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Anti-ischemic and anti-inflammatory activity of (S)-cis-verbenol

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Abstract

(S)-cis-verbenol, a natural metabolite from (-)-alpha-pinene of host pine tree, has been suggested to have anti-ischemic activity. However, the exact mechanism for the anti-ischemic activity of (S)-cis-verbenol remains unclear yet. In the present study, (S)-cis-verbenol reduced cerebral ischemic injury caused by 1.5-h middle cerebral artery occlusion followed by 24-h reperfusion. Furthermore, (S)-cis-verbenol significantly prevented neuronal cell death caused by oxygen-glucose deprivation (OGD, 1 h) and subsequent re-oxygenation (5 h). While (S)-cis-verbenol did not inhibit the NMDA-stimulated calcium influx, it reduced the intracellular level of reactive oxygen species (ROS) elevated by OGD/re-oxygenation. ORAC assay indicated that (S)-cis-verbenol potently eliminated peroxyl radicals. In DPPH and DHR123 fluorescence assays, however, (S)-cis-verbenol did not show a direct ROS scavenging effect. Furthermore, (S)-cis-verbenol reduced the expression levels of pro-inflammatory cytokines in ischemic brain and immunostimulated glial cells. The present results indicate that (S)-cis-verbenol may be a useful therapeutic agent due to its anti-oxidative and anti-inflammatory activities.

Keywords: *(S)-cis-verbenol, ischemia, OGD/re-oxygenation, ROS, peroxyl radicals, inflammation*

Introduction

(S)-cis-verbenol (Figure 1) is a natural metabolite to which bark beetles transformed from (-)-alpha-pinene of host pine tree. Essential oils containing (S)-cis-verbenol have been reported to have biological activity such as antimicrobial and anti-fungicidal [1–3]. Recently, (S)-cis-verbenol was reported to reduce ischemia/hypoxia-induced cell death in neuroblastoma SH-SY5Y cells [4]. However, the exact anti-ischemic/anti-hypoxic mechanism of (S)-cis-verbenol remains unclear.

During cerebral ischemia, neuronal cells are injured via various mechanisms. In the early period of cerebral ischemia, excitoxicity and oxidative stress are major factors injuring neuronal cells [5]. Especially, brain tissue is very vulnerable to oxidative stress, because it contains a large amount of unsaturated fatty acids that can be oxidized by oxygen free radicals [6]. Thus, many researchers have endeavoured to develop antioxidants for the treatment of cerebral ischemia [7]. One of the well known anti-oxidants, trolox, attenuated cerebral ischemic injury through reducing lipid peroxidation [8,9]. At the late period of cerebral ischemia, brain tissue is further damaged by inflammatory responses. Activation of glial cells, especially microglia, in the brain and infiltration of peripheral inflammatory cells such as macrophages and lymphocytes have been associated with delayed damage of the brain [10,11]. Activated inflammatory cells injure the brain tissue by production of inflammatory cytokines as well as reactive oxygen or nitrogen species [11–13].

In the present study, therefore, we investigated the cytoprotective mechanisms of (S)-cis-verbenol in *in vivo* and *in vitro* ischemia models. Here, we first demonstrate that (S)-cis-verbenol has strong anti-oxidant

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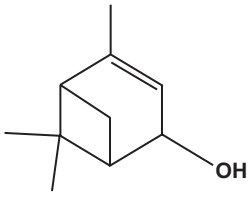

Figure 1. Structure of (S)-cis-verbenol.

activity, especially against peroxyl radicals, and ameliorates inflammatory responses.

Materials and methods

Materials

Minimum essential medium eagle (MEM), Dulbecco's modified Eagle's media (DMEM) and Ham's F12 medium were purchased from WelGENE Incorporation (Seoul, Korea). Foetal bovine serum (FBS) was purchased from Thermo Fisher Scientific Inc. (Hyclon Load Logan, UT). bis-[1,3-diethylthio-barbiturate]-trimethineoxonol ($DiBAC_4(3)$), fura2-AM, dihydrorhodamine 123 (DHR123) and 2,7-dichlorofluorescein (H_2DCF-DA) were purchased from Molecular Probes (Eugene, OR). (S)-cis-verbenol, triphenyltetrazolium chloride (TTC), 4-aminophenazone, 2,2-diphenyl-1- picrylhydrazyl (DPPH), 3-morpholinosydnonimine (SIN-1), hydrogen peroxide, catalase, SOD, fluorescein, 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), trolox, randomly methylated β-cyclodextrin (RMCD), lipopolysaccharide (LPS) and vitamin C were purchased from Sigma Chemical Co (St. Louis, MO). IFN-γ was purchased from Calbiochem (Darmstadt, Germany). TNF-α and IL-1β ELISA kit were purchased from Invitrogen Corp. (Carlsbad, CA). The antibodies of IL-1β and TNF-α were purchased from Abbiotec (San Diego, CA).

Animals

Male Sprague-Dawley (SD) rats were purchased from Charles River Laboratories (Seoul, Korea) and housed under conditions of controlled temperature (23 ± 1°C) and humidity (55 ± 2%). Animals had access to rat chow and water *ad libitum* and 220–230 g SD rats were used for middle cerebral artery occlusion (MCAO)/reperfusion experiments. All experimental procedures using animals were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Committee of Korea University College of Medicine.

Focal cerebral ischemia and drug treatment

For induction of cerebral ischemia, rats were initially anaesthetized with 3% isoflurane in a 70% N_2O and 30% O_2 (v/v) mixture via facemask. Anaesthesia was maintained with 2% isoflurane. A rectal temperature probe was introduced and a heating pad maintained the body temperature at 37°C during the whole surgery period. Focal cerebral ischemia was achieved by right-sided endovascular MCAO, as we described in a previous report [10]. After 1.5 h of MCAO, the suture was pulled back and the animal was allowed to recover. (S)-cis-verbenol (100 mg/kg), dissolved in saline solution containing DMSO (5%)/cremophor (10%), was injected intraperitoneally 2 h after starting MCAO (i.e. 30 min after starting reperfusion).

