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Propofol Prevents Amyloid-β-Induced Neurotoxicity through Suppression of Cytosolic Ca^{2+} and MAPK Signaling Pathway in SH-SY5Y Cells

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Abstract: Alzheimer's disease (AD) is one of the most common causes of dementia, characterized by the accumulation of amyloid-β (Aβ) peptide deposits in the brain. Within an aging society, elderly patients with preoperative dementia, or those who are affected by postoperative cognitive impairment, are a major health problem. Although inhalation anesthetics induce accumulation of Aβ protein and progression of AD, propofol, a short-acting intravenous anesthetic, has gained increasing attention for its neuroprotective effects following cerebral ischemia. However, the protective action of propofol against Aβ-induced neuronal damage remains unclear. Therefore, the aim of this study was to elucidate the mechanisms underlying the protective effect of propofol against Aβ-induced neurotoxicity. Neural damage was induced in human neuroblastoma cells (SH-SY5Y) using 2.5 μM Aβ(1−42). Cells were pretreated with propofol (1 μM) for 1 h, followed by further treatment with propofol for 20 h in combination with Aβ. In Aβ(1−42)-induced neural damage, caspase-3 activation was increased, as was phosphorylation of p38 mitogen-activated protein kinase (MAPK) and tau. Moreover, cell viability and the phosphorylation of Akt, cAMP response element-binding protein, and Bcl-2 decreased significantly with Aβ treatment. However, these responses were reversed by pretreatment with propofol and p38MAPK inhibitor. The Aβ(1−42)-induced increase in reactive oxygen species generation was inhibited by propofol pretreatment, but remained unchanged following pretreatment with the p38MAPK inhibitor. Furthermore, Aβ(1−42)-treated cells exhibited a significant increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$), but propofol pretreatment resulted in a significant decrease in $[Ca^{2+}]_i$ starting 30 s after exposure to Aβ(1−42). Our results indicate that the mechanism underlying the protective effect of propofol against Aβ-induced neurotoxicity is a decrease in $[Ca^{2+}]_i$, which subsequently suppresses oxidative stress, along with p38MAPK and tau phosphorylation. Thus, these findings suggest that propofol, at clinically relevant concentrations, is likely to be safe in elderly patients and in those with risk factors for AD.

Key words: amyloid-β, propofol, Alzheimer's disease, Ca^{2+}, mitogen-activated protein kinase (MAPK)

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Introduction

Dementia is a major medical problem worldwide, markedly reducing quality of life. Indeed, dementia is a disease accompanied by a variety of debilitating symptoms, including learning disabilities, memory loss, and poor judgment. Because dementia is a tremendous burden to a patient's family and caregivers, it can also be considered a serious problem at the societal level. Due to increasing life expectancies, there is a larger population of the elderly worldwide, with increasing numbers of dementia patients. Alzheimer's disease (AD) is the most common type of dementia and is the most common neurodegenerative disease among the elderly. AD causes many severe cognitive disorders involving memory, learning, and orientation impairment. AD is also characterized by the accumulation of amyloid-β (Aβ) peptide deposits and intraneuronal neurofibrillary tangles (NFTs) in the brain[1].

In an aging society, the number of elderly patients requiring general anesthesia is increasing. However, surgery and anesthesia in elderly patients have often been reported to cause cognitive disorders, including AD, as well as to reduce postoperative cognitive function, which is a serious health problem[2].

Inhaled anesthetic agents, such as halothane and isoflurane, have been reported to trigger Aβ aggregation, which promotes AD progression and the formation of amyloid plaques[3]. However, long-term treatment with the anesthetic propofol (2,6-diisopropylphenol) in aged mice has been reported to reduce Aβ production[4]. Furthermore, propofol treatment in humans[5], aged wild-type mice, or AD transgenic mice is shown to result in improved cognitive function[6]. Propofol is a widely used short-acting intravenous anesthetic agent that actives γ-aminobutyric acid $(GABA)_A$ receptors and inhibits *N*-methyl-D-aspartate (NMDA) receptors[7]. In addition to maintaining sedation as an anesthetic agent, propofol has antioxidative[8], antiemetic, antianxiety, and neuroprotective effects[9]. Furthermore, it has been reported that the cytoprotective action of propofol involves selective inhibition of Ca^{2+} influx through L-type calcium channels in smooth muscle cells[10] and the inhibition of Ca^{2+} release from intracellular stores into the cytoplasm[11].

One of the common factors underlying AD pathogenesis is the dysregulation of neuronal intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$). Aβ-induced increases in $[Ca^{2+}]_i$ lead to mitochondrial Ca^{2+} overload, the generation of reactive oxygen species (ROS), and increased activity of caspase and mitochondrial proteins, such as cytochrome *c*, that stimulate apoptosis, and have been shown to be associated with cell death and neurodegeneration[12].

The mitogen-activated protein kinase (MAPK) signaling pathway is known to be involved in the etiology of various neurodegenerative diseases, such as AD, Parkinson's disease, and amyotrophic lateral sclerosis[13]. Phosphorylation of p38MAPK, an MAPK that responds to stress stimuli, has been observed postmortem in the brains of early stage AD patients (Braak staging IV–V)[14]. Furthermore, p38MAPK activation has been reported to play various roles in AD pathology, such as mitochondrial dysfunction, apoptosis, tau phosphorylation, and synaptic dysfunction[15]. Therefore, inhibitors of p38MAPK may be a promising strategy for AD therapy. Indeed, propofol has been shown to reduce H_2O_2-induced neurotoxicity by inhibiting the

p38MAPK signaling pathway in PC12 cells[16].

However, the association of p38MAPK with the protective mechanism of action of propofol against Aβ-induced neurotoxicity has not been investigated. Therefore, the aims of the present study were to elucidate the mechanism underlying the protective effects of propofol against Aβ-induced neurotoxicity in SH-SY5Y cells and to evaluate propofol as a possible alternative anesthetic for elderly or early AD patients.

