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Propofol attenuates oxidative stress-induced PC12 cell injury via p38 MAP kinase dependent pathway[1]

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Key words

propofol; oxidative stress; PC12 cells; apoptosis; p38 MAP kinase

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Abstract

Aim: To investigate the neuroprotective effect of propofol and its intracellular mechanism on neurons *in vitro*. **Methods**: Cell viability was determined with 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reduction. Apoptotic cell death was determined by Hoechst 33258 staining and a fluorescence-activated cell sorter. The caspase-3 activity was measured by fluorometric assay. Mitogen-activated protein (MAP) kinase phosphorylation was detected with Western blotting. **Results:** The pretreatment of rat pheochromocytoma cell line PC12 with propofol (1–10 μmol/L) resulted in a significant recovery from hydrogen peroxide (H_2O_2)-induced cell death and the inhibition of H_2O_2 induced caspase-3 activation and PC12 cell apoptosis. Propofol inhibited the H_2O_2-induced p38 MAP kinase, but not c-Jun N-terminal kinase or extracellular signal-regulated kinase 1 and 2 activations. **Conclusion:** Propofol might attenuate H_2O_2-induced PC12 cell death through the inhibition of signaling pathways mediated by the p38 MAP kinase.

Introduction

Propofol (2,6-diisopropylphenol), the active ingredient of the common general anesthetic Diprivan, has shown direct antioxidant activity conferred by the phenolic hydroxyl group in its structure. This moiety scavenges free radicals and inhibits lipid peroxidation[1]. Since oxidative stress plays an important role in the brain ischemic injury[2], the antioxidant property of propofol suggests that it may serve as a good agent to combat ischemia. Certain hypotheses have been proposed, such as a reduction in cerebral metabolism[3], potentiation of γ-aminobutyric acid-mediated inhibition[4], and restoration of the glutamate uptake impaired during injury[5,6]. Our previous study showed that propofol protected cerebral cortical and hippocampal slices against hydrogen peroxide injury at low and mid concentrations[7], but its intracellular mechanism is still worth further investigation.

Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide radical, hydroxyl radical, and peroxynitrite increased, and probably would be involved in the pathogenesis of cerebral ischemic and reperfusion injury[8,9]. ROS-induced cellular events have been implicated, at least in part, in the activation of mitogen-activated protein (MAP) kinases[9,10]. Three subfamilies of MAP kinases sensitive to ROS have been identified: extracellular-signal regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), and the p38 kinase. The kinase of each subfamily modulated specific cell functions via certain signaling pathways. However, the hypothesis that ERK1/2 may be a protective signal and JNK-p38 a pro-apoptotic signal would not always hold true, and their effect may depend on the nature of the cell type, death stimulus, duration of its activation, and probably above all, the activity of other signaling pathways[11,12]. Therefore, the mechanism of these signal transduction pathways involved in ROS-mediated cell damage remains to be elucidated.

The concentration of H_2O_2 in healthy individuals is normally quite low. The elevation of H_2O_2 concentration upon cerebral ischemic and reperfusion injury could act as a significant signal[13]. H_2O_2 has been used frequently as an oxidative stimulus to identify redox-sensitive processes. Rat pheochromocytoma cell line PC12 is useful for studying the

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intracellular signaling mechanisms and are regarded as a model for catecholamine-containing neurons[14]. In the present study, we used PC12 cells injured by H_2O_2 as an oxidative stress model *in vitro* to examine the neuroprotective effect of propofol and its intracellular mechanism, especially focusing on the roles of MAP kinases in the events.

Materials and methods

Chemicals Propofol and H_2O_2 were purchased from Sigma Chemicals (St Louis, MO, USA). Anti-pan- and phospho-ERK1/2 (Thr^{202}/Tyr^{204}), p38 (Thr^{180}/Tyr^{182}), and JNK (Thr^{183}/Tyr^{185}) antibodies and SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole] were from Cell Signaling Technology (Beverly, MA, USA). All other chemicals were commercial products of reagent grade except where indicated.

