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Methanol Extract of *Paeonia Japonica* Root Protects Cultured Rat Cortical Neurons Against Oxidative Damage Induced by Hydrogen Peroxide

Min Su Park*, Ju Yeon Ban*, Ju Hyun Lee*, Kyung Sik Song, and Yeon Hee Seong*†**

**College of Veterinary Medicine and Research Institute of herbal Medicine, Chungbuk National University, Cheongju, Chungbuk 361-763, Korea.*

***College of Agriculture and Life-Sciences, Kyungpook National University Daegu 702-701, Korea.*

ABSTRACT : *Paeoniae* radix has been widely used for its anti-allergic, anti-inflammatory and analgesic effects, and demonstrated to have anticonvulsant, memory enhancing and anxiolytic activities. The present study was performed to examine the protective effect of methanol extract of *Paeoniae* radix (PR) from *Paeonia Japonica* Miyabe et Takeda (Paeoniaceae) on hydrogen peroxide (H_2O_2)-induced neurotoxicity using cultured rat cerebral cortical neuron. H_2O_2 produced a concentration-dependent reduction of neuronal viability. PR, over a concentration range of 10 to 100 ㎍/㎖ showed concentration-dependent decrease of the H_2O_2 (100 μM)-induced neuronal cell death, as assessed by a 3-[4,5-dimethylthiazol-2-yl]-2,5-di-phenyl-tetrazolium bromide assay and the number of apoptotic nuclei, evidenced by Hoechst 33342 staining. PR (100㎍/㎖) inhibited 100 μM H_2O_2-induced elevation of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$), which was measured by a fluorescent dye, fluo-4 AM. PR (50 ㎍/㎖) inhibited glutamate release into medium induced by 100 μM H_2O_2, which was measured by HPLC, and generation of reactive oxygen species (ROS). These results suggest that PR may mitigate the H_2O_2-induced neurotoxiciy by interfering with the increase of $[Ca^{2+}]_c$, and then inhibiting glutamate release and generation of ROS in cultured neurons.

Key words : *Paeoniae* radix, neurotoxicity, hydrogen peroxide, neuroprotection, cortical neurons

INTRODUCTION

Formation of reactive oxygen species (ROS) has been proposed to be an important step leading to neuronal death related to a variety of cerebral diseases, e.g. stroke, amyotropic lateral sclerosis, Alzheimer disease and Parkinson disease (Olanow, 1993). Therefore, in vitro hydrogen peroxide (H_2O_2) toxicity has become a well-established model for the oxidative stress on neurons. Many researches demonstrated the involvement of glutamate in H_2O_2-induced neurotoxicity in cultured neurons (Gardner *et al.*, 1996). H_2O_2 and O_2^- inhibited the uptake of glutamate and enhanced the release of glutamate, resulting in NMDA receptor overstimulation (Volterra *et al.*, 1994; Mailly *et al.*, 1999). The neurotoxic effects of H_2O_2 were strongly reduced by antagonists of NMDA receptors and enhanced in the absence of Mg^{2+} (Mailly *et al.*, 1999). There are some reports on H_2O_2-induced intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increase (Whittemore *et al.*, 1995). NMDA receptor is a ligand-gated/voltage-sensitive cation channel, especially highly permeable to Ca^{2+}. Calcium influx through NMDA receptor-coupled Ca^{2+} channel appears to be a critical role in the H_2O_2-induced neurotoxicity (Mailly *et al.*, 1999). Moreover, Ca^{2+} signals activate enzymes which lead to further ROS generation (e.g. xanthine oxidase, nitric oxide synthase, phospholipase A_2); conversely, ROS generation can facilitate $[Ca^{2+}]_i$ increases by damaging the $[Ca^{2+}]_i$ regulatory mechanism and activating Ca^{2+} release from intracellular Ca^{2+} stores (Duffy & MacViar 1996). However, the clinical benefit of NMDA receptor antagonists and direct blockers of neuronal Ca^{2+} channels is debatable, since they lack convincing effectiveness or have serious side-effects (Ferger & Krieglstein, 1996; Li *et al.*, 2002). There are much efforts to develop beneficial agents from medicinal plants to achieve neuroprotection.