Materials and methods

Drugs and reagents

Aβ(1-42) (human) was purchased from Peptide Institute (Osaka, Japan). Monomeric Aβ (1-42) was incubated for 24 h at 37°C to allow self-aggregation and oligomerization before treatment. Propofol (2,6-diisopropylphenol), all-*trans*-retinoic acid (ATRA), and Dulbecco's modified Eagle's medium (DMEM) / Ham's F-12 were purchased from Wako (Osaka, Japan). Fetal bovine serum (FBS) and inhibitors of p38MAPK (SB202190), c-Jun N-terminal kinase (JNK ; SP600125), and mitogen-activated protein kinase kinase (MEK) 1 / 2 (PD98059) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Penicillin G sodium, streptomycin sulfate, and amphotericin B were obtained from Life Technologies (Camarillo, CA, USA). All other chemicals used were the purest grade available commercially.

Cell culture and drug treatment

SH-SY5Y cells (human neuroblastoma, EC-94030304) were obtained from The European Collection of Authenticated Cell Cultures (London, UK). SH-SY5Y cells were cultured in DMEM / Ham's F-12 containing 10% FBS and antibiotic-antimycotic solution and maintained under a humidified atmosphere of 5% CO_2 and 95% air at 37°C. SH-SY5Y cells are comparable to neurons in their morphological and neurochemical properties, and have been widely used to evaluate neuronal injury or death in neurodegenerative diseases, cerebral ischemia-reperfusion, and epilepsy[17]. To evaluate the effects of Aβ, SH-SY5Y cells were first differentiated with ATRA. Differentiated cells were subsequently cultured with or without (control) Aβ for 20 h at 37°C.

In the present study, differentiated SH-SY5Y cells were pretreated with propofol (1, 2, or 5 μM) for 1 h before cotreatment with 2.5 μM Aβ for a further 20 h. In some experiments, differentiated cells were incubated with MAPK inhibitors (10 μM) for 15 min prior to treatment with propofol. All treatments were performed under sterile conditions.

Cell viability assay

Cell viability was measured based on the formation of blue formazan, which is metabolized from colorless 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) by mitochondrial dehydrogenases that are active only in live cells. A preliminary experiment was conducted in which SH-SY5Y cells were treated with Aβ (1, 2.5, 5, or 10 μM), and 2.5 μM Aβ was found to be a suitable concentration for inducing cytotoxicity in SH-SY5Y cells (Fig. 1A).

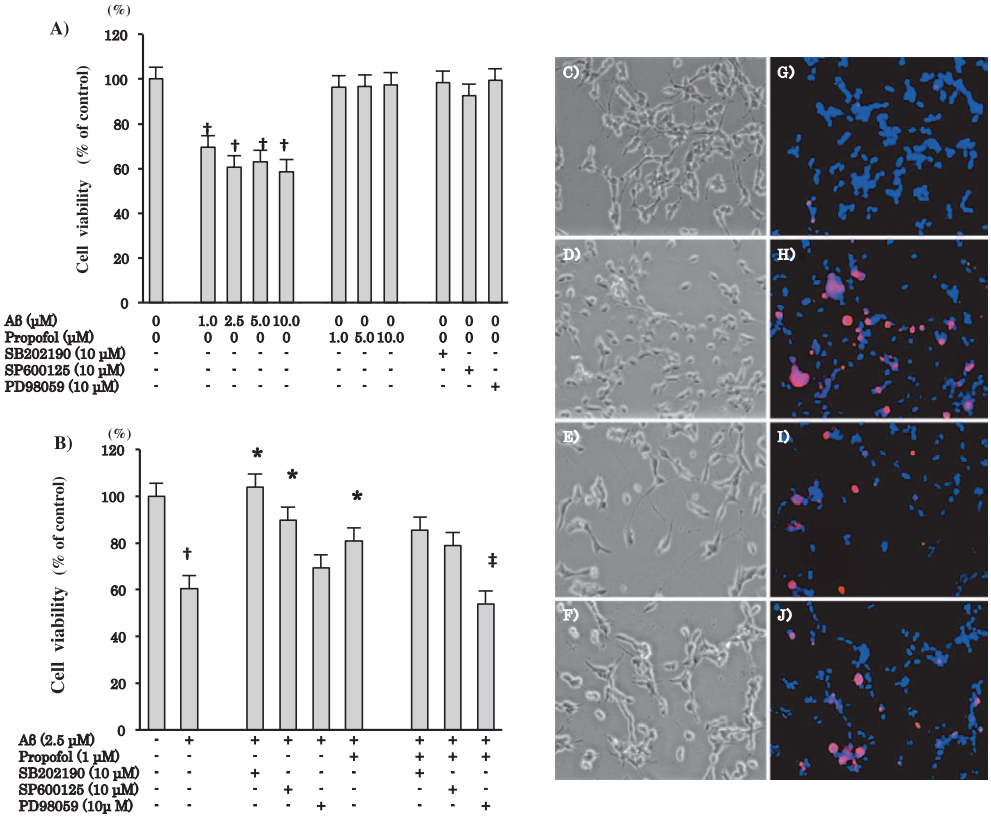

Fig. 1. Effects of propofol on cell viability and Annexin V and Hoechst 33342 staining in Aβ-stimulated SH-SY5Y cells. Cell viability was evaluated using the MTT assay and Annexin V and Hoechst 33342 staining. (A) Cell viability of SH-SY5Y cells after 20 h exposure to Aβ (1–10 μM) and propofol (1, 5 or 10 μM). (B) Effects of 2.5 μM Aβ and 1 μM propofol, in the presence or absence of inhibitors of p38MAPK (SB202190), JNK (SP600125), or MEK1/2 (PD98059), on the viability of SH-SY5Y cells. Data are the mean ± SEM. $^{\dagger}p < 0.05$ compared with control cells; $^{*}p < 0.05$ compared with 2.5 μM Aβ-treated cells; $^{\ddagger}p < 0.05$ compared with 2.5 μM Aβ + 1 μM propofol-treated cells. (C–J) Phase-contrast (C–F) and fluorescence (G–J) microscopy images of untreated SH-SY5Y cells (C, G) and SH-SY5Y cells treated with 2.5 μM Aβ (D, H), 2.5 μM Aβ + 1 μM propofol (E, I), and 2.5 μM Aβ + 1 μM propofol + p38MAPK inhibitor (F, J).