Cell cultures and determination of cell viability with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide-thiazolyl blue (MTT)reduction The PC12 cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (*v/v*) donor horse serum, 5% (*v/v*) newborn calf serum, and antibiotics (100 units/mL penicillin and 100 mg/mL streptomycin) in flasks precoated with collagen. The cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. The cells were grown to 70%–80% confluence in 60 and 100 mm dishes and the growth was arrested by incubation in serum-free DMEM for 24 h prior to use. All experiments were performed with growth-arrested cells to minimize basal MAP kinase activity. The neuroprotective effect of propofol on PC12 cell death induced by H_2O_2 was investigated by using [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidethiazolyl blue] (MTT) assay (Sigma, USA). The cells were treated with H_2O_2 (200 μmol/L) for 24 h with or without propofol, and the cellular MTT reduction was measured as described previously[15].

Determination of apoptotic cell death with Hoechst 33258 staining and a fluorescence-activated cell sorter To further evaluate the effect of propofol on the H_2O_2-induced apoptosis of PC12 cells, we examined apoptotic nuclei staining and DNA fragmentation in H_2O_2-administered PC12 cells. After treatment with H_2O_2 (200 μmol/L) for 24 h with or without propofol, the cells were stained with Hoechst 33258 (Molecular Probes, Eugene, OR, USA) and visualized by fluorescence microscopy to evaluate morphological changes of the nuclei as a measure of apoptosis. In addition, separate groups of cells were also harvested and stained with Annexin V and PI (propidium iodide) (BD Biosciences, San Jose, CA, USA) to evaluate the percentage of apoptotic cells using flow cytometry.

Measurement of caspase-3 activity The PC12 cells in serum-free DMEM (60 mm collagen-coated dishes) were pre-incubated with different concentrations of propofol for 30 min, then incubated in the presence of H_2O_2 (200 μmol/L) for 4 h. The assay was performed according to the manufacturer's protocol. In brief, the lysates were centrifuged at 25 000×*g* for 3 min at 4 °C to precipitate cell debris. 50 μL of 2×reaction buffer/Dithiothreitol mix was added to the supernatant, and finally, 5 μL of 1 mmol/L caspase-3 substrate Asp-Glu-Val-Asp (DEVD) conjugated to 7-amino-4-trifluoromethylcoumarin (AFC) was added to each tube and incubated for 1 h at 37 °C. After transferring the samples and standard solution to a 96-well microplate, we measured fluorescent activity using a fluorimeter with a 400 nm excitation filter and a 505 nm emission filter.

Measurement of the p38 MAP kinase, JNK, and ERK1/2 phosphorylations in PC12 cells MAP kinase phosphorylation induced by H_2O_2 in PC12 cells were performed by Western blotting. ERK1/2, JNK, and p38 activations in the cells were determined by using phospho-ERK1/2, phospho-p38 and phospho-JNK antibodies. Total ERK1/2, p38 and JNK protein expressions were measured in the presence of pan-ERK1/2, p38, and JNK antibodies. The PC12 cells in serum-free DMEM were treated with or without propofol and then incubated for 30 min. After incubation with H_2O_2 for 10 min, the cells were collected and lysed for the immunoblot analysis. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL, Pierce, Rockford, IL, USA) and were quantified by densitometry in the linear range of film exposure using a UMAX Astra 2200 scanner and NIH Image Version 1.60 software (NIH Division of Computer Research and Technology).

Statistical analysis Data were expressed as mean±SD. Differences were analyzed for significance by Student's *t*-test. The results were considered significant at $P<0.05$.

Results

Inhibition of propofol on H_2O_2-induced PC12 cell death First, we examined the effect of propofol on H_2O_2-induced PC12 cell death by measuring MTT reduction. As shown in Figure 1, the application of H_2O_2 (200 μmol/L) to PC12 cell for 24 h resulted in about 35% death. Pretreatment of cells with propofol resulted in inhibition of H_2O_2-induced cell death in a concentration-dependent manner. No effect of propofol on cell viability was observed (data not shown).

Effect of propofol on H_2O_2-induced PC12 cell apoptosis To evaluate the effect of propofol on the apoptosis of PC12 cells, we examined the staining of apoptotic nuclei and DNA fragmentation in H_2O_2-administered PC12 cells with or

