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Ganoderma atrum Polysaccharide Protects Cardiomyocytes Against Anoxia/Reoxygenation-Induced Oxidative Stress by Mitochondrial Pathway

Wen-Juan Li,[1] Shao-Ping Nie,[1] Yi Chen,[1] Ming-Yong Xie,[1*] Ming He,[1,2] Qiang Yu,[1] and Yan Yan[1]

[1] *State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang 330047, China*

[2] *Department of Pharmaceutical Science, Nanchang University, Nanchang 330006, China*

ABSTRACT

It is now well established that oxidative stress plays a causative role in the pathogenesis of anoxia/reoxygenation (A/R) injury. *Ganoderma atrum* polysaccharide (PSG-1), the most abundant component isolated from *G. atrum*, has been shown to possess potent antioxidant activity. The goals of this study were to investigate the effect of PSG-1 against oxidative stress induced by A/R injury and the possible mechanisms in cardiomyocytes. In this work, primary cultures of neonatal rat cardiomyocytes pretreated with PSG-1 were subjected to A/R and subsequently monitored for cell viability by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The levels of intracellular reactive oxygen species (ROS), apoptosis, and mitochondrial membrane potential ($\Delta\psi_m$) were determined by flow cytometry. Western blot analysis was used to measure the expression of cytochrome *c*, Bcl-2 family, and manganese superoxide dismutase (MnSOD) proteins, and the activities of caspase-3 and caspase-9 were determined by a colorimetric method. The results showed that PSG-1 protected against cell death caused by A/R injury in cardiomyocytes. PSG-1 reduced the A/R-induced ROS generation, the loss of mitochondrial membrane potential ($\Delta\psi_m$), and the release of cytochrome *c* from the mitochondria into cytosol. PSG-1 inhibited the A/R-stimulated activation of caspase-9 and caspase-3 and alteration of Bcl-2 family proteins. Moreover, PSG-1 significantly increased the protein expression of MnSOD in cardiomyocytes. These findings suggest that PSG-1 significantly attenuates A/R-induced oxidative stress and improves cell survival in cardiomyocytes through mitochondrial pathway. J. Cell. Biochem. 110: 191–200, 2010.

KEY WORDS: *Ganoderma atrum* POLYSACCHARIDE; ANOXIA/REOXYGENATION; OXIDATIVE STRESS; CARDIOMYOCYTE; APOPTOSIS

Cardiovascular disease is a leading cause of death worldwide and remains one of the major killers in modern society. It can be initiated by multiple factors including ischemia/reperfusion injury. It is now generally accepted that oxidative stress resulting from reactive oxygen species (ROS) that are generated in cardiomyocytes subjected to ischemia/reperfusion plays an important role in the development of heart failure and may contribute to promote cell death (apoptosis) [Takano et al., 2003; Zhao, 2004]. During ischemia/reperfusion, ROS can alter the structural and functional integrity of cells by a variety of mechanisms, including lipid peroxidation, proteolysis, and shearing of the nuclear material [Ferrari et al., 1991; Zhang and Herman, 2006.]. Appropriate antioxidant strategies could be particularly useful to limit ROS production and ROS-induced alterations and hence to protect ischemic reperfused myocardium [Moens et al., 2005; Shao et al., 2009; Zhu et al., 2009]. Thus, there is continued interest in defining and discovering new antioxidants.

Ganoderma atrum is one of the most popular medicinal/nutritional fungi with a long history in Asian countries [Gao et al., 2005]. Scientific information demonstrating the effective delay in aging and extension of lifespan encompasses a variety of biological functions such as antioxidation and cardiovascular support. The polysaccharide is regarded as the major bioactive substances in *G. atrum* [El-Mekkawy et al., 1998; Paterson, 2006; Chen et al., 2007b; Hsu et al., 2009]. However, there is rare information about the protection of polysaccharide from *G. atrum*

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*Correspondence to: Prof. Ming-Yong Xie, State Key Laboratory of Food Science and Technology, Nanchang University, 235 Nanjing East Road, Nanchang 330047, China. E-mail: myxie@ncu.edu.cn

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against oxidative stress-related ischemia/reperfusion injury and the mechanism in this pathological condition. Recently, a polysaccharide, named PSG-1 with a purity of >99.8%, was isolated from *G. atrum* in our laboratory. Its primary structural features and molecular weight were characterized and has been shown to possess potent antioxidant activity [Chen et al., 2008].