To study the protective effects of propofol against Aβ-induced cytotoxicity, SH-SY5Y cells were pretreated with 1 μM propofol for 1 h, followed by treatment with Aβ + propofol for 20 h. After incubation, the supernatant was removed and the formation of the formazan was measured at 540 nm using Cell Proliferation Kit I (Roche Diagnostics, Mannheim, Germany) and a microplate reader (Spectra Max i3; Molecular Devices, San Jose, CA, USA).

Staining with Annexin V and Hoechst 33342

SH-SY5Y cells cultured in six-well plates were treated with 2.5 μM Aβ and 1 μM propofol for 20 h, after which they were stained using the DNA dye Hoechst 33342 (Wako) to visualize nuclear morphology. Stained cells were then washed in phosphate-buffered saline (PBS), and

the specific binding of Annexin V-Cy3 (Annexin V-Cy3 Apoptosis Detection Kit; Medical & Biological Laboratories, Nagoya, Japan) was investigated by incubating the cells for 5 min at room temperature in binding buffer containing Annexin V. The Annexin V-Cy3 Apoptosis Detection Kit detects the distribution of phosphatidylserine in the outer monolayer of cell membranes, which is present in the early stages of apoptosis, using fluorescence emitted from specific Cy3-labeled Annexin V. After 20 h incubation with Aβ, cells were stained according to the manufacturer's instructions and examined under a fluorescence microscope (DIAPHOT TMD 300; Nikon, Tokyo, Japan). When SH-SY5Y cells were viewed under the fluorescence microscope, staining of apoptotic cells in the early stages was observed.

Caspase-3 activity

Caspase-3 activity was determined fluorometrically using appropriate synthetic peptide substrates provided by Kamiya Biomedical Company (Seattle, WA, USA). SH-SY5Y cells were incubated with or without 1 μM propofol for 1 h, followed by treatment with 2.5 μM Aβ + propofol for 20 h. After incubation, cells were rinsed with cold PBS and resuspended in chilled cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA), incubated for 10 min on ice, and then centrifuged at 10,000 × *g* for 3 min at 4°C. The supernatant was then added to reaction buffer containing 10 μM dithiothreitol (DTT; Medical & Biological Laboratories, Aichi, Japan) and respective specific peptide substrates, and incubated at 37°C for 60 min. The substrate for caspase-3 (Kamiya Biochemical Company) was Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (DEVD-AFC). AFC released by the enzymatic reaction was measured spectrophotometrically at an excitation wavelength of 405 nm and an emission wavelength of 505 nm using a Spectra Max i3 (Molecular Devices).

MAPK phosphorylation

The role of MAPK in Aβ-induced neurotoxicity in SH-SY5Y cells was examined by investigating MAPK phosphorylation. The phosphorylation of p38MAPK, JNK, and ERK1/2 was determined using cell-based p38MAPK (Thr^{180}/Tyr^{182}), JNK (Thr^{183}/Tyr^{185}), and ERK1/2 (Thr^{202}/Tyr^{204}) ELISA kits (Ray Biotech, Norcross, GA, USA), respectively. SH-SY5Y cells were incubated with or without 1 μM propofol for 1 h, followed by treatment with Aβ + propofol for 30 min. Cells were then fixed and blocked according to the manufacturer's instructions, and then incubated for 2 h with anti-phosphorylated (p)-p38MAPK (Thr^{180}/Tyr^{182}) or anti-p38MAPK (primary antibody), anti-p-JNK (Thr^{183}/Tyr^{185}) or anti-JNK (primary antibody), and anti-p-ERK1/2 (Thr^{202}/Tyr^{204}) or anti-ERK1/2 (primary antibody), respectively. Then, the cells were washed with washing buffer in the assay kit again, after which horseradish peroxidase (HRP)-conjugated anti-mouse IgG (secondary antibody) was added. The cells were washed with washing buffer in the assay kit again and 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate solution was added, resulting in the development of color in proportion to the amount of protein. When the reaction was stopped by the addition of the stop solution in the assay kit, the color changed from blue to yellow, and the intensity of the color was measured at 450 nm.

Detection of Akt and tau phosphorylation

SH-SY5Y cells were incubated with or without 1 μM propofol for 1 h, followed by treatment with Aβ + propofol for 20 h. Akt (protein kinase B) is a serine / threonine-specific protein kinase that plays an important role in many cellular processes, such as apoptosis, cell proliferation, and tau phosphorylation[18]. To evaluate Akt phosphorylation, SH-SY5Y cells were extracted with a cell lysis buffer (Cell Signaling Technology), incubated for 10 min on ice, and then centrifuged at 10,000 × *g* for 3 min at 4°C. Akt phosphorylation was then determined in cell lysate samples using the p-Akt (pSer^{473}) / pan-Akt ELISA kit (Sigma-Aldrich).

After incubation for 1 h, the phosphorylation of tau in the cell was determined using a Human Tau[pS396] Assay ELISA kit (Invitrogen, Carlsbad, CA, USA).

Detection of cAMP response element-binding protein phosphorylation and Bcl-2 assay

cAMP response element-binding protein (CREB), which is activated by the phosphorylation of residue Ser^{133}, is one of the best characterized transcription factors ; CREB phosphorylation is an event induced by various extracellular signals[19]. In the present study, SH-SY5Y cells were lysed in a cytoplasmic extraction buffer in the assay kit. Phosphorylation of CREB was determined using the CREB (Total) InstantOne ELISA kit and the CREB (Phospho) InstantOne ELISA kit (Affymetrix, Santa Clara, CA, USA). Protein concentrations in the samples were then determined using the DC Protein Assay (Bio-Rad Laboratories).

After 20 h exposure to Aβ, with or without 1 h pretreatment with propofol or a p38MAPK inhibitor, SH-SY5Y cells were extracted with the cell lysis buffer provided in the Human Bcl-2 Platinum ELISA kit (eBioscience, Vienna, Austria) for the quantitative determination of Bcl-2 in SH-SY5Y cell lysates.