Paeoniae radix (PR), the root of the genus *Paeonia* (Paeoniaceae), has been widely used for its antispasmodic, anti-inflammatory, and analgesic effects (Huang, 1999; Murakami *et al.*, 1996). In the central nervous system (CNS), PR has been characterized pharmacologically to exhibit anticonvulsant, antianxiety and memory enhancing activities in animals (Sugaya *et al.*, 1991; Watanabe *et al*, 1991; Ohta *et al.*, 1993; Nizami & Jafri, 2005). Paeoniflorin and paeonol, as strong bioactive principles of PR, have been demonstrated to have cognitive enhancing and anxiolytic effects (Ohta *et al.*, 1993; Watanabe, 1997; Mi *et al.*, 2005). Antioxidative effect of PR has been also reported in various cell types (Liu & Ng, 2000; Okubo *et al.*, 2000; Lee *et al.*, 2003). Therefore, to extend the

† Corresponding author: (Phone) +82-43-261-2968 (E-mail) vepharm@chungbuk.ac.kr
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knowledge on the pharmacological action of PR in the CNS, the present study was performed to examine the protective effect of PR, the root of *Paeonia Japonica* Miyabe et Takeda (Paeoniaceae), on H_2O_2-induced neurotoxicity using cultured rat cerebral cortical neuron.

MATERIALS AND METHODS

Materials

PR was purchased from an oriental drug store in Taegu, Korea, and identified by professor K.-S. Song, Kyungpook National University. H_2O_2, 2-mercaptoethanol, trypsin (from bovine pancreas), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), *o*-phthaldialdehyde (OPA), Dulbecco's modified Eagle's medium (DMEM), Joklik-modified Eagle's medium, poly-L-lysine and amino acids for HPLC standard were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fluo-4 AM, 2',7'-dichlorodihydrofluorescin diacetate (H_2DCF-DA) and Hoechst 33342 dye were purchased from Molecular Probes Inc. (Eugene, OR, USA). Fetal bovine serum was purchased from JRH Biosciences (Lenexa, KS, USA). All other chemicals used were of the highest grade available.

Preparation of methanol extract of PR

PR (300 g) was extracted three times in a reflux condenser for 24 h each with 2 ℓ of methanol. The solution was combined, filtered through Whatman NO. 1 filter paper, concentrated using a rotary vacuum evaporator, and freeze-dried. The yield was about 10% (w/w).

Primary culture of cortical neurons

Primary cortical neuronal cultures were prepared using SD rat fetuses on embryonic day 15 ± 1, according to the previously described methods (Ban *et al.*, 2005). Briefly, fetuses were isolated from a dam anaesthetized with ether. Cortical hemispheres were dissected under sterile conditions and placed into Joklik-modified Eagle's medium containing trypsin (0.25 ㎎/㎖). After slight trituration through a 5-㎖ pipette 5 to 6 times, the cells were incubated for 10 min at 37 ℃ Dissociated cells were collected by centrifugation (1,500 rpm, 5 min) and resuspended in DMEM supplemented with sodium pyruvate (0.9 mM), L-glutamine (3.64 mM), sodium bicarbonate (44 mM), glucose (22.73 mM), penicillin (40 U/㎖), gentamicin (50 ㎍/㎖), KCl (5 mM) and 10% fetal bovine serum at a density of about 2×10^6 cells/㎖. Cells were plated onto poly-L-lysine coated 12 well-plates (Corning 3512, NY, USA) for the measurements of cell death and glutamate release, and coverslips (Fisher Scientific 12CIR, Pittsburgh, PA, USA) for the measurements of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$), ROS and apoptosis. After 2 day' incubation, the medium was replaced with a new growth medium in which the concentrations of fetal bovine serum and KCl were changed to 5% and 15 mM, respectively. Cultures were kept at 37 ℃ in a 5% CO_2 atmosphere, changing the medium twice a week. Immunochemical staining of cultures, when grown for 5-7 days *in vitro*, with anti-microtubule associated protein-2 (MAP-2) antibody and anti-glial fibrillary acidic protein (GFAP) antibody revealed that the culture method used in this study provided cell cultures containing about 90% neurons.