Numerous studies have demonstrated that many natural products could attenuate oxidative stress in cardiomyocytes under anoxia/reoxygenation (A/R) conditions as an in vitro ischemia/reperfusion model [Karliner, 2004; Psotová et al., 2004]. However, molecular mechanisms for the cardioprotection effect of natural products are far from clear. Apoptosis is a physiologic process of cell death that plays a critical role in a variety of biologic systems, which has been identified as providing an important molecular basis for both the initiation and progression of ischemia/reperfusion injury [Saitoh et al., 2003; Stein, 2005; Lv et al., 2008; Pratap et al., 2009]. There are distinct mechanisms that execute apoptosis according to various different apoptotic stimuli, and these are classified into the mitochondria-dependent pathway (intrinsic pathway) and the death receptor-dependent pathway (extrinsic pathway) [Hou and Hsu, 2005]. The mitochondria-dependent apoptotic pathway is stimulated in ischemia/reperfusion injury and induces generation of ROS, loss of mitochondrial transmembrane potential ($\Delta\Psi_m$), release of cytochrome *c* from the impaired mitochondria to cytosol, and activation caspase-9. Activated caspase-9 leads to the activation of caspase-3, which subsequently contributes to apoptosis [Yao et al., 2008; Li et al., 2009].

Based on these observations, we hypothesize that PSG-1 can protect cardiomyocytes from oxidative stress-induced apoptosis and may have benefits in the treatment of ischemia/reperfusion with oxidative stress. Therefore, the present study is designed to investigate the protective effect of PSG-1 against oxidative stress induced by A/R and further examine the molecular mechanisms in cardiomyocytes.

MATERIALS AND METHODS

ANIMALS

One to 3-day-old Sprague–Dawley rats (Grade II, Certificate Number SCXK (GAN) 2006-0001) were purchased from Jiangxi College of Traditional Chinese Medicine, Jiangxi, China. All animals used in this study were cared for in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health (NIH Publication No. 85-23, revised 1996), and all procedures were approved by Nanchang University Medical College Animal Care Review Committee.

CHEMICALS AND REAGENTS

Cell culture products were obtained from Life Technologies (Paisley, Scotland); 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and Rhodamine-123 (Rho123) were purchased from Molecular Probes, Inc. (OR); Annexin V-FITC apoptosis detection kits were obtained from BD Biosciences (SD); Anti-Bcl-2, anti-Bax, anti-cytochrome *c*, and anti-β-actin primary antibodies, as well as the HRP-linked secondary antibody, were from Santa Cruz Biotechnology (Santa Cruz, CA).

ISOLATION AND CHEMICAL ANALYSIS OF PSG-1

Standard procedure was followed for the isolation of the polysaccharide from *G. atrum* (PSG-1) with a purity of >99.8%. Briefly, the polysaccharide fractions were prepared from *G. atrum*, which were collected from Ganzhou, Jiangxi Province, China. All extracts were finally pooled, and the polysaccharide-enriched fractions were precipitated by the addition of 80% (v/v) ethanol. The polysaccharide fraction was further purified by gel filtration chromatography. Its primary structural features and molecular weight were characterized by infrared spectrometry, gas chromatography, size exclusion chromatography, amino acid analyzer, and high-performance liquid chromatography (HPLC). The data obtained indicated that PSG-1 contains 10.1% of protein and 17 general amino acids and it is rich in glutamic acid, asparagic acid, alanine, glycine, threonine, and serine. It was mainly composed of mannose, galactose, and glucose in a molar ratio of 1:1.28:4.91, with an average molecular weight of about 1,013 kDa [Chen et al., 2008].

PRIMARY CULTURE OF NEONATAL RAT CARDIOMYOCYTES AND A/R INJURY MODEL

Primary cultures of neonatal rat cardiomyocytes were prepared from the ventricles of 1- to 3-day-old Sprague–Dawley rats according to Reinecke et al. [1999], with some modifications. Briefly, the hearts were harvested and placed in calcium- and bicarbonate-free Hanks' buffer with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The ventricles were minced. The tissue fragments were dissociated by treatment with 0.1% trypsin 5–6 times (37°C), then filtered and centrifuged for 8 min (120*g*, 4°C), and finally resuspended in a nutritive medium consisting of Eagle's minimum essential medium supplemented with 15% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Resuspended cells were placed in a Petri dish in a humidified incubator (5% CO_2, 37°C) for 2 h to promote early adherence of fibroblasts. Nonadherent cells were counted with a hemocytometer, and the final myocyte cultures were found to contain >90 cardiomyocytes. Approximately 2×10^6 cells in the nutritive medium were pipetted into six-well gelatin-coated plates and incubated for 3–4 days before the experiment. The in vitro model of A/R used in the present study was in accordance with a previously reported method [Chen et al., 2009].