Detection of ROS

To study the effect of Aβ treatment on H_2O_2 production, we used the chloromethyl derivative of 2', 7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), a useful indicator for ROS detection. SH-SY5Y cells were seeded in 96-well plates at a density of 1×10^5 cells / ml and incubated as described above in the Cell Culture section. A Spectra Max i3 (Molecular Devices) was used to determine the fluorescence intensity at excitation and emission wavelengths of 488 and 525 nm, respectively.

Measurement of $[Ca^{2+}]_i$ by Meta Xpress Image Acquisition

After pretreatment with 1 μM propofol, 1 μM memantine, or 10 μM nicardipine, SH-SY5Y cells were exposed with 2.5 μM Aβ. $[Ca^{2+}]_i$ was analyzed using a FLIPR Calcium 5 Assay Kit (Molecular Devices). SH-SY5Y cells were loaded with the FLIPR reagent diluted in Hank's balanced salt solution + 20 mM HEPES buffer (pH 7.4) for 1 h at 37°C. After SH-SY5Y cells had been incubated with the FLIPR reagent, changes in $[Ca^{2+}]_i$ were measured by monitoring the fluorescence signals of FLIPR with excitation and emission wavelengths of 485 and 525 nm, respectively, using Meta Xpress Image Acquisition (Molecular Devices).

Statistical analysis

Each measurement was repeated three times. Results are expressed as the mean ± SEM. The effects of various treatments were compared with untreated control cells using one-way analysis of variance (ANOVA) and Dunnett's post hoc test, with $p < 0.05$ considered statistically significant.

Results

Effects of propofol on cell viability and Annexin V and Hoechst 33342 staining in Aβ-stimulated SH-SY5Y cells

First, we examined the effect of Aβ on cell viability (Fig. 1A, B). MTT assays revealed that 20-h exposure of cells to Aβ (1, 2.5, 5, or 10 μM) dose-dependently decreased cell viability. The viability of cells pretreated with propofol (1, 5, or 10 μM) or MAPK inhibitors (10 μM) did not differ significantly from that of control cells (Fig. 1A). Based on these results, we selected 2.5 μM Aβ and 1 μM propofol for use in subsequent experiments. Pretreatment of cells with 1 μM propofol or MAPK (p38MAPK or JNK) inhibitors for 1 h significantly recovered the cell viability that was reduced by treatment with 2.5 μM Aβ alone (Fig. 1B). Furthermore, cell viability was significantly suppressed when SH-SY5Y cells were pretreated with the MEK1/2 inhibitor (PD98059) compared with cells treated with Aβ + propofol. Pretreatment with the p38MAPK (SB202190) or JNK (SP600125) inhibitor induced a significant increase in cell viability (Fig. 1B).

Phase-contrast images of cells are shown in Fig. 1C–F, with control cells shown in Fig. 1C. Treatment of SH-SY5Y cells with 2.5 μM Aβ induced extensive damage to dendrite morphology, as revealed by the shortened dendrites or branchless forms (Fig. 1D). However, propofol pretreatment suppressed the dendrite damage caused by 2.5 μM Aβ (Fig. 1E). A well-known feature of the early apoptotic process is the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane bilayer. Results of the Annexin V-Cy3 binding assay of untreated and treated cells are shown in Fig. 1G–J. Emission of red fluorescence and nuclear condensation indicate the induction of apoptosis in Aβ-treated cells (Fig. 1H). Both red and bright blue fluorescence was attenuated in propofol-pretreated cells compared with Aβ-treated cells (Fig. 1I). However, pretreatment with the p38MAPK inhibitor (SB202190) did not result in any considerable change in Annexin V-positive cells (Fig. 1I) compared with that in the Aβ + propofol-treated group (Fig. 1J).

Effects of propofol on caspase-3 activity

Caspases play critical roles in the apoptosis of SH-SY5Y cells; therefore, caspase-3 activity was measured in the present study using a synthetic fluorometric substrate (Fig. 2). After incubation of SH-SY5Y cells with Aβ (2.5, 5, or 10 μM) for 20 h, caspase-3 activity increased significantly compared with that in untreated control cells. Incubation of cells with propofol (1, 5, or 10 μM) or 10 μM p38MAPK inhibitor (SB202190) had no significant effect on caspase-3 activity. However, pretreatment of cells with propofol (1 μM) or 10 μM p38MAPK inhibitor (SB202190)

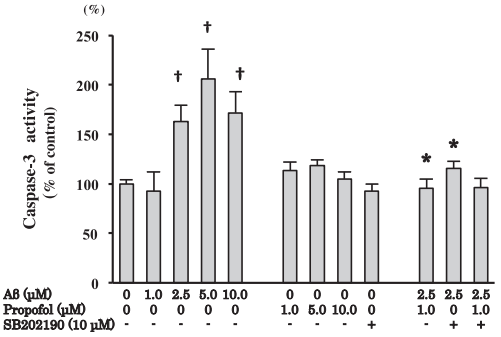

Fig. 2. Effects of propofol on caspase-3 activity in Aβ-stimulated SH-SY5Y cells. Caspase-3 activity was measured using the peptide substrate (DEVD-AFC) in SH-SY5Y cells treated with Aβ (1–10 μM) or propofol (1, 5, or 10 μM) for 20 h. In addition, caspase-3 activity was determined in SH-SY5Y cells incubated with or without 1-h pretreatment with 1 μM propofol or 10 μM p38MAPK inhibitor (SB202190), followed by 20 h incubation with 2.5 μM Aβ. Caspase-3 activity of control cells was 60.54 ± 4.35 nmol / mg protein per hour. Data are the mean ± SEM. $^{\dagger}p < 0.05$ compared with control cells; $^{*}p < 0.05$ compared with 2.5 μM Aβ-treated cells.