Neurotoxicity experiments

H_2O_2 was diluted freshly with the incubation buffer described below, for every experiment. PR was dissolved in methanol with the concentration of 100 ㎎/㎖ and further diluted with experimental buffers. The final concentration of methanol was 0.1%, which did not affect neuronal cell viability. Neurotoxicity experiments were performed on neurons grown for 5-7 days *in vitro*. The culture medium was removed and neurons were washed with a HEPES-buffered solution (incubation buffer) containing 8.6 mM HEPES, 154 mM NaCl, 5.6 mM KCl and 2.3 mM $CaCl_2$, at pH 7.4. They were then incubated for 30 min in the same medium, and incubated for a further 15 min (unless otherwise indicated) in the presence of H_2O_2 at 37 ℃. Exposed to H_2O_2, the neurons were washed and post-incubated in H_2O_2-free and serum-free growth medium for 15 h for the measurement of cell viability. PR was pretreated 20 min prior to the H_2O_2 treatment, and added during the H_2O_2 exposure period and the post-incubation period.

Analysis of cell viability

MTT colorimetric assay

This method is based on the reduction of the tetrazolium salt MTT into a crystalline blue formazan product by the cellular oxidoreductase (Ban *et al.*, 2005). Therefore, the amount of formazan produced is proportional to the number of viable cells. At the end of the incubation, the culture medium was replaced by a solution of MTT (0.5 ㎎/㎖) in serum-free growth medium. After a 4 h incubation at 37 ℃, this solution was removed, and the resulting blue formazan was solubilized in 0.4 ㎖ of acid-isopropanol (0.04 N HCl in isopropanol), and the optical density was read at 570 ㎚ using microplate reader (Bio-Tek EL_X808, Vermont, USA). Serum-free growth medium was used as a blank solution.

Measurement of apoptotic cell death

The bis-benzimidazole dye, Hoechst 33342, penetrates the plasma membrane and stains DNA in cells without permeabili-

zation. In contrast to normal cells, the nuclei of apoptotic cells have highly condensed chromatin that is uniformly stained by Hoechst 33342. These morphological changes in the nuclei of apoptotic cells may be visualized by fluorescence microscopy. After the post-incubation as described above, neurons on coverslips were fixed in 4% paraformaldehyde at room temperature for 20 min, then stained with Hoechst 33342 dye at the concentration of 1 ㎍/㎖ in the incubation buffer for 15 min. The morphological change was examined under UV illumination using a fluorescence microscope (Olympus IX70-FL, Tokyo, Japan). The dye was excited at 340 ㎚, and emission was filtered with a 510 ㎚ barrier filter. To quantify the apoptotic process, neurons with fragmented or condensed DNA and normal DNA were counted. Data were shown as apoptotic cells as a percentage of total neurons.

Measurement of $[Ca^{2+}]_c$

Neurons grown on coverslips were loaded with 3 μM fluo-4 AM (dissolved in dimethylsulfoxide (DMSO)) in serum-free growth medium for 45 min at 37℃ in the CO_2 incubator, and washed with the incubation buffer. The coverslips containing fluo-4 AM labeled neurons were mounted on a perfusion chamber containing incubation buffer, subjected to a laser scanning confocal microscope (Carl Zeiss LSM 510, Oberkochen, Germany), and then scanned every 2 second with a 488 excitation argon laser and a 515 ㎚ longpass emission filter. After the baseline of $[Ca^{2+}]_c$ was observed for 50 sec, 100 μM H_2O_2 was added to the perfusion chamber for the measurement of $[Ca^{2+}]_c$ change. In order to test the effect of PR on the H_2O_2-induced $[Ca^{2+}]_c$ change, neurons were pretreated with PR (100 ㎍/㎖) 15 min before the treatment with 100 μM H_2O_2 after being loaded with fluo-4 AM and washed. PR was also present in the perfusion chamber during the $[Ca^{2+}]_c$ measurement period. All images, about 100 images from the scanning, were processed to analyze changes of $[Ca^{2+}]_c$ in a single cell level. The results were expressed as the relative fluorescence intensity (RFI) (Lee *et al.*, 1998).