EXPERIMENTAL GROUPS AND PROTOCOLS

A known number of cultured neonatal rat cardiomyocytes were randomly and homogeneously distributed into different experimental groups as follows, and each group included two parallel wells for four replicate experiments: (i) *Control group*: cardiomyocytes were incubated in aerobic tyrode solution during the entire experimental period; (ii) *A/R group*: cardiomyocytes were incubated with anaerobic simulated ischemia buffer for 3 h of anoxia followed by reoxygenation for 2 h as described above; (iii) *PSG-1-20 group*: PSG-1 (20 μg/ml) was applied 30 min prior to A/R and maintained throughout the experiment; (iv) *PSG-1-50 group*: PSG-1 (50 μg/ml) was applied 30 min prior to A/R and maintained throughout the experiment; (v) *PSG-1-100 group*: PSG-1 (100 μg/ml) was applied 30 min prior to A/R and maintained throughout the experiment.

CELL VIABILITY ASSAY

Cardiomyocytes were seeded in 96-well tissue culture plates at an initial concentration of 1×10^5 cells/ml and incubated with different concentrations of PSG-1 alone (0, 3.13, 6.25, 12.5, 25, 50, 75, 100, 150, and 200 μg/ml) or pretreated with different concentrations of PSG-1 (0, 3.13, 6.25, 12.5, 25, 50, 75, 100, 150, and 200 μg/ml) for 30 min and treated with A/R. After incubation for the indicated time, cells were treated by the addition of 20 μl 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (5 mg/ml) dye to each well. After additional 4 h incubation, the growth medium was removed and the formazan crystals, formed by oxidation of the MTT dye, were dissolved with 150 μl DMSO. The absorbance was measured at 490 nm and the cell survival ratio was expressed as a percentage of the control.

FLOW CYTOMETRIC ANALYSIS OF APOPTOSIS

Apoptosis was determined by Annexin V and PI double staining. Cardiomyocytes were centrifuged to remove the medium, washed with PBS, and stained with Annexin V and PI in binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$). Ten thousand events were collected for each sample. Stained cells were analyzed using a FACStar Plus flow cytometry in the FL1-H and FL2-H channels.

MEASUREMENT OF ROS GENERATION

Cardiomyocytes were treated with different concentrations of PSG-1 and incubated for prescribed period. After incubation period, cardiomyocytes were harvested and washed with cold phosphate-buffered saline. Washed cells were further incubated with10 μM of DCFH-DA at 37°C for 20 min in dark. Cells were washed in PBS, resuspended in FACS buffer (phosphate-buffered saline with 1% bovine serum albumin and 0.01% sodium azide), and analyzed by flow cytometry with FACSort cell sorter (Becton Dickinson).

DETERMINATION OF MITOCHONDRIAL MEMBRANE POTENTIAL ($\Delta\psi_m$)

To determine the change of $\Delta\psi_m$ in cardiomyocytes, flow cytometry was applied using Rho123 staining. The cardiomyocytes (1×10^6 cells) were harvested and washed with ice-cold PBS twice by centrifugation at 1,000*g* for 5 min, and then Rho123 (10 μg/ml) was added to the cells. The tubes were vortexed gently and incubated at 37°C for 20 min. Rho123 fluorescence was measured using a FACStar Plus cell sorter with excitation and emission wavelengths of 488 and 530 nm, respectively.

PREPARATION OF MITOCHONDRIAL AND CYTOSOLIC FRACTIONS

Mitochondrial and cytosolic fractions of cells were prepared using a mitochondrial/cytosolic fractionation kit (Biovision, CA). Cells at 1×10^7 with or without A/R injury were harvested by centrifugation at 700*g* for 5 min and washed twice with cold phosphate-buffered saline. Afterwards, the cells were resuspended in a 250 μl of extraction buffer containing protease inhibitor mixture and dithiothreitol. After incubation on ice for 30 min, the cells were homogenized using a Kontes Dounce tissue grinder on ice. Homogenizations were centrifuged at 700*g* for 10 min at 4°C, and the supernatant was collected. Then the collected supernatant was centrifuged again at 10,000*g* for 30 min at 4°C. The resulting supernatants were harvested and designated as cytosolic fractions, and the pellets were resuspended in an appropriate buffer and designated as mitochondrial fractions.

PREPARATION OF TOTAL CELL EXTRACTS

Upon completion of the experimental periods, cardiomyocytes were rinsed with ice-cold PBS and then lysed in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 1 mM NaF, 1 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The cells were incubated for 45 min at 4°C in lysis buffer followed by centrifugation (12,000*g*, 4°C for 10 min). The supernatants were used as total cell extracts.