Measurement of infarct volume

Rats were anaesthetized with chloral hydrate and decapitated at 1 day after MCAO. Rat brains were cut into coronal slices of 2 mm in thickness using a rat brain matrix (Ted Pella, Redding, CA). The brain slices were then incubated in 2% triphenyltetrazolium chloride (TTC) at 37°C for 30 min to reveal the ischemic infarction. After TTC reaction, the brain slices were fixed with 4% paraformaldehyde (pH 7.4) in 0.1 M phosphate buffer (PB) for 1 day and subsequently cryoprotected in PB containing 30% sucrose at 4°C for 2 days. The cross-sectional area of infarction between the bregma levels of +4 μm (anterior) and −6 μm (posterior) were determined with a computer-assisted image analysis program (OPTIMAS 5.1, BioScan Ins.). On each slice, brain infarct size was measured manually by outlining the margins of infarct areas, and the infarct volume was calculated according to the slice thickness of 2 mm per section. Each side of the brain slices was measured separately and mean values were calculated. The total volume of infarction was determined by integrating six chosen sections and expressed as a percentage of the total brain volume. Because post-ischemic brain oedema will increase brain volume in the infracted area, the corrected infarct volumes were calculated to compensate for brain oedema, as previously described by Yang et al. [14]. Thereafter the tissues were frozen and cut into 10 or 30 μm coronal sections on a cryostat (Leica 3050, Leica, Germany) and stored at −20°C.

Measurement of rectal temperature

Rats were anaesthetized and maintained with a mixture of 2% isoflurane and nitrous oxide/oxygen (7:3). Rectal temperature was measured using a temperature controller system (TR-100; FST, Germany). Body temperature was measured at 30 min before peritoneal injection of saline or (S)-cis-verbenol (100 mg/kg) and then at 30 min intervals for 1.5 h. After each measurement, rats had free access to food and water.

Immunohistochemistry

Immunohistochemical reaction was performed using ABC methods. The sections were treated with 0.3% hydroperoxide in PBS (pH 7.3) for 30 min and then incubated in 10% normal horse serum-supplemented PBS for 30 min. The sections were then incubated at room temperature with primary antibodies in PBS containing 0.3% triton X-100 and 1% normal horse serum overnight. Primary antibodies were used at the following dilutions: rabbit anti-IL-1β (1:200, Abbiotec, San Diego, CA); rabbit anti-TNF-α (1:200, Abbiotec). After washing three times for 10 min each with PBS, sections were incubated sequentially with biotinylated anti-mouse IgG, anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and then with peroxidase-conjugated streptavidin (Vector Laboratories), diluted 1:200 in the same solution as the primary antiserum. To establish the specificity of each primary antibody, control sections were similarly treated in the absence of primary antibodies. The antigens were visualized by 5 min of incubation in 0.05 M Tris-HCl buffer containing 0.02% 3,3-diaminobenzidine and 0.0045% hydrogen peroxide at 37°C. Finally, all sections were mounted on slides coated with gelatin solution, dehydrated, coverslipped, and then analysed under a bright-field microscope (Olympus).

Cell cultures

Cortical neuronal cells were prepared from embryonic 17–18 days old foetal Sprague–Dawley rats. In brief, meninges-free brain tissues were dissociated by triturating through a Pasteur pipette. Neuronal cells (5×10^5 cells/ml) were added onto the culture plates, pre-coated with poly-D-lysine (100 μg/ml)/laminin (4 μg/ml) and maintained in 10% heat-inactivated FBS/10% Ham's F12 medium-supplemented Dulbecco's modified Eagle's medium (DMEM) in a humidified 95% air/5% CO_2 atmosphere at 37°C. Cells were replaced with growth media every 4 days during culture days.

Mixed glial cells were prepared from the pre-frontal cortices of to 1 day-old Sprague-Dawley rat pups. Meninges-free cerebral tissues were dissected and softly triturated through a Pasteur pipette. Cells were then plated onto a poly-*D*-lysine (1 μg/ml)-coated 75-cm^2 T-flask and kept for 1 week in Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated FBS and penicillin/streptomycin (100 μg/ml). Detached glial cells by trypsinization were washed and then re-plated (1×10^4 cells/ml) in the growth medium onto poly-D-lysine (10 μg/ml)-coated 24-well plates. Cells were used for the experiments 7 days later.

Oxygen-glucose deprivation/re-oxygenation

For *in vitro* hypoxic-ischemic insult, cells were placed in an anaerobic chamber (partial pressure of oxygen <2 mmHg) and the culture medium was replaced with a glucose-free DMEM bubbled with an anaerobic gas mix (95% N_2, 5% CO_2) for 30 min to remove residual oxygen. Cells were left in an anaerobic chamber at 37°C for 1 h to induce oxygen deprivation. Control cells, not exposed to OGD, were maintained in glucose (25 mM)-containing DMEM aerated with an aerobic gas mix (95% air, 5% CO_2). OGD was stopped by replacing with oxygenated DMEM supplemented containing 25 mM glucose and returning the cells to the incubator under normoxic conditions. Cells were treated with (S)-cis-verbenol at 30 min before/during OGD/reoxygenation.

N-methyl-D-aspartate-induced excitotoxicity

At 16–18 days after neuronal culture, cells were exposed to NMDA (100 μM, 10 min) in nominally Mg^{2+}-free Earle's balanced salt solution (EBSS) containing 1.8 μM $CaCl_2$ and 10 μM glycine. After NMDA exposure, cells were washed with EBSS and then maintained in glucose (25 mM)-containing DMEM in the incubator of 37°C, 5% CO_2. At times indicated elsewhere, aliquots were collected to determine the activity of lactate dehydrogenase (LDH), a cell death or injury marker. All drugs were treated at 30 min before/during NMDA treatment.

Immunostimulation

Glial cells were immunostimulated by the treatment with lipopolysaccharides (LPS, 1 μg/ml) and interferon-gamma (IFN-γ, 100 U/ml) for 48 h. (S)-cis-Verbenol was pre-treated for 30 min before immunostimulation at all experiments.

Assessment of cell injury or death

Cell injury or death was assessed by morphological examination using phase-contrast microscopy (DM IL, Leica, Germany) and quantified by measuring the amount of lactate dehydrogenase (LDH) released into the bathing medium. LDH activity was measured using a diagnostic kit (Sigma Chemical Co., St. Louis, MO). Cell viability was expressed as a percentage of total LDH, which was measured in sister cultures frozen and thawed after the experiments.