Measurement of glutamate concentration

Washed and equilibrated for 20 min with the incubation buffer, neurons were incubated with the buffer containing 100 μM H_2O_2 for 15 min and incubated with H_2O_2-free incubation buffer for a further 2 h (post-incubation) at 37℃. PR was pretreated 20 min prior to the H_2O_2 treatment and added during the H_2O_2 exposure period and post-incubation period. At the end of the incubation, glutamate secreted into the medium from the treated cells was quantified by high performance liquid chromatography (HPLC) with an electrochemical detector (ECD) (BAS MF series, IN, USA) (Ban *et al.*, 2005). Briefly, after a small aliquot was collected from the culture wells, glutamate was separated on an analytical column (ODS2; particle size, 5 ㎛; 4.6×100 ㎜) after pre-derivatization with OPA/2-mercaptoethanol. Derivatives were detected by electrochemistry at 0.1 μA/V, and the reference electrode was set at 0.7 V. The column was eluted with mobile phase (pH 5.2) containing 0.1 M sodium phosphate buffer with 37% (v/v) HPLC-grade methanol at a flow rate of 0.5 ㎖/min.

Measurement of ROS generation

The microfluorescence assay of 2',7'-dichlorofluorescin (DCF), the fluorescent product of H_2DCF-DA, was used to monitor the generation of ROS. Neurons grown on coverslips were washed with phenol red-free DMEM 3 times and incubated with the buffer for 30 min at 37℃. Then, the buffer was changed into the incubation buffer containing 100 μM H_2O_2, and cells were incubated for a further 20 min. In order to test the effect of PR on H_2O_2-induced generation of ROS, PR was added 20 min prior to the treatment with H_2O_2. The uptake of H_2DCF-DA (final concentration, 5 μM) dissolved in DMSO was carried out for the last 10 min of the incubation with 100 μM H_2O_2. After being washed, coverslips containing cortical neurons loaded with H_2DCF-DA were mounted on the confocal microscope stage, and the neurons were observed by a laser scanning confocal microscope (Bio-Rad, MRC1024ES, Maylands, UK) using 488 ㎚ excitation and 510 ㎚ emission filters. The average pixel intensity of fluorescence was measured within each cell in the field and expressed in the relative units of DCF fluorescence. Values for the average staining intensity per cell were obtained using the image analyzing software supplied by the manufacturer. Challenge of H_2DCF-DA and measurement of fluorescence intensity was performed in the dark.

Statistical analysis

Data were expressed as mean ± SEM and statistical significance was assessed by one-way analysis of variance (ANOVA) with subsequent Turkey's tests. P values of <0.05 were considered to be significant.

RESULTS

PR protects neurons against neuronal death induced by H_2O_2.

To assess H_2O_2-induced neuronal cell death, the MTT assay was performed. MTT assay is extensively used as a sensitive, quantitative and reliable colorimetric assay for cell viability. Fig. 1 shows concentration-dependent decrease of MTT reduction rate by H_2O_2. The capacity of MTT reduction of cells

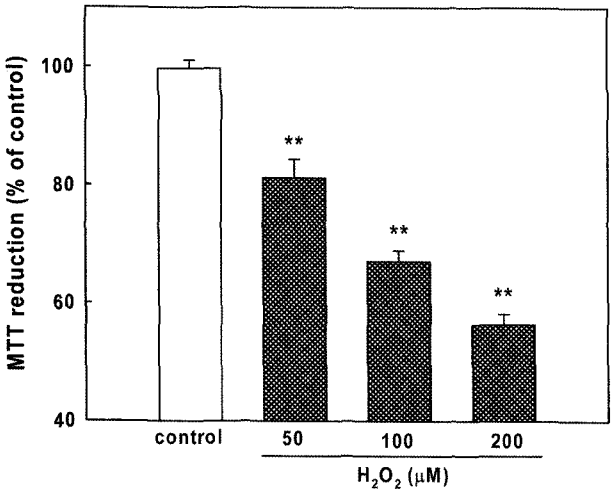

Fig. 1. Concentration-response of H_2O_2 on cell viability of cultured cortical neurons. Cell viability was measured by the MTT assay. The absorbance of non-treated cells was regarded as 100%. Results are expressed as mean ± SEM values of the data obtained from three independent experiments performed in triplicate. ** $p < 0.01$ compared to 100 μM H_2O_2.