WESTERN BLOT ANALYSIS

Samples were mixed with sample buffer (Laemmli sample buffer, Bio-Rad) and then boiled for 5 min and loaded onto 12% or 15% SDS–polyacrylamide gels. After they were electroblotted onto PVDF membranes, the sample blots were blocked for 2 h with 5% nonfat milk powder in TBST solution (25 mM Tris–HCl [pH 7.6], 0.2 M NaCl, and 0.1%, v/v, Tween-20) at room temperature. Incubated with the antibodies overnight at 4°C, the membranes were blotted with different antibodies in Tris-buffered saline (pH 7.6) containing 0.1% Tween-20 (TBST). To assure equivalent protein loading, the membranes were also incubated with anti-β-actin antiboby, and subsequently with a corresponding horseradish peroxidase-conjugated second antibody IgG and developed using Chemiluminescence Reagent Plus. The scan densitometric analysis was carried out using GDS-8000 UVP photo scanner and LAB WOEK45 Image software (Bio-Rad).

MEASUREMENT OF CASPASE ACTIVITIES

Caspase activities were evaluated by the use of caspase-3 and caspase-9 colorimetric assay kit (Biovision). The assay is based on spectrophotometric detection of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the labeled substrate DEVD-*p*NA. The absorbance at 405 nm of the released *p*NA was monitored in a spectrophotometer.

STATISTICAL ANALYSIS

Values are expressed as means ± SEM. One-way analysis of variance followed by the Student–Newman–Keuls test was applied to calculate the statistical significance between various groups. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

EFFECTS OF PSG-1 ON A/R-INDUCED CELL DAMAGE IN CARDIOMYOCYTES

The quantitative release of LDH and measures of cell viability are usually used as indexes of cardiomyocyte injury. To observe the effects of PSG-1 on cardiomyocytes injury induced by A/R, we performed a series of dose–response assays to determine the working concentration that led to a consistent degree of cytotoxicity. The cells were exposed to different concentrations of PSG-1 (0, 3.13,

6.25, 12.5, 25, 50, 75, 100, 150, and 200 μg/ml) for 24 h, and the MTT assay showed no loss of cell viability (data not shown). The cardiomyocytes pretreated with PSG-1 (0–200 μg/ml) significantly prevented the loss of viability that resulted from A/R in a dose-dependent manner up to 100 μg/ml (Fig. 1A) and the effective half maximal concentration for protection (EC50) was 20 μg/ml; Therefore, we used concentrations of PSG-1 (20, 50, or 100 μg/ml) for our subsequent studies. Moreover, cardiomyocytes incubated with PSG-1 (20, 50, or 100 μg/ml) significantly suppressed the release of LDH activity that result from A/R (Fig. 1B).

To examine whether PSG-1 protects against A/R-induced apoptosis in cardiomyocytes, flow cytometric analysis was used to quantify the rate of cell apoptosis using double staining of Annexin V and PI (Fig. 2). A significant increase of apoptosis was observed in cardiomyocytes treated with A/R, compared with control cells. However, treatment with PSG-1 (20, 50, or 100 μg/ml) showed a significant resistance in apoptosis in cardiomyocytes undergoing A/R. These results suggest that PSG-1 is a potent cardioprotective agent against A/R injury.

