2+} in a concentration-dependent fashion. Bax and Bcl-2, the two main members of Bcl-2 family, can affect mitochondrial membrane permeability [45]. Bax, a pore-forming, cytoplasmic protein, could translocate to the outer mitochondrial membrane and influences its permeability by inducing cytochrome c release from the intermembrane space of the mitochondria into the cytosol, and subsequently leads to apoptosis. Bcl-2, as an anti-apoptotic protein, is associated with the outer mitochondrial membrane where it stabilizes membrane permeability. Thus, Bcl-2 could preserve mitochondrial integrity, suppress the release of cytochrome c and inhibit apoptosis. Any shift in the balance of pro- and anti-apoptotic proteins would affect the fate of the cell. The Bax/Bcl-2 ratio is believed to be a better predictor of apoptotic fate than the absolute concentrations of either Bax or Bcl-2 alone. In this study, isoborneol can prevent the 6-OHDA-induced increases in the Bax/Bcl-2 ratio, suggesting that isoborneol reduce the apoptosis induced by 6-OHDA, at least in part by regulating Bax and Bcl-2 expression. Cytochrome c release is known to play a critical role in apoptosis [46]. From our observations, increases in cytochrome c release correlate well with an increase in the Bax/Bcl-2 ratio, as pro-apoptotic Bax is thought to be upstream of cytochrome c release in the mitochondria-mediated apoptosis pathway. In this study, our results show that an increase in caspase-3 cleavage correlates well with induction of cytochrome c release. At present, the prospects are favorable for developing an anti-apoptotic compound that modifies PD progression. Our results on the effects of isoborneol resemble those of neuroprotective drugs, such as ladostigil, epigallocatechin-3-gallate, and rasagilin [47-49], which similarly alter Bcl-2 and Bax expression.

It's well known that oxidative stress seems to be one of the major stimuli for the MAPK cascade, which ultimately leads to cell survival/death, and MAPK pathways play a crucial role as transducers of extracellular stimuli into a series of intracellular phosphorylation cascades [50-54]. Moreover, PKC plays a fundamental role in the regulation of cell survival, programmed cell death and neuronal differentiation [32, 56-57]. So a number of intracellular signaling pathways, such as MAPKs, PKC, and phosphatidylinositol-3-kinase-Akt pathways are thought to be involved in antioxidant-promoted neuronal protection [58, 59]. For instance, a rapid loss of neuronal PKC activity was observed after ischemic brain damage [60]. Additionally, the neuroprotective effect of epigallocatechin gallate, a natural antioxidant, against 6-OHDA involves the intracellular signaling mediator PKC [47]. Consistent with this, we also found that isoborneol significantly increased 6-OHDA suppressed PKC activity in SY5Y cells. These actions of isoborneol result in cell protection from 6-OHDA induced apoptosis in SY5Y cells.

In summary, our studies provide evidence that isoborneol-mediated cytoprotection is due, at least in part, to inhibition of the oxidative stress resulting from the mitochondrial apoptotic pathway. As apoptosis and intracellular oxidation of DA play critical roles in the degeneration of DA neurons, our studies using DA neuroblastoma cells suggest that isoborneol may be effective in treating neurodegenerative diseases associated with oxidative stress.

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