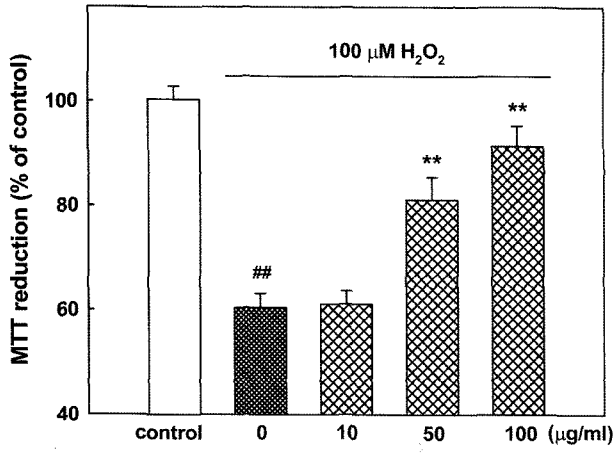

Fig. 2. Inhibitory effect of PR on H_2O_2-induced cell death in cultured cortical neurons. Cell viability was measured by the MTT assay. The absorbance of non-treated cells was regarded as 100%. Results are expressed as mean ± SEM values of the data obtained from three independent experiments performed in triplicate. ** $p < 0.01$ compared to control. ** $p < 0.01$ compared to 100 μM H_2O_2.

decreased to 81.2 ± 3.2, 67.0 ± 1.9, and 56.3 ± 1.8% by 50, 100 and 200 μM of H_2O_2, respectively. Therefore, the concentration of 100 μM was used for the determination of the H_2O_2-induced neuronal cell damage in the following experiments. Fig. 2 shows the effect of PR (10, 50 and 100 ㎍/㎖) on the H_2O_2-induced decrease of MTT reduction rate. MTT reduction

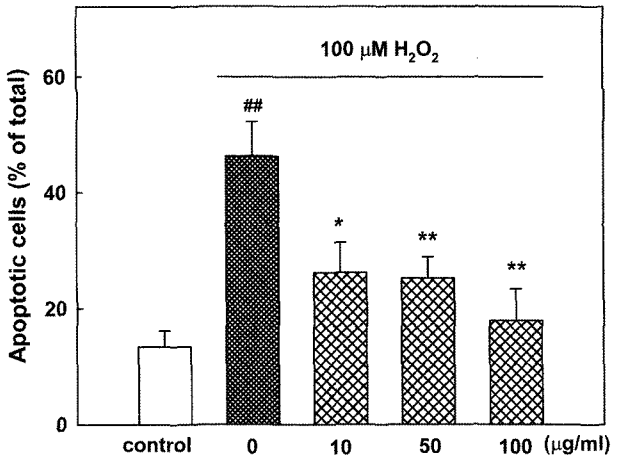

Fig. 3. Inhibitory effect of PR on H_2O_2-induced apoptosis of cultured cortical neurons as measured by Hoechst 33342 staining. Apoptotic cells produced by 100 μM H_2O_2 were counted from five to six fields per well. Results are shown as apoptotic cells as a percentage of total number of cells and expressed as mean ± SEM values of the data obtained from four independent experiments performed in two to three wells. ** $p < 0.01$ compared to control. * $p < 0.05$, ** $p < 0.01$ compared to 100 μM H_2O_2.

rate decreased to 60.4 ± 2.7% with the treatment with 100 μM H_2O_2. PR showed concentration-dependent inhibition on the H_2O_2-induced neuronal cell death, recovering cell death to 81.1 ± 4.4 and 91.4 ± 3.9% by 50 and 100 ㎍/㎖, respectively.