Measurement of plasma membrane potential

Changes in the membrane potential of neurons were monitored with the fluorescent dye $DiBAC_4(3)$ (bis-[1,3-diethyl-thio-barbiturate]-trimethineoxonol), which is a lipophilic anion distributed across the membrane. Thus, the increase of bisoxonol fluorescence

indicates that the membrane has been depolarized, allowing more of this negatively charged dye to enter the cells [15]. Plated neurons were pre-incubated with 1 μM $DiBAC_4(3)$ for 20 min and then fluorescence intensity (Ex 540 nm, Em 565 nm) was time-dependently measured after NMDA exposure at a fluorescence plate reader (SpectraMAX GeminiEM, Molecular Device, CA). All drugs were treated 30 min before NMDA treatment.

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

$[Ca^{2+}]_i$ was measured fluorometrically according to the fura-2 method described by Lim et al. [16]. In brief, the plated cortical neurons on round coverslips (diameter: 13 mm) in culture plates were incubated with 1 μM fura-2AM for 30 min at 37°C and then washed three times with HBSS buffer. A coverslip was diagonally placed inside the cuvette and the bathing medium was stirred with a teflon-coated magnetic bar to allow adequate mixing of added drugs. In initial studies, inclusion of 2.5 mM probenecid blocked leakage of the fluorescent dye (<2% leakage over 3 h) in both control and drug-treated groups. The fluorescence (Em. 510 nm, Ex. 340 nm for Ca^{2+}-bound form and Ex. 380 nm for Ca^{2+}-free form) was measured with a spectrofluorophotometer (RF-5301PC, Shimadzu, Tokyo, Japan). The amount of intracellular calcium were calculated by using computer operated software (D 2060) according to the formula: $[Ca^{2+}]_i = K_d(R - R_{min})/(R_{max} - R) \times S_{f2}/S_{b2}$, where R is the 340/380 wavelength ratio of the sample, R_{min} is the ratio obtained with fura-2 in the absence of Ca^{2+} and S_{f2}/S_{b2} is the ratio of fluorescence values at 380 nm for free and Ca^{2+}-bound dye. Constants were determined for each coverslip with 0.1% Triton-X100 for R_{max} and 40 mM EGTA for R_{min}. A K_d value of 224 nM was used in this study [17]. All drugs were treated at 30 min before/during NMDA treatment.

Intracellular ROS production: DCF fluorescence

2,7-dihydrodichlorofluorescein diacetate (H_2DCF-DA) diffuses and then changes to non-fluorescent form dichlorofluorescein (DCFH) by intracellular esterases. Free radicals (hydrogen peroxide, hydroxyl radical, peroxyl radical and peroxynitrite) effectively convert DCFH to the highly fluorescent form dichlorofluorescein (DCF) [18,19]. In brief, 30 μM H_2DCF-DA was treated for 10 min in EBSS buffer containing 0.1% BSA and 2.5 mM probenecid. After washing out H_2DCF-DA supernatant, fluorescence was measured at appropriate time points. Wavelengths of excitation and emission were 488 and 525 nm, respectively. DCF fluorescence was measured by using a fluorescence microscope (DM IL HC Fluo, Leica, Germany) equipped with a digital camera (DFC420C, Leica, Germany). Three different cultures were used in this study and groups of confluent cells were randomly selected from the image for each sample. The program of image analyser (TOMORO ScopeEye 3.5, Seoul, Korea) was used for quantification of fluorescence.

ORAC assay

The oxygen radical absorbance capacity (ORAC) assay was modified according to the method of Huang et al. [20]. AAPH (60 mM) and fluorescein (50 nM) were used as a peroxyl radical generator and a fluorescent probe, respectively. In the ORAC assay, based on hydrogen atom transfer (HAT) reaction, antioxidant reacts to peroxyl radical competitively with fluorescin. All reagents were prepared at 75 mM phosphate buffer (pH 7.4), with the exception of sample or trolox standards, which were made in 7% RMCD solvent (acetone:water = 1:1). RMCD was used to enhance the solubility of lipophilic samples [20]. The wells of a 96-well plate were sequentially filled with fluorescein solution (66 nM, 190 μl) and 30 μl of (S)-cis-verbenol (blank or standards) and then shaken for 5 s. AAPH (500 mM, 30 μl) was quickly added to each well with a multi-channel pipette, finally making the total volume amount to 250 μL. The decreasing fluorescence was measured every 5 min for 3 h after pre-incubation of the plate at 37°C for 10 min. All ORAC analyses were performed on a fluorescence microplate reader at 37°C with an excitation wavelength of 485 nm and an emission wavelength of 530 nm (SpectraMAX GeminiEM, Molecular Device, CA). with the area-under-the-curve (AUC) approach for quantification of scavenging capacity to peroxyl radical, net AUC was calculated from kinetic curves of different concentrations. $AUC = (0.5 + f_5/f_0 + f_{10}/f_0 + f_{15}/f_0 + \ldots + f_{110}/f_0 + f_{115}/f_0 + f_{120}/f_0) \times 5$, where f_0 is the initial fluorescence reading at 0 min and time i. The net AUC of the sample was calculated by subtracting the AUC of the blank.

DPPH assay

The stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used to analyse the scavenging capacity of (S)-cis-verbenol [21]. DPPH free radical gives a strong absorption maximum at 517 nm, which transformed purple colour to yellow. In brief, (S)-cis-verbenol (100, 50, 25, 10, 1 and 0.1 μM in ethanol) were mixed with DPPH solution (23.6 μg/ml in ethanol) and then incubated for 30 min at 37°C in the dark. The absorbance was measured by an ELISA microplate reader (SPECTRAmax 340PC, Molecular Devices, CA). The scavenging activities were expressed as a percentage of DPPH•SCAVENGED vs