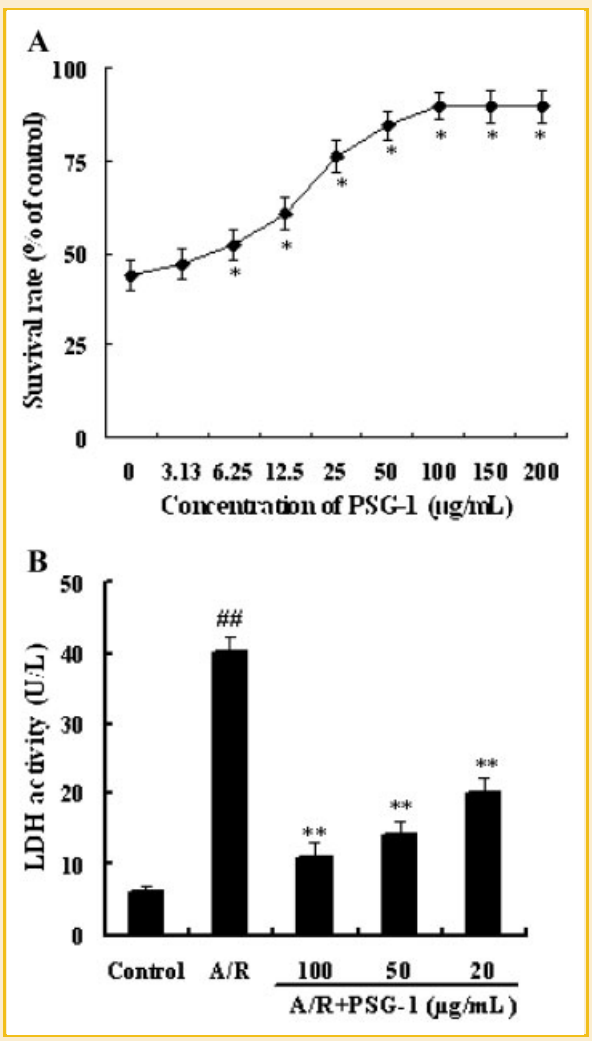

Fig. 1. PSG-1 protects against A/R-induced cell death in cardiomyocytes. A: After the cardiomyocytes were incubated with PSG-1 treatment (0, 3.13, 6.25, 12.5, 25, 50, 75, 100, 150, and 200 μg/ml) undergoing A/R, the viability was measured by MTT assay. B: Effects of PSG-1 (20, 50, or 100 μg/ml) on LDH activity in cardiomyocytes subjected to A/R. Eight independent experiments were done and all gave similar results. $^{*}P < 0.05$ versus untreated control group; $^{\#\#}P < 0.01$ versus control group; $^{**}P < 0.01$ versus A/R group.

EFFECTS OF PSG-1 ON INTRACELLULAR ROS LEVELS IN CARDIOMYOCYTES UNDERGOING A/R

Mitochondrial production of ROS also seems to play a role in cell death [Wang et al., 2000]. ROS, the byproducts of normal cellular oxidative processes, have been suggested to regulate the process involved in the initiation of apoptotic signaling. We conducted experiments to analyze ROS of cardiomyocytes. Intracellular ROS levels were assessed by measuring DCF fluorescence intensity. As shown in Figure 3A,B, A/R caused a rapid and significant increase in DCF fluorescence. However, when PSG-1 (20, 50, or 100 μg/ml) was applied 30 min prior to A/R and maintained throughout the experiment, it caused an attenuation in DCF fluorescence in a dose-dependent manner. These findings indicate that PSG-1 has a significant ability to scavenge ROS.

EFFECTS OF PSG-1 ON $\Delta\psi_m$ AND THE RELEASE OF MITOCHONDRIAL CYTOCHROME *c* INTO THE CYTOSOL IN CARDIOMYOCYTES UNDERGOING A/R

Recent studies have suggested that mitochondria play an essential role in death signal transduction [Kluck et al., 1997]. Mitochondrial changes, including permeability transition pore opening and the collapse of the $\Delta\psi_m$, result in the release of cytochrome *c* into the cytosol, which subsequently causes apoptosis by the activation of caspases. To examine whether the $\Delta\psi_m$ is involved in A/R-induced apoptosis or changed by PSG-1 in the presence of A/R, $\Delta\psi_m$ was detected with Rho123 staining by flow cytometric analysis. Mitochondrial energization induces quenching of Rho123 fluorescence and the rate of fluorescence decay is proportional to the mitochondrial membrane potential. As shown in Figure 3C,D, A/R caused a marked loss of Rho123 fluorescence ($P < 0.01$). The collapse of $\Delta\psi_m$ was reversed by PSG-1 pretreatment group in Rho123 fluorescence in a dose-dependent manner. These results suggest that the A/R is capable of inducing mitochondrial dysfunction and that $\Delta\psi_m$ loss was reversed by PSG-1 pretreatment.

Mitochondrial dysfunction releases several apoptogenic proteins, most notably cytochrome *c*, from the mitochondria into the cytosol. Western blot revealed that A/R led to an accumulation of cytochrome *c* in the cytosol (Fig. 4). The observed cytochrome *c* in the cytosol was significantly reduced when the cells were pretreated with PSG-1 before A/R treatment. These results indicate that A/R causes a mitochondrial dysfunction via the mitochondrial pathway and PSG-1 is protective against such dysfunction.