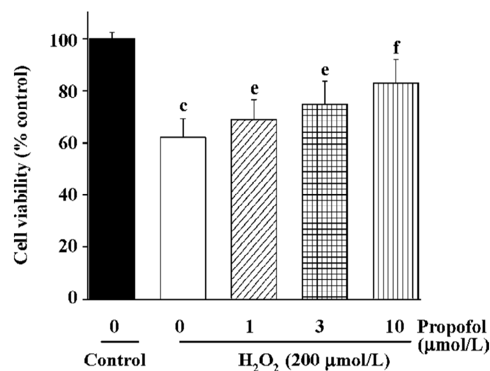

Figure 1. Propofol inhibited H_2O_2-induced PC12 cell death. Cell viability was evaluated with MTT reduction. Cells were treated with or without H_2O_2 (200 μmol/L) for 24 h and then assayed. Propofol at the indicated concentrations was added to the incubation medium 30 min prior to H_2O_2 stimulation. Values are expressed as mean±SD of 5 experiments preformed in triplicate. $^cP<0.01$ *vs* control, $^eP<0.05$, $^fP<0.01$ *vs* H_2O_2 (200 μmol/L) alone.

without propofol pretreatment. As shown in Figure 2A, staining with Hoechst 33258 revealed that PC12 cells showed apoptotic nuclei after treatment with H_2O_2 (200 μmol/L) for 24 h. Pretreatment with propofol decreases the rate of apoptotic nuclei. As shown in Figure 2B, in the determination of apoptotic cell death by a fluorescence-activated cell sorter (FACS), 22% of cells showed apoptosis. Pretreatment of PC12 cells with propofol (3 μmol/L) resulted in the inhibition of H_2O_2-induced apoptosis to 14% of total cells. The observation was consistent with that of the MTT test and the results suggested that propofol attenuates H_2O_2-induced PC12 cell death, including apoptosis.

Effects of propofol on H_2O_2-induced caspase-3 activation in PC12 cells Since caspase-3 has been shown to be an important regulator of apoptotic cell death, we next examined the effect of H_2O_2 (200 μmol/L) on caspase-3 activity in PC12 cells. As shown in Figure 3, the incubation of PC12 cells with H_2O_2 (200 μmol/L) for 4 h significantly increased caspases-3 activity. Pre-incubation with different concentrations of propofol for 30 min significantly inhibited H_2O_2-induced caspase-3 activation in a concentration-dependent manner (Figure 3).

Effect of propofol on H_2O_2-induced ERK1/2, JNK, and p38 activation in PC12 cells To clarify the effect of propofol on H_2O_2-induced MAP kinase phosphorylation, the PC12 cells were incubated in the presence of H_2O_2 (200 μmol/L) for 10 min following pretreatment with indicated concentrations of propofol for 30 min. We found that H_2O_2-induced phosphorylation of p38 instead of JNK and ERK1/2 was