An additional experiment was performed with Hoechst 33342 staining to assess the neurotoxicity of H_2O_2. Cell nuclei stained by Hoechst 33342 enables the occurrence of DNA condensate to be detected, a feature of apoptosis. In neurons treated with 100 μM H_2O_2, chromatin condensation and nuclear fragmentation were observed, whereas the control culture had round blue nuclei of viable cells. The proportion of apoptotic cells was calculated and shown in Fig. 3. The treatment of neurons with 100 μM H_2O_2 induced apoptosis of 46.3 ± 5.9% of total population of cultured cerebral cortical neurons, as compared with 13.4 ± 2.8% of apoptotic neurons in control cultures. PR, 10, 50 and 100 ㎍/㎖, decreased the H_2O_2-induced apoptotic cell death to 26.2 ± 5.2, 25.2 ± 3.7 and 17.9 ± 5.5%, respectively. PR alone did not affect cell viability (data not shown).

PR inhibits H_2O_2-induced elevation of $[Ca^{2+}]_c$

The increase of $[Ca^{2+}]_c$ has been postulated to be associated with H_2O_2-induced cell death in many studies. In cultured cortical neurons, treatment with 100 μM H_2O_2 produced relatively slow and gradual increase of $[Ca^{2+}]_c$. A maximal fluorescence intensity of about 170, compared to a base of 100, with the

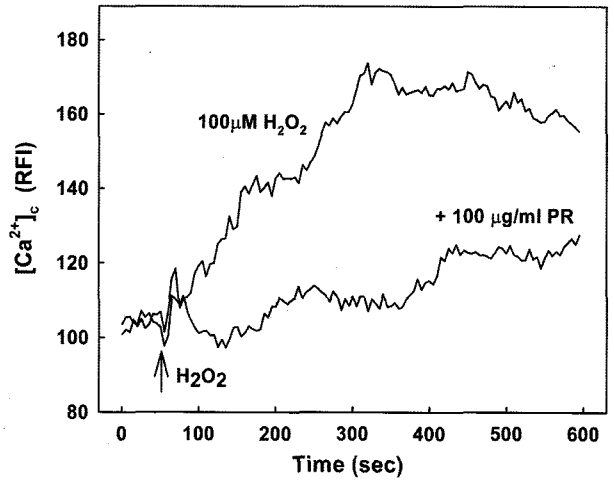

Fig. 4. Change of $[Ca^{2+}]_c$ in response to H_2O_2 in the presence or absence of PR in cultured cortical neurons. $[Ca^{2+}]_c$ was monitored using a laser scanning confocal microscope. Results are expressed as the relative fluorescence intensity (RFI). Each trace is a single cell representative from at least three independent experiments.

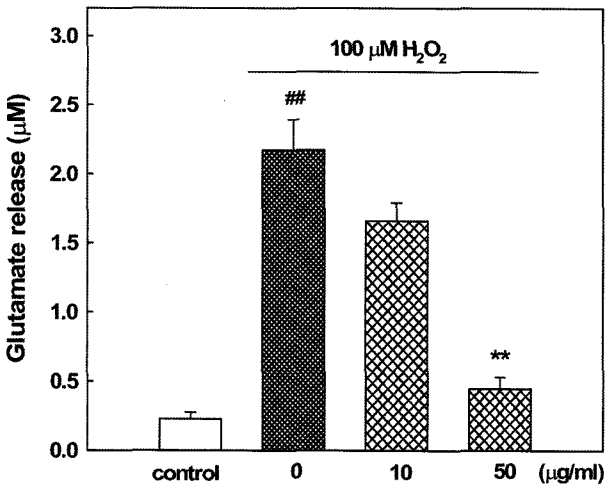

Fig. 5. Inhibitory effect of PR on H_2O_2-induced glutamate release in cultured cortical neurons. The amount of released glutamate was measured by HPLC with ECD. Results are expressed as mean ± SEM values of the data obtained in three independent experiments performed in two to three wells. ** $p < 0.01$ compared to control. ** $p < 0.01$ compared to 100 μM H_2O_2.

$[Ca^{2+}]_c$ elevation was measured about 5 min after the H_2O_2 (100 μM) application. After peaking, the fluorescence level decreased gradually. In contrast, 100 μM H_2O_2 failed to produce the similar elevation of $[Ca^{2+}]_c$ in the presence of PR (100 ㎍/㎖) throughout the measurement period (Fig. 4). PR did not affect basal $[Ca^{2+}]_c$ (data not shown).