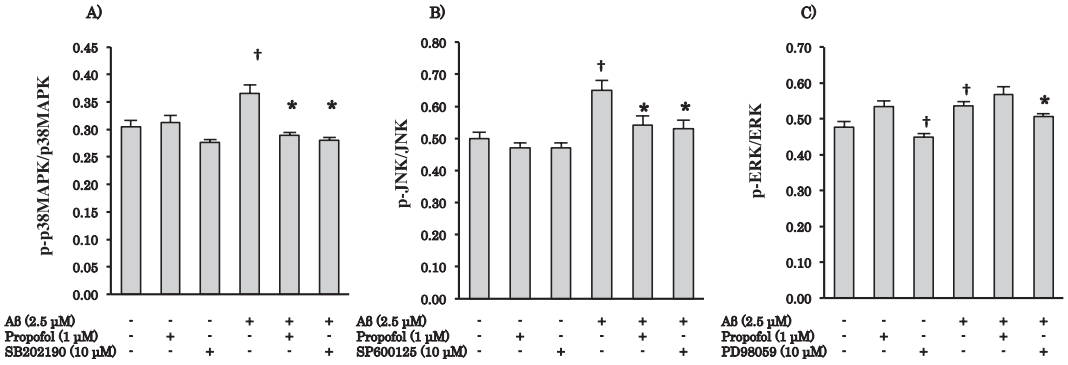

Fig. 3. Effects of propofol on phosphorylation of (A) p38MAPK, (B) JNK, and (C) ERK1 / 2 in Aβ-stimulated SH-SY5Y cells. p38MAPK, JNK, and ERK1 / 2 phosphorylation was examined using ELISA kits, as described in the Materials and Methods. SH-SY5Y cells were pretreated or not for 1 h with 1 μM propofol, followed by exposure to 2.5 μM Aβ for 30 min. The effects of p38MAPK, JNK, and ERK1 / 2 inhibitors (SB202190, SP600125, and PD98059, respectively) were also examined. Data are the mean ± SEM. $^{\dagger}p < 0.05$ compared with control cells; $^{*}p < 0.05$ compared with 2.5 μM Aβ-treated cells.

significantly suppressed the increase in caspase-3 activity seen in cells treated with 2.5 μM Aβ alone (Fig. 2).

Effects of propofol on MAPK phosphorylation in Aβ-stimulated SH-SY5Y cells

Phosphorylation of p38MAPK, JNK, and ERK was compared among untreated and 2.5 μM Aβ-treated SH-SY5Y cells, with or without 1 μM propofol pretreatment (Fig. 3A). Phosphorylation of the three MAPKs increased significantly in Aβ-treated SH-SY5Y cells compared with untreated cells. Propofol pretreatment decreased the Aβ-induced increase in the phosphorylation of p38MAPK and JNK (Fig. 3A, B), but had no significant effect on ERK1 / 2 phosphorylation (Fig. 3C). On the basis of these results, it is reasonable to speculate that p38MAPK is activated in Aβ-treated SH-SY5Y cells, whereas ERK does not seem to play a major role. Thus, subsequent studies were performed on p38MAPK.

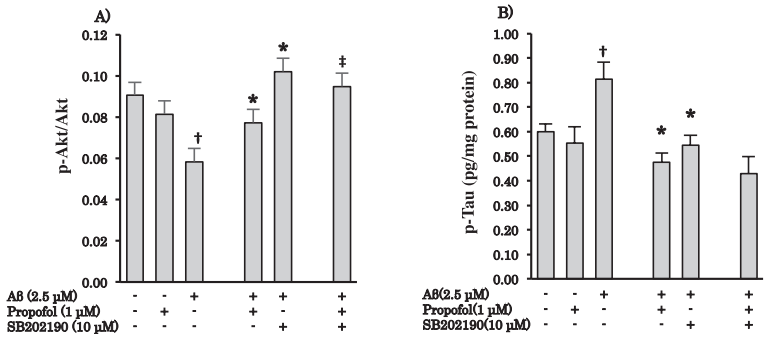

Fig. 4. Effects of propofol and p38MAPK inhibitor (SB202190) on (A) Akt and (B) tau phosphorylation in Aβ-stimulated SH-SY5Y cells. Akt and tau phosphorylation was determined in SH-SY5Y cells pretreated or not for 1 h with 1 μM propofol or 10 μM p38MAPK inhibitor, followed by 20 h incubation with 2.5 μM Aβ. Data are the mean ± SEM. $^{\dagger}p < 0.05$ compared with control cells; $^{*}p < 0.05$ compared with 2.5 μM Aβ-treated cells; $^{\ddagger}p < 0.05$ compared with 2.5 μM Aβ + 1 μM propofol-treated cells.

Effects of propofol on Akt and tau phosphorylation in Aβ-stimulated SH-SY5Y cells

To study the effects of propofol on the phosphorylation of Akt in Aβ-induced neurotoxicity, we first examined how propofol affected levels of p-Akt.

As shown in Fig. 4A, the p-Akt / total-Akt ratio was decreased in Aβ-treated compared with untreated SH-SY5Y cells; however, the Aβ-induced reduction in this ratio was recovered by pretreatment of cells with 1 μM propofol. Furthermore, pretreatment of cells with 10 μM p38MAPK inhibitor (SB202190) resulted in a significant increase in Akt phosphorylation compared with that in the Aβ + propofol-treated group (Fig. 4A).

It has been demonstrated that NFTs are formed by hyperphosphorylated tau protein, which is the pathological hallmark of AD[19]. In the present study, tau phosphorylation was increased significantly in Aβ-treated compared with untreated cells (Fig. 4B). However, the Aβ-induced increase in p-tau was recovered by pretreatment with 1 μM propofol or 10 μM p38MAPK inhibitor (SB202190).

Effects of propofol on CREB phosphorylation and Bcl-2 levels in Aβ-stimulated SH-SY5Y cells

CREB phosphorylation and levels of the anti-apoptotic protein Bcl-2 were investigated in SH-SY5Y cells.