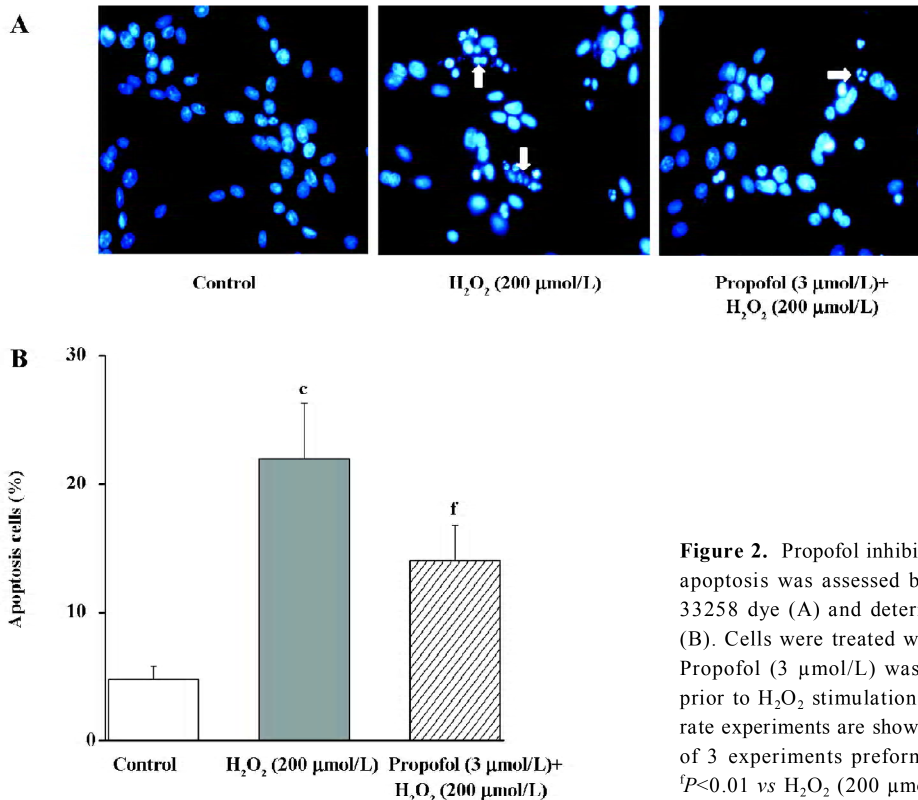

Figure 2. Propofol inhibited H_2O_2-induced PC12 cell apoptosis. Cell apoptosis was assessed by apoptotic nuclei staining with Hoechst 33258 dye (A) and determination of apoptotic cell death by FACS (B). Cells were treated with or without 200 μmol/L H_2O_2 for 24 h. Propofol (3 μmol/L) was added to the incubation medium 30 min prior to H_2O_2 stimulation. Representative photographs from 3 separate experiments are shown in (A). Values are expressed as mean±SD of 3 experiments preformed in triplicate (B). $^cP<0.01$ *vs* control, $^fP<0.01$ *vs* H_2O_2 (200 μmol/L) alone

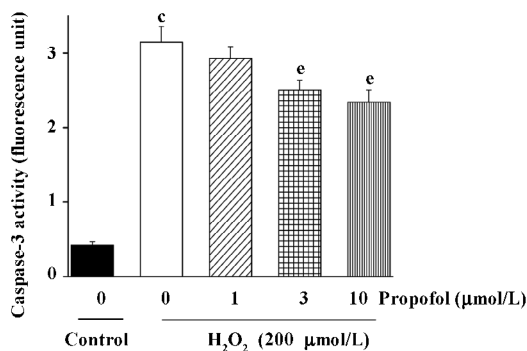

Figure 3. Propofol inhibited H_2O_2-induced caspase-3 activation in PC12 cells. After 30 min pre-incubation with propofol at the indicated concentrations, PC12 cells were incubated in presence of H_2O_2 (200 μmol/L) for 4 h. Caspase-3 activity was measured. Values are expressed as mean±SD. n=5. $^cP<0.01$ *vs* control, $^eP<0.05$ *vs* H_2O_2 (200 μmol/L) alone.

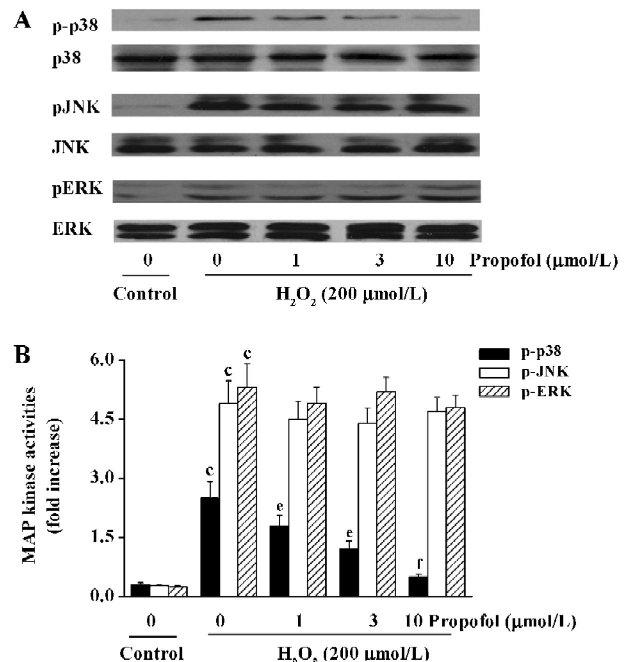

Figure 4. Inhibitory effect of propofol on H_2O_2-induced p38 MAP kinase activation, but not JNK nor ERK1/2 in PC12 cells. Cells were pretreated with or without propofol (at the indicated concentrations) for 30 min. After 10 min incubation with H_2O_2 (200 μmol/L), the phosphorylations of p38 MAP kinase, JNK, and ERK1/2 were measured. No significant differences in the amounts of p38 MAP kinase, JNK, and ERK1/2 were observed in the samples by the immunoblot analysis with the anti-p38 MAP kinase, JNK, and ERK1/2 antibodies (data not shown). The blot depicted is representative of three similar ones (A). Values were normalized by arbitrarily setting the densitometry of the control cells. Values are expressed as mean±SD. n=3. $^cP<0.01$ *vs* control, $^eP<0.05$, $^fP<0.01$ *vs* H_2O_2 (200 μmol/L) alone (B).

inhibited by the treatment of propofol (Figure 4), which indicated that the H_2O_2-induced p38 MAP kinase phosphorylation in PC12 cells would be specifically sensitive to propofol.