Table I. Conditions for PCR of target genes

Gene	Primer sequence	No. cycles	Product size	Accession no.
TGF-β1	For 5'-CTG AGT GGC TGT CTT TTG AC-3' Rev 5'-TTG CGA CCC ACG TAG TAG AC-3'	26	540	NM_021578
IL-1β	For 5'-GTG TGG ATC CCA AAC AAT AC-3' Rev 5'-CCA TAC ACA CGG ACA ACT AG-3'	26	555	NM_031512
IL-6	For 5'-TGT TCT CAG GGA GAT CTT GG-3' Rev 5'-TCT GAC CAC AGT GAG GAA TG-3'	28	517	NM_012589
TNF-α	For 5'-CAC GCT CTT CTG TCT ACT GA-3' Rev 5'-GGA CTC CGT GAT GTC TAA GT-3'	27	541	AJ002278
GAPDH	For 5'-ACT CCC TCA AGA TTG TCA GC-3' Rev 5'-CAT ACT TGG CAG GTT TCT CC-3'	25	342	NM_017008

TGF-β1, Transforming growth factor-beta1; IL-1β, Interlukin-1 beta; IL-6, Interlukin-6; TNF-α, Tumor necrosis factor-alpha; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. For, forward; Rev, reverse.

concentration of the standard anti-oxidant (100 μM of Vitamin C).

DHR123 fluorescence

The non-fluorescent Dihydrorhodamine 123 (DHR123) was oxidized to rhodamine123 (RH123) by free radicals such as hydrogen peroxide, peroxynitrite and hypochlorous acid. In the non-cell system, to measure direct scavenge activity, 3-morpholinosydnonimine (SIN-1; 200 μM) or hydrogen peroxide (H_2O_2; 1 mM) was added into PBS buffer containing DHR123 (10 μM) in the absence and presence of (S)-cis-verbenol. After 10 min incubation of DHR123

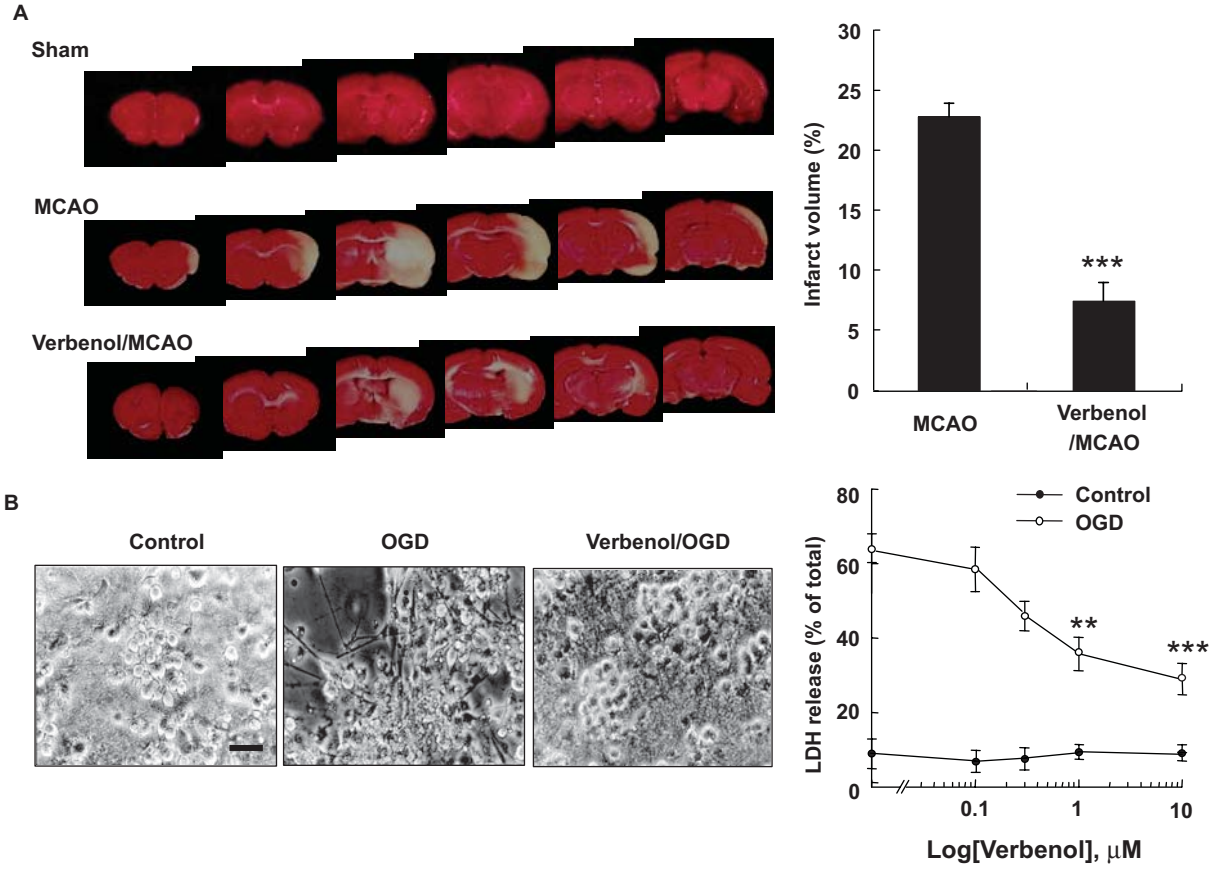

Figure 2. Protection by (S)-cis-verbenol in ischemic/hypoxic model. (A) For focal cerebral ischemia, middle cerebral artery was occluded for 1.5 h and reperfused for 24 h. (S)-cis-verbenol was treated intraperitoneally 2 h after starting MCA occlusion, as described in Materials and methods. Representative TTC-stained coronal brain sections and quantification of infarct volumes. Each bar represents the mean ± SD of 10 rats. ***$p < 0.001$. (B) Cortical neuronal cells were exposed to oxygen-glucose deprivation (OGD, 1 h) and subsequent re-oxygenation (5 h). (S)-cis-verbenol (0.1–10 μM) were pre-treated for 30 min before exposure of OGD/re-oxygenation. Neuronal death was assessed by measuring the activity of LDH released from injured cells into culture medium. Each bar represents mean ± SD from five independent experiments. **$p < 0.01$, ***$p < 0.001$: significantly different from the OGD/reoxygenation group.