EFFECTS OF PSG-1 ON THE ACTIVITIES OF CASPASE-9 AND CASPASE-3 INDUCED BY A/R IN CARDIOMYOCYTES

Caspases are believed to play a central role in mediating various apoptotic responses. To examine whether caspases are activated in A/R-induced apoptosis, we examined the activities of caspase-9 and caspase-3. Caspases were measured using the synthetic caspase substrates DEVD-*p*NA and LEHD-*p*NA. Both caspase-3

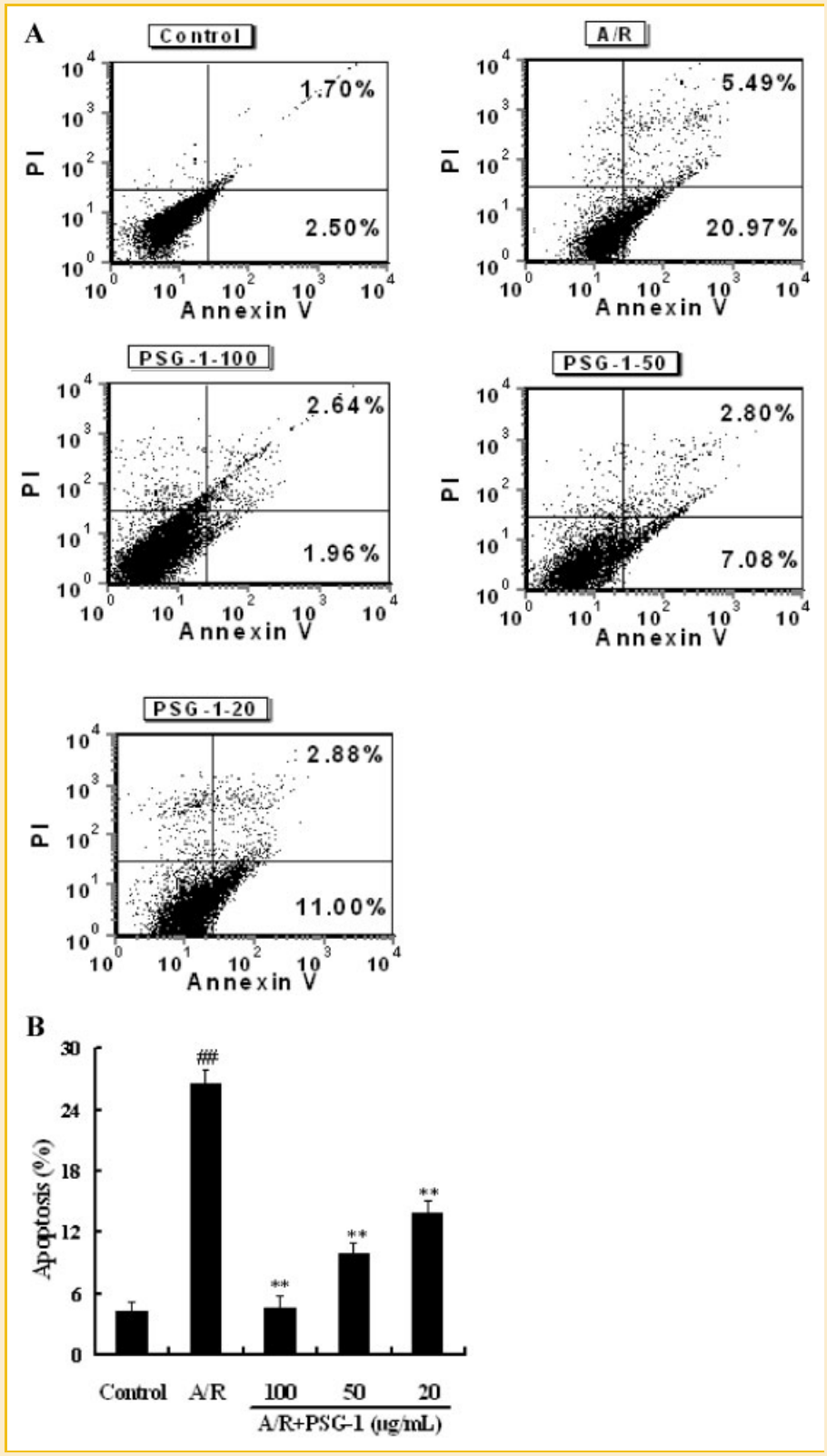

Fig. 2. Effects of PSG-1 on the apoptosis in cardiomyocytes subjected to A/R. A: Detection of apoptotic cells by AnnexinV and PI double staining. Cardiomyocytes were treated with or without 20, 50, or 100 μg/ml PSG-1, respectively, stained with AnnexinV and PI labeling and analyzed by flow cytometry. B: Column bar graph of apoptosis. Eight independent experiments were done and all gave similar results. $^{\#\#}P < 0.01$ versus control group; $^{**}P < 0.01$ versus A/R group.