To study the effects of propofol on CREB phosphorylation in Aβ-induced neurotoxicity, we first examined how propofol affected total CREB and p-CREB levels. As shown in Fig. 5A, CREB phosphorylation was increased significantly in propofol-treated compared with untreated SH-SY5Y cells. Exposure of SH-SY5Y cells to Aβ for 20 h led to a decrease in p-CREB / total-CREB ratio. However, the Aβ-induced reduction in this ratio was recovered by pretreatment with 1 μM propofol. CREB phosphorylation in cells pretreated with the p38MAPK

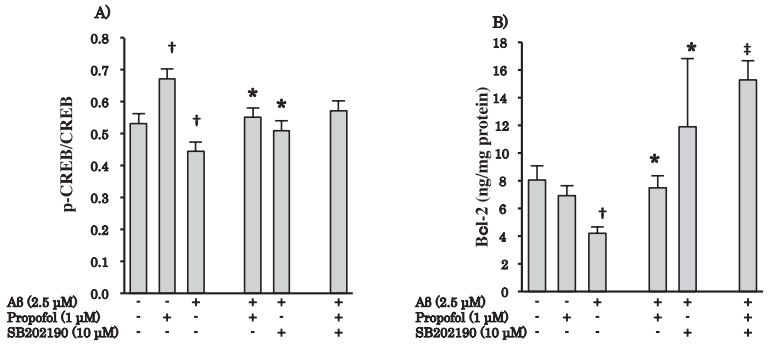

Fig. 5. Effects of propofol and p38MAPK inhibitor (SB202190) on (A) CREB phosphorylation and (B) Bcl-2 levels in Aβ-stimulated SH-SY5Y cells pretreated or not for 1 h with 1 μM propofol or 10 μM p38MAPK inhibitor, followed by 20 h incubation with 2.5 μM Aβ. Data are the mean ± SEM. $^{\dagger}p < 0.05$ compared with control cells; $^{*}p < 0.05$ compared with 2.5 μM Aβ-treated cells; $^{\ddagger}p < 0.05$ compared with 2.5 μM Aβ + 1 μM propofol-treated cells.

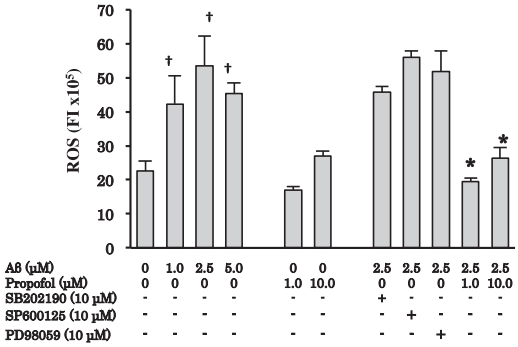

Fig. 6. Effects of propofol on ROS generation in Aβ-stimulated SH-SY5Y cells. ROS generation was evaluated using CM-H_2DCFDA in SH-SY5Y cells treated with Aβ (1–5 μM) or propofol (1 or 10 μM) for 1 h, as well as in cells pretreated for 1 h with propofol or inhibitors of p38MAPK, JNK, and ERK1/2 (SB202190, SP600125, and PD98059, respectively) followed by exposure to Aβ for 1 h. Data are the mean ± SEM. $^{\dagger}p < 0.05$ compared with control cells; $^{*}p < 0.05$ compared with 2.5 μM Aβ-treated cells.

inhibitor (SB202190) did not differ significantly from that in the propofol + Aβ-treated cells.

Bcl-2 levels were investigated in untreated or 2.5 μM Aβ-treated SH-SY5Y cells, with or without pretreatment with 1 μM propofol or 10 μM p38MAPK inhibitor (SB202190; Fig. 5B). Bcl-2 levels were significantly decreased in Aβ-treated cells, but this reduction in Bcl-2 was significantly suppressed by pretreatment with 1 μM propofol or 10 μM p38MAPK inhibitor (SB202190). Pretreatment with the p38MAPK inhibitor (SB202190) significantly increased Bcl-2 levels compared with levels in the Aβ + propofol-treated group (Fig. 5B).

Effects of propofol on ROS production in Aβ-stimulated SH-SY5Y cells

CM-H_2DCFDA, a ROS-sensitive dye, was used in the present study as an indicator of ROS formation to investigate of the effects of Aβ treatment on hydrogen peroxide production. As shown in Fig. 6, ROS production increased dose dependently in SH-SY5Y cells treated with Aβ

(1–5 μM) compared with control cells. However, propofol pretreatment of cells (1 and 10 μM) significantly decreased the 2.5 μM Aβ-induced increase in dichlorofluorescein (DCF) fluorescence. Furthermore, ROS production was not significantly suppressed in SH-SY5Y cells pretreated with MAPK inhibitors compared with cells treated with 2.5 μM Aβ alone.

Changes in $[Ca^{2+}]_i$ following propofol treatment

We next investigated the effects of 2.5 μM Aβ and propofol + Aβ on $[Ca^{2+}]_i$ homeostasis using Meta Xpress Image Acquisition (Fig. 7). $[Ca^{2+}]_i$ increased significantly in SH-SY5Y cells from 10 s after exposure to Aβ (2.5 μM), and was maintained at high levels for at least 5 min of the observation period (the duration of the observation period). In contrast, pretreatment with memantine (1 μM) resulted in a marked decrease in the Aβ-induced increase in $[Ca^{2+}]_i$. Memantine modulates the glutamatergic system, likely by blocking Ca^{2+} influx through NMDA receptors. In order to investigate whether the Ca^{2+} influx that follows Aβ stimulation was coupled to dihydropyridine-sensitive channels, the effects of nicardipine, a non-specific L-type voltage-dependent calcium channel (VDCC) blocker, on $[Ca^{2+}]_i$ were examined. Pretreatment with nicardipine (10 μM) markedly reduced the effects of Aβ on $[Ca^{2+}]_i$. Pretreatment with memantine (10–110 s ; $p < 0.01$), nicardipine (10–120 min ; $p < 0.01$), or memantine + nicardipine (10–120 min ; $p < 0.01$) significantly decreased the 2.5 μM Aβ-induced increase in $[Ca^{2+}]_i$. These findings suggest that Aβ promotes calcium influx through L-type VDCC and NMDA receptors in SH-SY5Y cells.