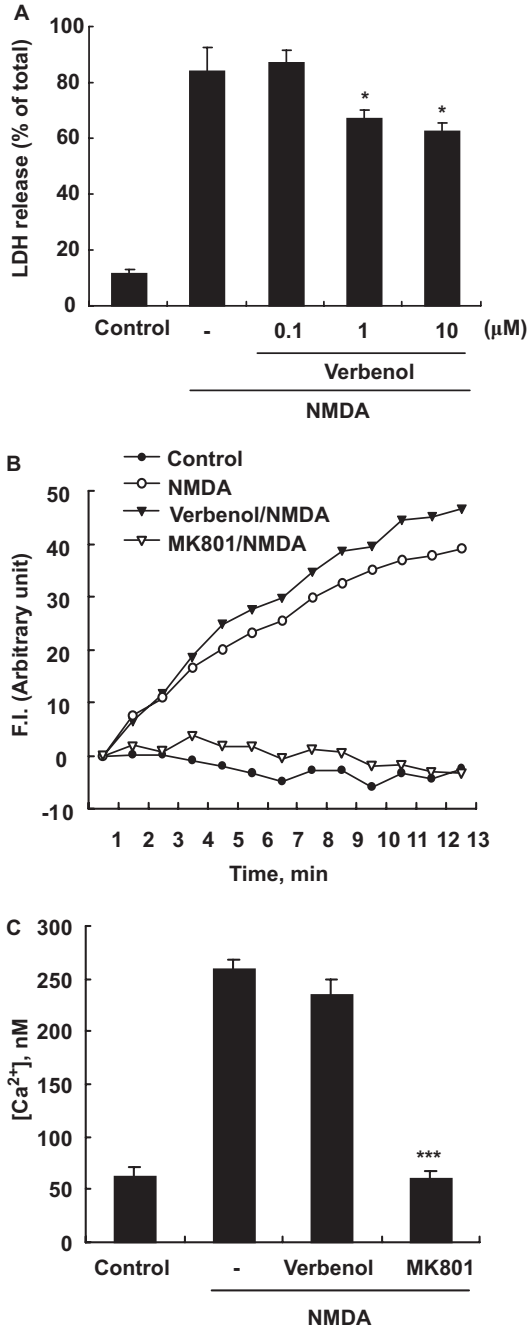

Figure 3. Effect of (S)-cis-verbenol in excitotoxicity. Cortical neurons were treated with NMDA (100 μM) for 10 min and then incubated for 5 h. (A) Supernatants were taken at 5 h for LDH assay. (B) Plasma membrane potential was measured by using a potential-sensitive fluorescent dye $DiBAC_3(4)$. $n = 4$. (C) Intracellular Calcium level was measured by a calcium-sensitive fluorescent dye Fura2-AM. Each bar represents mean ± SD of peak calcium levels obtained from four independent experiments. $*p < 0.05$, $***p < 0.001$: significantly different from the NMDA-treated group.

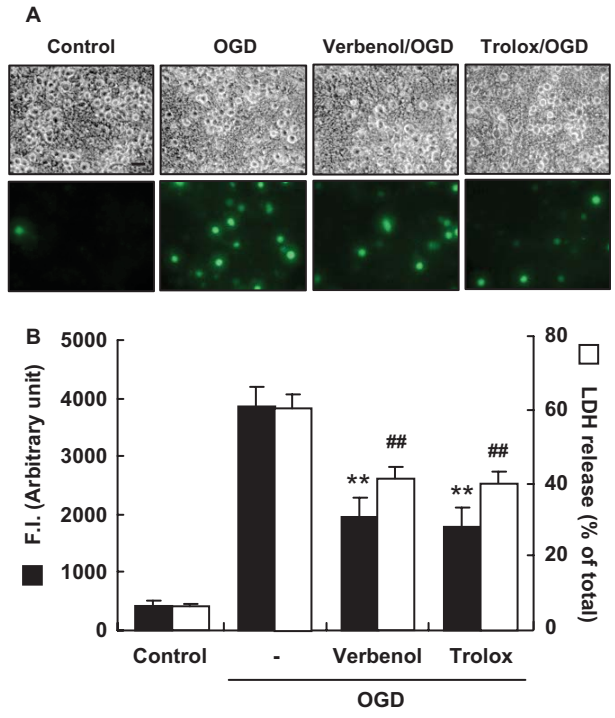

Figure 4. Anti-oxidant effect of (S)-cis-verbenol. Cortical neurons were exposed to OGD (1 h) followed by re-oxygenation. H_2DCF-DA was loaded 1 h after starting reoxygenation and then DCF fluorescence was determined at 3 h after starting reoxygenation. (S)-cis-verbenol (10 μM) or trolox (25 μM) was pre-treated for 30 min before OGD. (A) Representative microphotographs. Scale bar = 50 μm. (B) Quantification of DCF fluorescence intensities (F.I.) and LDH release after OGD (1 h)/reoxygenation (3 h). $**p < 0.01$ and $^{\#\#}p < 0.01$: significantly different from the OGD/reoxygenation group.

at room temperature, RH123 fluorescence (Ex 490 nm and Em 530 nm) was detected by using a fluorescence microplate reader (SpectraMAX GeminiEM, Molecular Device, CA). To distinguish the type of free radicals, we added catalase (50 Unit/ml) and SOD (50 Unit/ml) for 10 min before SIN-1 or H_2O_2 treatment.

Reverse transcription-PCR

Total RNA was extracted from cultured primary cells with a RNeasy mini kit from Qiagen (Hilden, Germany). For RT reaction, total RNA (1 μg) was reverse transcribed in a reaction mixture containing 1 U RNase inhibitor, 500 ng random primers, 3 mM $MgCl_2$, 0.5 mM dNTP, 1 X RT buffer, and 1 U reverse transcriptase (Qiagen, Hilden, Germany). The synthesized cDNA was used as a template for PCR reaction using a HotStar Taq Master Mix kit (Qiagen, Hilden, Germany) and primers for target genes. Single-band PCR products were separated by electrophoresis through 1% agarose gels. Table I presents the primers sequences, cycles, product sizes and accession numbers of target genes such as GAPDH, IL-1β, IL-6, TNF-α and TGF-β1. PCR was performed as follows; denaturation at 95°C for 15 min; then denaturation at 94°C for 50 s, annealing at 57°C for 50 s and extension at 72°C for 60 s for each cycle of target genes; finally additional extension at 72°C for 10 min.