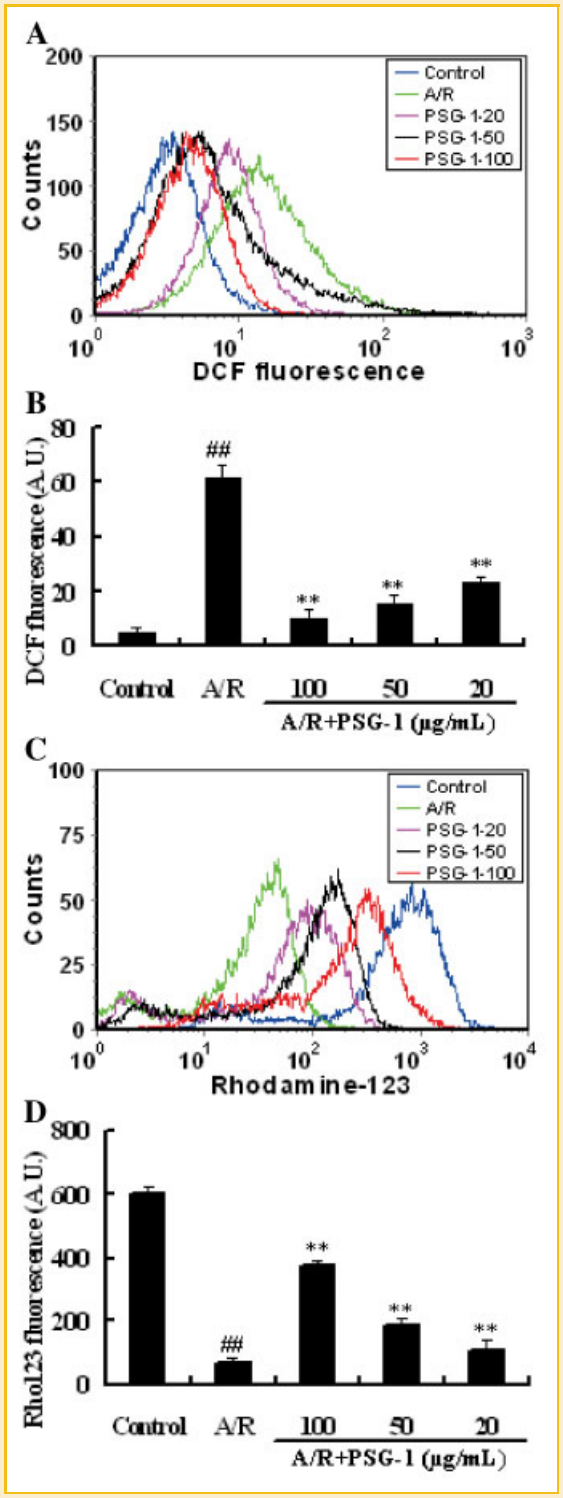

Fig. 3. Effect of PSG-1 on the generation of ROS and the loss of $\Delta\psi_m$ in cardiomyocytes exposed to anoxia/reoxygenation (A/R). A,B: Flow cytometric analysis of ROS generation as estimated by the fluorescence of 2′,7′-dichloro-fluorescein (DCF). A: Flow cytometric histograms of DCF fluorescence in cardiomyocytes. B: Column bar graph of cell fluorescence for DCF. C,D: Flow cytometric analysis of $\Delta\psi_m$ as estimated by the Rho123 fluorescence. C: Flow cytometric analysis of $\Delta\psi_m$ as estimated by the Rho123 intensity. D: Column bar graph of cell fluorescence for Rho123. Eight independent experiments were done and all gave similar results. $^{\#\#}P < 0.01$ versus control group; $^{**}P < 0.01$ versus A/R group.