Discussion

Oxidative stress has been implicated as a potential contributor to the pathogenesis of acute central nervous system injury. After brain injury by ischemic or hemorrhagic stroke or trauma, the increased ROS production may lead to tissue damage via different cellular molecular pathways. ROS can cause damage to cardinal cellular components, such as lipids, proteins, and DNA, resulting in subsequent cell death by necrosis or apoptosis. In the present study, the examined concentrations of H_2O_2 were in the range of those reached under pathophysiological conditions, such as during transient cerebral ischemia[16]. Propofol has been shown to cause protective action on neurons[7], including antioxidative effects[17], inhibitory effects on lipid peroxidation[18], and direct anti-excitotoxic properties[19]. Other studies showed that propofol had a potential to enhance neurological outcome and decrease the infarct size in experimental animal models of stroke[20,21]. However, no evidence has been reported concerning the direct effect of propofol on neuronal-like PC12 cells and their effect on MAP kinase activity. For the first time, this study showed that propofol protected PC12 cells from H_2O_2-induced cell death, including apoptosis, by measuring MTT reduction, apoptotic nuclei staining, FACS, and caspase-3 activity. We also observed that propofol specifically inhibited H_2O_2-induced p38 activation, but not ERK1/2 and JNK activation in PC12 cells.

Caspases are a family of specific cysteine proteases whose activation is critical for the intracellular execution of apoptotic death[22]. Among the 10+ caspases that have been identified, caspase-3 is a common effecter to which several procaspases and caspases converge, and therefore, can serve as an apoptotic marker[23] induced by a variety of stimuli. It has been shown that caspase-3 can be activated by H_2O_2 exposure as a final effecter in apoptotic death *in vitro*[24,25]. In agreement with this view, our results showed that H_2O_2 markedly increased caspase-3 activity in PC12 cells. Moreover, we investigated the effect of propofol on the H_2O_2-induced effect, where we found that propofol inhibited H_2O_2-induced caspase-3 activation, as shown in Figure 3. However, since the inhibitory effect of 10 μmol/L propofol on H_2O_2-induced caspase-3 activation was incomplete (Figure 3), the involvement of other mechanisms of propofol protective effects against H_2O_2-induced PC12 cells death can not be

denied. Further studies are required to define the exact role of caspase-3 in the mechanism of H_2O_2-induced PC12 cell death.

Cellular signaling pathways are regulated by the intracellular redox state of the cell. ROS may play an important role as a second messenger in signal transduction cascades and may lead to the activation of MAP kinases[26]. Previous studies have shown that H_2O_2 rapidly activated ERK and p38 proteins in PC12 cells, and the mediation of cell death by ROS may be closely related to MAP kinase signaling[27,28]. In line with these studies, we found in the present study that 3 MAP kinases were rapidly and significantly activated by oxidative stress in cultured PC12 cells (Figure 3). The phosphorylation of the 3 MAP kinases almost simultaneously reached their maximum at 10 min, and then the activity of the 3 MAP kinases decreased gradually (data not shown). Therefore, we investigated the effect of propofol on the activity of the 3 MAP kinases at 10 min. We found that propofol suppressed p38 activation, but not JNK nor ERK1/2 phosphorylations (Figure 4), indicating that p38 is specifically sensitive to propofol, which may contribute to its cellular protective effect under oxidative stress. Previous studies showed that the activation of the p38 MAP kinase induced apoptotic death in neuroblastoma cells[29], while inhibitors of the p38 MAP kinases promoted neuronal survival *in vitro* and reduced cerebral infarction *in vivo*[30,31]. In line with these studies, we also found that SB203580, a specific p38 inhibitor, attenuated H_2O_2-induced PC12 cell death and caspase-3 activity (data not shown), which further verified the role of p38 in oxidative stress-mediated cell death.

In conclusion, this study confirmed a neuroprotective effect of propofol, at clinically relevant concentrations, on H_2O_2-induced PC12 cell death, including apoptosis. It could be assumed that this beneficial effect of propofol on PC12 cell viability is mediated, at least in part, by an inhibition of the p38 pathway, but not that of ERK1/2 or JNK, which are dramatically activated by H_2O_2. The results of the present study may shed light on the pharmacological basis for the clinical application of some anesthetic compounds, such as propofol in cerebral ischemic and/or reperfusion injury.

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