Enzyme-linked immunosorbent assay (ELISA)

Glial cells were treated with lipopolysaccharide (LPS: 1 μg/ml)/interferon-γ (IFN-γ: 100 U/ml) for 18 h and then supernatants were collected in immunostimulated glial cells. The concentrations of IL-1β and TNF-α were measured by enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies and the procedure recommended by the supplier (BD Bioscience, CA).

Statistical analysis

Data were expressed as mean and standard deviation (SD) and analysed for statistical significance using repeated measures of ANOVA or two-way ANOVA method.

Results

First, we determined physiological parameters, as we described before [10]. (S)-cis-verbenol did not itself change physiological parameters including mean arterial pressure, pH, arterial partial CO_2 and O_2 pressures and blood glucose concentration (data not shown). Treatment of (S)-cis-verbenol once at 2 h after starting MCA occlusion reduced 67.2 ± 5.89% MCAO/reperfusion-evoked brain injury (Figure 2A). Some drugs are known to reduce the infarct volume due to their hypothermic effects [22,23]. However, we found that (S)-cis-verbenol did not decrease the rectal temperature (data not shown).

We further tested whether (S)-cis-verbenol could inhibit neuronal cell death in an *in vitro* OGD/re-oxygenation model. LDH measurement and morphological examination showed that neuronal cells

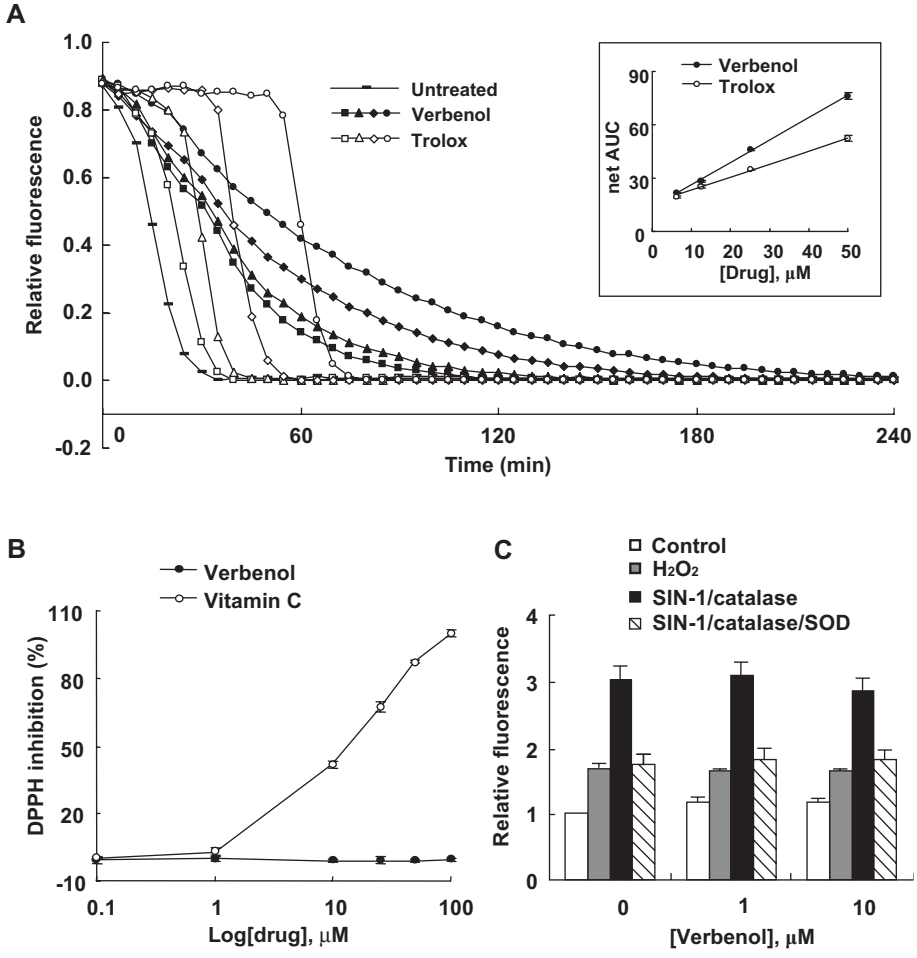

Figure 5. Scavenging effect of (S)-cis-verbenol to free radicals. (A) ORAC assay. AAPH-induced fluorescence decay curve in the presence of (S)-cis-verbenol or trolox at different concentrations (□, ■ = 6.25 μM; ▲, △ = 12.5 μM; ◊, ◆ = 25 μM; ○, ● = 50 μM). Plots are representatives of four separate experiments. Inset presents best-fit lines between net AUC and different concentrations of (S)-cis-verbenol or trolox. The net AUC = $AUC_{sample} - AUC_{blank}$. The linear coefficients (r^2) for (S)-cis-verbenol and trolox are 0.9987 and 0.9981, respectively. Data represent mean ± SD. $n = 4$. (B) DPPH reduction assay. Each bar represents mean ± SD of four independent experiments. (C) DHR123 oxidation. (S)-cis-verbenol did not scavenge hydrogen peroxide and peroxynitrite donated from SIN-1. Fluorescence intensity (F.I.) was corrected by subtracting with autofluorescence (i.e. fluorescence of cells which is not loaded with DHR123). The fluorescence intensity of control group that was not treated with any drug was assigned to 1.0. Each bar represents mean ± SD of three independent experiments.

are significantly injured by the experimental condition used here (i.e. OGD (1 h)/re-oxygenation (5 h)) (Figure 2B). (S)-cis-verbenol prevented the ischemia-induced neuronal injury/death in a concentration-dependent manner (Figure 2B). In general, glial cells are not vulnerable to OGD/re-oxygenation [16,24]. Similarly, we also found that a significant LDH release was not observed in cultured mixed glial cells under the present experimental condition (data not shown).

Our further study showed that, although (S)-cis-verbenol slightly reduced NMDA (100 μM)-evoked neuronal death (Figure 3A) it did not inhibit the NMDA-induced plasma membrane depolarization (Figure 3B) and intracellular calcium uptake (Figure 3C). Thus, the neuroprotective effect of (S)-cis-verbenol on NMDA receptor-mediated excitotoxicity may not be mediated by direct inhibition of NMDA receptor-coupled channel activity.