Pretreatment of SH-SY5Y cells with propofol (1 μM) resulted a significant decrease in Aβ-induced $[Ca^{2+}]_i$ for up to 140 s ; thereafter, $[Ca^{2+}]_i$ became almost constant and similar to that in cells treated with Aβ alone. These results suggest that propofol attenuates Aβ-induced neurotoxicity possibly by inhibiting the Aβ-induced increase in $[Ca^{2+}]_i$.

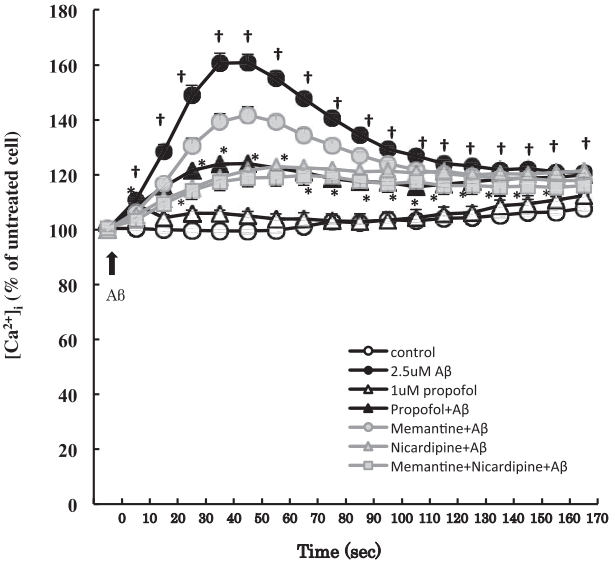

Fig. 7. Effects of propofol on $[Ca^{2+}]_i$ in SH-SY5Y cells. Changes in $[Ca^{2+}]_i$ were determined in untreated SH-SY5Y cells, as well as in cells treated with 2.5 μM Aβ, 1 μM propofol, or 2.5 μM Aβ + 1 μM propofol. In addition, some SH-SY5Y cells were pretreated or not with 1 μM propofol, 1 μM memantine, or 10 μM nicardipine for 1 h, followed by exposure to Aβ. Arrows indicate the addition of Aβ to the SH-SY5Y cells. Significant decreases were seen in the Aβ-induced increase in $[Ca^{2+}]_i$ following pretreatment of cells with memantine (10–110 s ; $p < 0.01$), nicardipine (10–120 min ; $p < 0.01$), and memantine + nicardipine (10–120 min ; $p < 0.01$). Data are the mean ± SEM of 200–300 cells. $^{\dagger}p < 0.05$ compared with control cells ; $^{*}p < 0.05$ compared with 2.5 μM Aβ-treated cells.

Discussion

In the present study we demonstrated that propofol, a short-acting intravenous anesthetic, has a protective effect against Aβ-induced neurotoxicity in SH-SY5Y cells.

Preclinical, animal, and *in vitro* cell culture studies have reported that anesthetics induce neuronal apoptosis, caspase activation, neurodegeneration, accumulation and oligomerization of Aβ, and, ultimately, cognitive decline. Recent studies have shown that 25% of patients over 65 years of age develop delirium after surgery and short- and long-term postoperative cognitive impairment following cardiac and non-cardiac operations[20]. Exposure to anesthesia during surgery can cause the onset or progression of AD in patients with mild cognitive impairment (MCI) or AD. However, in the present study propofol treatment reduced Aβ-induced neurocytotoxicity in SH-SY5Y cells (Figs. 1, 2). These results suggest that the use of propofol in patients with MCI or dementia has the potential to prevent the progression of AD by inhibiting neuron death due to Aβ.

In Aβ-exposed SH-SY5Y cells, Bcl-2 and Akt phosphorylation was decreased, ROS production was increased, and p38MAPK and tau phosphorylation was increased (Figs. 2, 3B, 4B, 6). Pretreatment of SH-SY5Y cells with a p38MAPK inhibitor (SB202190) significantly inhibited these effects of Aβ, with the exception of ROS generation, which did not change significantly; thus, p38MAPK does not appear to regulate ROS generation (Fig. 6). These results suggest that Aβ-induced p38MAPK activation occurs downstream of ROS generation. Recent AD research using cellular and animal models has shown that p38MAPK regulates various AD-related phenomena, such as tau phosphorylation, neurotoxicity, neuroinflammation, and synaptic dysfunction[15]. Furthermore, examination of brains of AD patients postmortem suggests high p38MAPK expression in regions of the brain associated with learning and memory. Thus, p38MAPK is likely an important factor of higher brain function. NFTs in an AD brain were reported to have formed aggregates of hyperphosphorylated tau protein via p38MAPK[13].

Aβ leads to decreased Akt-dependent Ser^9 phosphorylation of glycogen synthase kinase (GSK) 3β, promoting GSK3β activity and resulting in increased tau phosphorylation. Furthermore, Aβ reduced Bcl-2 and activated caspase-3 to promote apoptosis, and reduced the viability of SH-SY5Y cells (Fig. 8). However, pretreatment with propofol inhibited Aβ-induced neurotoxicity. Propofol has demonstrated cytoprotective effects by inhibiting excessive ROS generation in both *in vitro* and *in vivo* ischemia-reperfusion models[21], and suppresses H_2O_2-induced p38MAPK activation in neurons and human umbilical vein endothelial cells (HUVEC)[16]. In the present study, propofol significantly inhibited the Aβ-induced increase in ROS production, caspase-3 activity, and p38MAPK and tau phosphorylation, as well as the Aβ-induced decrease in Bcl-2 and Akt phosphorylation.