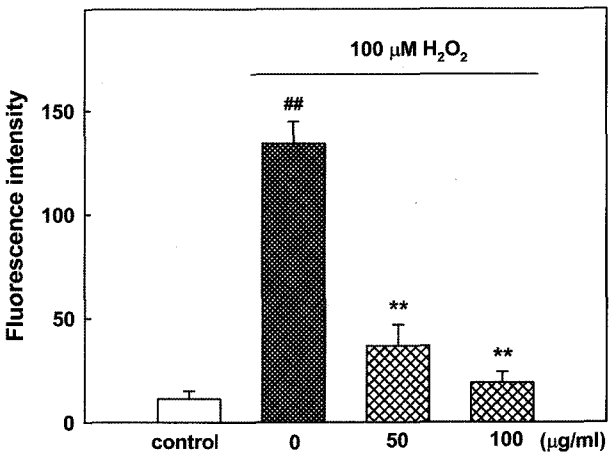

Fig. 6. Inhibitory effect of PR on H_2O_2-induced ROS generation in cultured cortical neurons. Results are expressed as mean ± SEM of relative fluorescence intensity obtained from three independent experiments performed in two to three wells. ** $p < 0.01$ compared to control. ** $p < 0.01$ compared to 100 μM H_2O_2.

PR inhibits H_2O_2-induced elevation of glutamate release

Glutamate released into the extracellular medium for 2 h was quantified 15 min after the incubation of cells with 100 μM H_2O_2. As shown in Fig. 5, 100 μM H_2O_2 markedly elevated the basal glutamate level from 0.23 ± 0.05 to 2.17 ± 0.22 μM. PR (50 ㎍/㎖) strongly blocked the H_2O_2-induced elevation of glutamate release showing 0.45 ± 0.08 μM. However, the inhibitory effect by 100 μM PR was not detected (data not shown).

PR inhibits H_2O_2-induced ROS generation

H_2O_2 increased the glutamate release and the concentration of $[Ca^{2+}]_c$. Furthermore, the pathological condition induced by H_2O_2 is associated with accelerated formation of ROS. In H_2DCF-DA-loaded cortical neurons, 100 μM H_2O_2 increased the fluorescence intensity, indicating the generation of ROS. The fluorescence intensity in H_2O_2-treated cells increased to 134.0 ± 10.5 by about 12 folds compared to control cells of 11.4 ± 3.7. The H_2O_2-induced increase of generation was significantly inhibited by PR showing 36.6 ± 10.2 and 19.0 ± 5.1 of fluorescence intensity with 50 and 100 ㎍/㎖, respectively (Fig. 6). PR alone did not show direct reaction with H_2DCF-DA to generate fluorescence.

DISCUSSION

H_2O_2 produces neuronal death by inducing a delayed accumulation of extracellular glutamate and NMDA receptor stim-

ulation, with both effects being mediated by · OH (Mailly *et al.*, 1999; Halliwell, 1992). Exposure to H_2O_2 causes an increase in $[Ca^{2+}]_i$, which is blocked by Na^+ channel antagonists as well as Ca^{2+} channel antagonists, and produces ROS concomitant with NMDA receptor stimulation in cultured neuronal cells (Mailly *et al.*, 1999; Su *et al.*, 1999; Wang & Joseph, 2000). Thus, the neurotoxic effect of H_2O_2 was strongly reduced by antagonists of NMDA receptors and Ca^{2+} channel antagonists (Mailly *et al.*, 1999). We also demonstrated the protections of MK-801, verapamil, an L-type Ca^{2+} channel blocker, and N^G-nitro-L-arginine methyl ester ($_L$-NAME), a nitric oxide synthase inhibitor against the H_2O_2-induced neuronal death in cultured rat neurons (Koh *et al.*, 2003; Lee *et al.*, 2005). Confirming these reports, the present study demonstrated that H_2O_2 stimulated glutamate release, elevation of $[Ca^{2+}]_c$ and ROS generation which in turn led to neuronal cell death in cultured cortical neurons. Many researchers have demonstrated that the neuronal loss following a transient exposure to H_2O_2 of cultured neuronal cells results from an apoptotic process (Whittemore *et al.*, 1995; Gardner *et al.*, 1996; Mailly *et al.*, 1999). Cultured cortical neurons exposed to H_2O_2 transiently followed by 15 h post-incubation with H_2O_2-free medium showed increased chromatin condensation, a typical feature of apoptotic cell death, in the present work.