In addition to excitoxicity, oxidative stress is another main cause of neuronal cell death in the initial period of ischemia [5]. A fluorescence dye H_2DCF-DA can specifically interact with ROS including peroxyl radical, peroxynitrite or hydrogen peroxide [25]. Thus, we further determined the level of ROS by using H_2DCF-DA. Like a well-known antioxidant trolox, (S)-cis-verbenol significantly decreased the DCF fluorescence and inhibited LDH release (Figure 4).

In general, anti-oxidant capacities can be determined by using different types of chemical reactions: hydrogen atom transfer-based assay (i.e. ORAC assay) and single electron transfer-based assay (i.e. DPPH assay) (For a review of antioxidant capacity assays, see Huang et al. [26].) In ORAC assay, (S)-cis-verbenol significantly delayed the decay rate of AAPH-induced fluorescin fluorescence (Figure 5A). However, many anti-oxidants that react with peroxyl radicals may react slowly with or even be inert to DPPH radicals [26]. In the present study, we found that (S)-cis-verbenol did not react with DPPH radicals (Figure 5B). In addition, (S)-cis-verbenol did not reduce the fluorescence intensity of DHR123 increased by H_2O_2 or SIN-1 that produces superoxide and nitric oxide and then quickly generates peroxynitrite [25] (Figure 5C). The results indicate that (S)-cis-verbenol directly scavenge peroxyl radicals, not H_2O_2 or peroxynitrite.

In the delayed stage of ischemic stroke, inflammatory cells are infiltrating into the ischemic lesion and damage the brain tissue via over-production of inflammatory mediators including pro-inflammatory cytokines [5,10]. Thus, we further examined whether (S)-cis-verbenol could modulate the production of inflammatory cytokines. Immunohistochemical studies showed that MCAO/reperfusion highly increased the protein expression levels of IL-1β and TNF-α. However, (S)-cis-verbenol significantly reduced their expressions (Figure 6). Furthermore, (S)-cis-verbenol

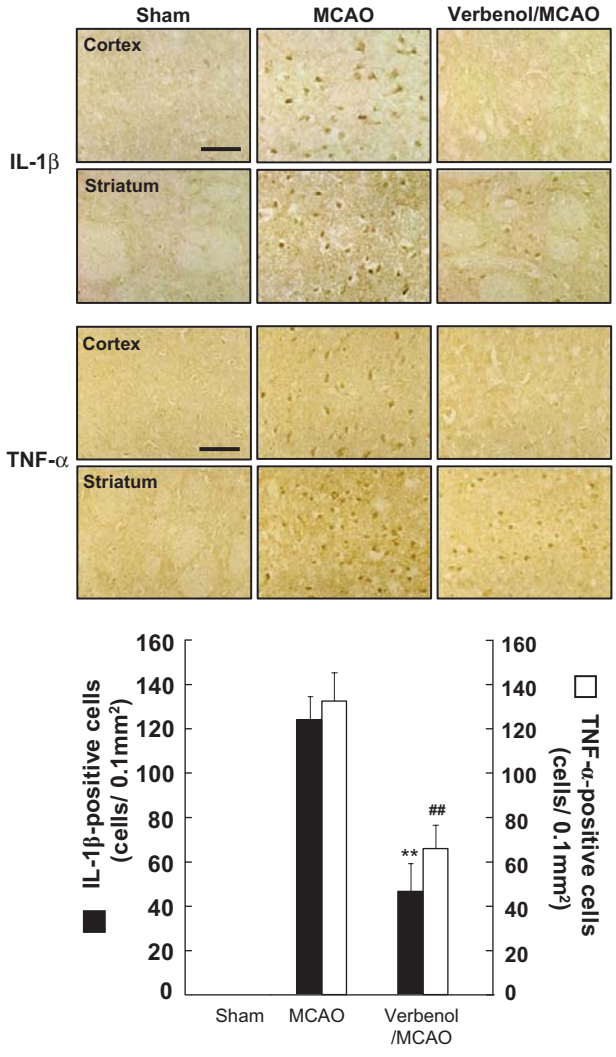

Figure 6. Effects of (S)-cis-verbenol on expressions of IL-1β and TNF-α in MCAO/reperfusion-evoked brain injury. (S)-cis-verbenol (100 mg/kg) was injected at 30 min after starting reperfusion. Brain slices were stained with anti-IL-1β or anti-TNF-α antibodies. Scale bar = 50 μm. For quantitative analysis, IL-1β- and TNF-α-positive cells were counted at three random-selected regions per independent experiment. Each bar represents mean ± SD of five independent experiments. $^{**}p < 0.01$, $^{\#\#}p < 0.01$, significantly different from MCAO/reperfusion group. $n = 5$.

significantly decreased the expression of mRNA and proteins of pro-inflammatory cytokines such as IL-1β and TNF-α in mixed glial cells stimulated by LPS and IFN-γ for 48 h (Figure 7). In contrast, however, the mRNA expression of TGF-β, a well-known anti-inflammatory cytokine, was not changed by (S)-cis-verbenol (Figure 7).

Discussion

Previously, (S)-cis-verbenol was reported to inhibit OGD/re-oxygenation-induced toxicity in cultured neuroblastoma SH-SY5Y cells [4]. However, little has been known of the exact cytoprotective mechanism of (S)-cis-verbenol. In the present study,