Propofol has been shown to protect hippocampal neurons in ischemic brain injury and against H_2O_2-induced oxidative stress[22] via the Akt signaling pathway. Because pretreatment with the p38MAPK inhibitor also increased Bcl-2 and Akt phosphorylation, inhibition of p38MAPK activity by propofol may have increased Akt phosphorylation and Bcl-2 levels and reduced tau

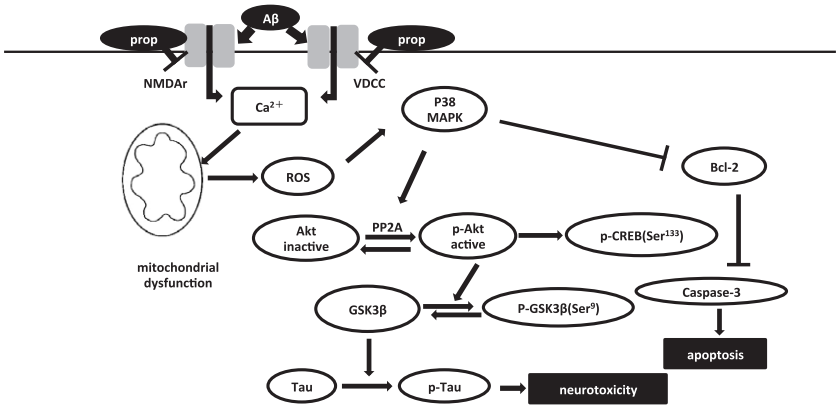

Fig. 8. Schematic diagram showing suppression of Aβ-induced neurotoxicity by propofol (prop) mediated via inhibition of $[Ca^{2+}]_i$-dependent p38MAPK activation. NMDAr, *N*-methyl-D-aspartate receptor; VDCC, voltage-dependent calcium channel; ROS, reactive oxygen species; p-, phosphorylated; PP2A, protein phosphatase 2A; CREB, cAMP response element-binding protein; GSK3β, glycogen synthase kinase 3β.

phosphorylation and caspase-3 activation to inhibit neuronal damage.

Although we used propofol at low concentrations in the present study, several previous studies have indicated that treatment with propofol at high concentrations promotes apoptosis. For example, a study in which macrophages were exposed to 140 μM propofol for 24 h reported that the treatment promoted apoptosis[23]. Other studies have shown that treatment of human glioblastoma cells with 300–500 μM propofol increases $[Ca^{2+}]_i$ in a dose-dependent manner as part of the induction of apoptosis[24].

Typically, 1.0–2.5 mg / kg propofol is administered to patients as an intravenous bolus injection to induce general anesthesia. In using a target control infusion (TCI) pump, the target plasma propofol concentrations are maintained between 2.0 and 5.0 μg / ml. However, propofol binds to plasma proteins (mean 97%–98% bound)[25], with 50% bound to erythrocytes and 48% bound to serum proteins, almost exclusively to human serum albumin. At a total concentration of 0.5–32 μg / ml, the free propofol fraction in the blood is 1.2%–1.7%. Based on these data, we used 1 μM propofol in a FBS-free culture medium in the present study.

Intracellular Ca^{2+} signal transmission has a ubiquitous role. The collapse of $[Ca^{2+}]_i$ homeostasis is involved in a variety of disease processes, and is a primary focal point of various studies on multifactorial neurodegenerative diseases such as AD. The Aβ-induced increase in $[Ca^{2+}]_i$ causes mitochondrial Ca^{2+} overload and promotes the generation of superoxide radicals. Released mitochondrial proteins that induce apoptosis, such as cytochrome *c*, and the increase in $[Ca^{2+}]_i$ have been suggested as the primary factors responsible for neuronal death or neurodegeneration in several AD models[12].

Aβ promotes Ca^{2+} influx through the plasma membrane into neurons, and this is widely known as the primary mechanism of neurodegeneration. Aβ induces a sustained increase in

$[Ca^{2+}]_i$ by activating NMDA receptors on the cell membrane, which is believed to be one of the major causes of neurodegeneration in AD[26]. Direct injection of Aβ(1-40) into the hippocampus also induces neural loss in the CA1 region, whereas treatment with memantine, an NMDA receptor antagonist, reduces neurodegeneration[27]. In the present study, memantine pretreatment also suppressed the Aβ-induced increase in $[Ca^{2+}]_i$ (Fig. 7). Therefore, the results of the present study support the notion that the NMDA receptor plays a role in Aβ-induced neurotoxicity.

Aβ also causes Ca^{2+} influx into neurons via VDCC. In the central nervous system there are two main types of L-type calcium channels, Cav1.2 and Cav1.3. In a recent study, expression of Cav1.2 and Cav1.3 was reportedly increased by Aβ(25-35) in rat hippocampal and human kidney cells (HEK293)[28]. In the present study, nicardipine pretreatment suppressed Aβ(1-42)-induced increases in $[Ca^{2+}]_i$ in SH-SY5Y cells (Fig. 7). These results may suggest that Aβ(1-42) promotes Ca^{2+} influx into neurons by way of the NMDA receptor and L-type VDCC. It is likely that the increase in $[Ca^{2+}]_i$ induced by Aβ results in mitochondrial dysfunction, increases ROS generation, and triggers p38MAPK activation.

Furthermore, in the present study, the Aβ-induced increase in $[Ca^{2+}]_i$ was reduced in propofol-pretreated cells (Fig. 7). A study using a human astrocytic cell line showed that propofol inhibited Ca^{2+} influx by targeting VDCC[29]. In neurons, propofol has been shown to impair the phosphorylation of NR1 subunits of the NMDA receptor, reducing NMDA-induced changes in $[Ca^{2+}]_i$[30].

In conclusion, the present study demonstrated that propofol inhibits Aβ-induced increases in $[Ca^{2+}]_i$ and p38MAPK activation in SH-SY5Y cells, thus protecting against neurodegeneration by suppressing Aβ-induced upregulation of tau phosphorylation and downregulation of Bcl-2 and CREB (Fig. 8). Thus, propofol is believed to be a beneficial anesthetic that works by protecting against oxidative stress, mitochondrial dysfunction, and apoptosis, and may serve as an anesthetic agent to prevent neuronal damage.

Therefore, it is suggested that the use of propofol at clinically relevant concentrations appears safe for elderly patients and for patients with risk factors for AD.

Conflict of interest disclosure

The authors declare that they have no conflict of interest.

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