The present study provides evidence that H_2O_2-induced injury to rat cortical neurons can be prevented by PR. PR was able to reduce the H_2O_2-induced neuronal apoptotic death, $[Ca^{2+}]_c$ increase, glutamate release and ROS generation. Neuronal cells exposed to H_2O_2 produce membrane depolarization depending on the increased permeability to Na_+ ions. Na_+ ions increased by the depolarization further increase the opening of Na_+ channels, and subsequently cause a great Ca_{2+} influx via the voltage-dependent Ca^{2+} channels (VDCC) (Halliwell, 1992; Wang & Joseph, 2000). In the present study, H_2O_2 elicited gradual and significant $[Ca^{2+}]_c$ increase, which was blocked by PR. Therefore, it is strongly suggested that the neuroprotective effect of PR is mainly due to the inhibition on the H_2O_2-induced increase of $[Ca^{2+}]_c$ through VDCC. In support of this hypothesis, the inhibitory action of L-type Ca^{2+} current by paeoniflorin, a major constituent of Paeony root, in NG108-15 neuronal cells has been reported (Tsai *et al.*, 2005). PR also significantly inhibited the H_2O_2-induced glutamate elevation. This result indicates that the sustained inhibition on $[Ca^{2+}]_c$ elevation by PR resulted in the decrease of the H_2O_2-induced glutamate release. The elucidation of the variety of events occurring downstream of neuronal Ca^{2+} overloading is still a matter for further research. ROS generation undoubtedly takes place in glutamate neurotoxicity and is likely due to Ca^{2+} influx in the cytosol (Pereira & Oliveira, 2000). PR decreased the H_2O_2-induced increase of ROS generation. It was not elucidated whether PR suppressed ROS generation through the inhibition of $[Ca^{2+}]_c$ increase, or vice versa, in the present study. In a previous report in my laboratory, it was demonstrated that $_L$-NAME, an inhibitor of ROS generation, failed to show an inhibition on the H_2O_2-induced $[Ca^{2+}]_c$ increase in the earlier 20 min after the treatment, while verapamil, a Ca^{2+} channel antagonist, completely blocked ROS generation for the 20 min incubation in cultured cerebellar granule neurons (Koh *et al.*, 2003). Therefore, it is suggested that PR inhibited the H_2O_2-induced ROS generation via the blockade of $[Ca^{2+}]_c$ increase. It is thus concluded that PR may prevent the H_2O_2-induced apoptosis of neuronal cell by interfering with the increase of $[Ca^{2+}]_c$, and then by inhibiting glutamate release and generation of ROS.

PR have been reported to have anticonvulsant, anxiolytic, and memory enhancing effects in animals (Sugaya *et al.*, 1991; Watanabe *et al*, 1991; Ohta *et al.*, 1993; Nizami & Jafri, 2005). These results imply the possibility of PR having neuroprotective effect. Furthermore, PR inhibited the H_2O_2-induced apoptosis of a peripheral cells (Lee *et al.*, 2003). PR completely blocked H_2O_2-induced neuronal cell death in the present study. This PR-induced neuroprotection might be related to the prevention of oxidative damage in neurodegenerative diseases such as stroke, Alzheimer disease and Parkinson disease. PR has been known to contain many pharmacologically active components including oxypaeoniflorin, albiflorin, benzoylpaeoniflorin, paeonol and paeoniflorin. It, however, must be made further studies to clarify the active components to which PR-induced neuroprotection is attributable.

In conclusion, it was demonstrated a novel pharmacological action of PR and its mechanism in the present study. The protection against H_2O_2-induced neurotoxicity by PR may help to explain its inhibitory action on oxidative injury-linked various neurodegenerative pathophysiological conditions.

ACKNOWLEDGEMENTS

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