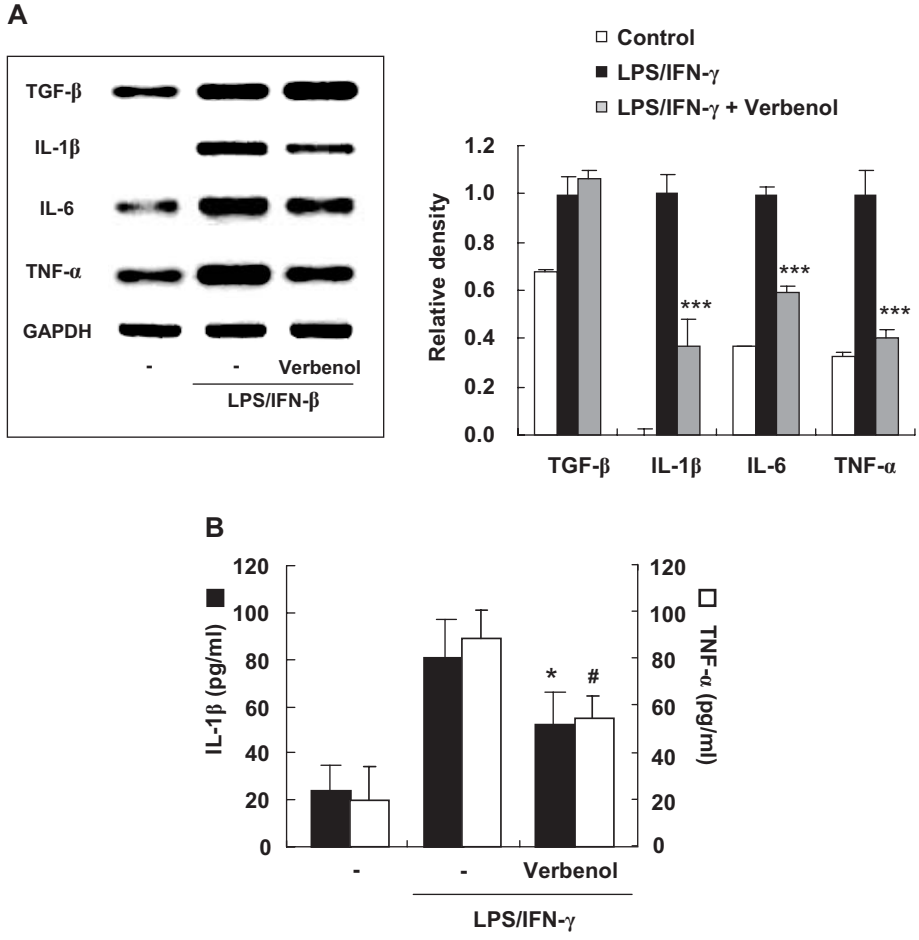

Figure 7. Effects of (S)-cis-verbenol on expressions of inflammatory cytokines in immunostimulated glia. Glial cells were treated with LPS/IFN-γ for 48 h. (A) RT-PCR. (S)-cis-verbenol (10 μM) decreased mRNA expressions of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) with little change of the expression of anti-inflammatory cytokine TGF-β. For quantification, the mRNA expression level was normalized with respect to GAPDH and then expressed as relative fold changes in comparison to appropriate LPS/IFN-γ-treated groups, which were assigned to 1.0. Data represent mean ± SD. $n = 4$. ***$p < 0.001$; significantly different from the LPS/IFN-γ-treated group. (B) Protein levels of IL-1β and TNF-α. (S)-cis-verbenol (10 μM) decreased pro-inflammatory cytokines (IL-1β and TNF-α). Data represent mean ± SD. $n = 5$. *$p < 0.05$; significantly different from the IL-1β (or TNF-α)-treated group.

we first show that (S)-cis-verbenol decreases the infarct volume in a transient ischemic rat model. Also, we demonstrate that (S)-cis-verbenol alleviates oxidative stress in OGD/re-oxygenation-treated neuronal cells and reduces the production of pro-inflammatory cytokines in ischemic brain lesions and immunostimulated glial cells.

The mechanisms responsible for the neuronal cell death caused by OGD/reoxygenation are mainly excitotoxicity and radical burst [27–29]. Although (S)-cis-verbenol significantly decreased NMDA receptor-mediated neuronal death, the cytoprotective effect was not greater than that observed in the OGD/re-oxygenation model. Also, the NMDA-induced plasma membrane depolarization and calcium influx were not blocked by (S)-cis-verbenol, indicating that (S)-cis-verbenol has a NMDA receptor-independent cytoprotective mechanism.

In respect of oxidative stress, (S)-cis-verbenol significantly decreased the level of intracellular ROS elevated by OGD/re-oxygenation, to a similar degree obtained with trolox. Trolox was previously reported to protect the brain in focal or global ischemic models through reducing lipid-peroxidation [8,9]. A simple decrease of intracellular ROS level does not provide direct evidence of cause or result of cytoprotection by (S)-cis-verbenol against oxidative stress. The present study, however, manifests that (S)-cis-verbenol has a direct scavenging capacity against AAPH-generated peroxyl radicals. As assessed by measuring net AUC in an ORAC assay, (S)-cis-verbenol presents a more potent antioxidative capacity (ORAC value is ~1.7 TE), in comparison with those of vitamin C (0.9 TE) [30] and vitamin E (0.5 TE) [20]. The decay curve of fluorescin fluorescence illustrates the different shapes between (S)-cis-verbenol- and trolox-treated groups (Figure 4A). Previously, the decay curves obtained with reduced glutathione (GSH) and melatonin also did not show an initial lagging period [31,32].

Compared with trolox, although (S)-cis-verbenol slowly reacted with peroxyl radicals, the prolonged activity might increase its anti-oxidant capacity against peroxyl radicals.

In the delayed stage of ischemic stroke, neuroinflammation plays critical roles in ischemic injury [5,10]. We found that MCAO/reperfusion increased the number of amoeboid cells in the entire ipsilateral hemisphere including the cortex and striatum shown in Figure 6. Most of those amoeboid cells are thought to be macrophages/monocytes recruited from the periphery and some activated microglia to be morphologically transformed into amoeboid form, as we described before [10]. In response to neuronal injury, macrophages/monocytes and microglia become activated and secrete inflammation mediators such as cytokines as well as the potential cytotoxic mediator NO and ROS [11–13]. (S)-cis-verbenol largely reduced the number of amoeboid cells as well as the proinflammatory cytokines expression levels of those cells in ischemic brain lesions. Furthermore, we found that (S)-cis-verbenol also suppressed RNA/protein expressions of proinflammatory cytokines in immunostimulated glial cells. Further studies are needed to delineate how (S)-cis-verbenol modulates RNA/protein expressions of proinflammatory cytokines.

In the present study, our results indicate that (S)-cis-verbenol may be a useful compound for the treatment of cerebral ischemic injury. Further understanding the cytoprotective mechanism of (S)-cis-verbenol and its chemical derivatization may provide new therapeutic agents for the treatment of neurodegenerative diseases caused by oxidative stress and/or inflammation.

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