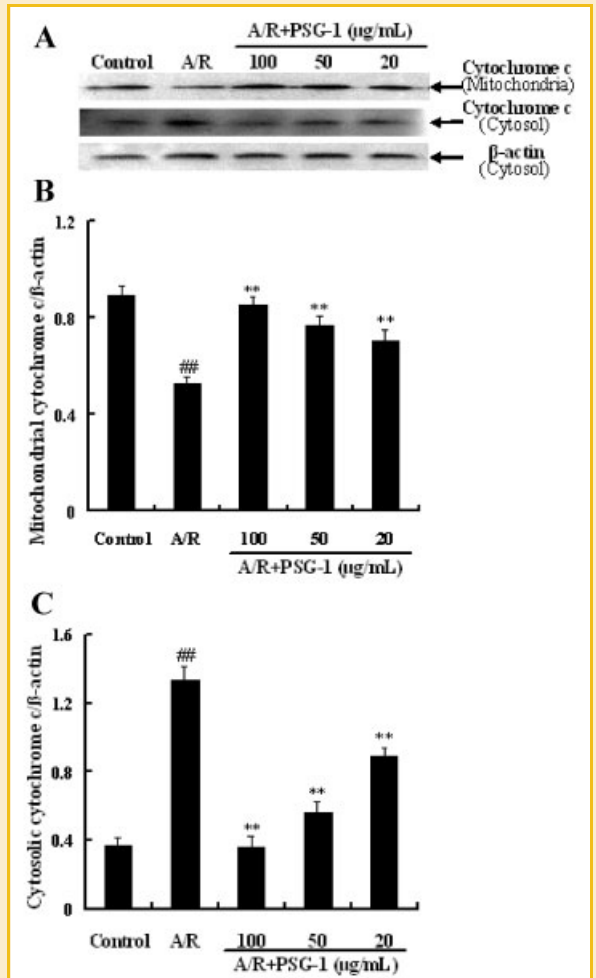

Fig. 4. Effect of PSG-1 on cytochrome *c* release in cardiomyocytes exposed to A/R. A: The cytosolic and mitochondrial proteins were analyzed by Western blot with anti-cytochrome *c* and anti-β-actin antibodies. B, C: Blots were scanned and expression of cytosolic cytochrome *c*, mitochondrial cytochrome *c*, and β-actin was quantified by densitometric analysis. The ratios for these proteins are shown. Eight independent experiments were done and all gave similar results. $^{\#\#}P < 0.01$ versus control group; $^{**}P < 0.01$ versus A/R group.

and caspase-9 increased during A/R. However, PSG-1 significantly blocked the activation of caspase-9 and caspase-3 induced by A/R in dose-dependent manner (Fig. 5). These results suggest that PSG-1 protects against A/R-induced apoptosis associated with the inhibition of caspase-3 and caspase-9 in cardiomyocytes.

EFFECTS OF PSG-1 ON THE EXPRESSION OF Bcl-2 FAMILY PROTEINS IN CARDIOMYOCYTES UNDERGOING A/R

To determine whether PSG-1 protects against A/R-induced apoptosis in cardiomyocytes by modulating Bcl-2 family, the protein levels of Bax and Bcl-2 were examined by Western blot analysis (Fig. 6). A/R induced the loss of Bcl-2 from the mitochondria. PSG-1 pretreatment blocked the loss of Bcl-2 from the mitochondria. The pro-apoptotic Bax molecule translocates and homodimerizes in the mitochondrial membrane, thereby promoting

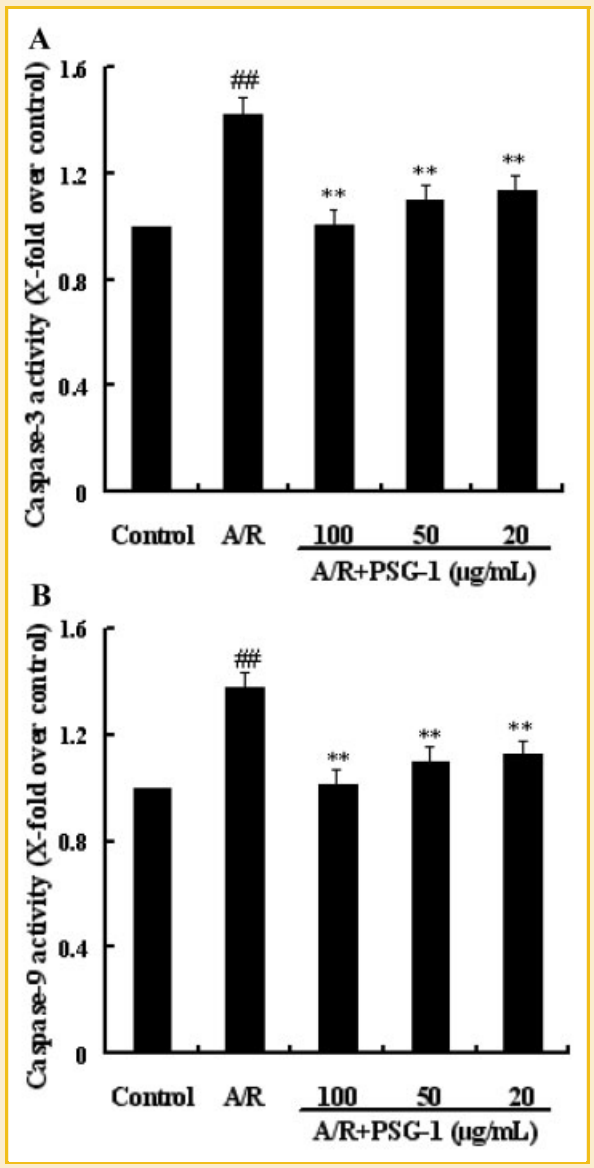

Fig. 5. Effect of PSG-1 on caspase-3 and caspase-9 activities in cardiomyocytes exposed to A/R. A,B: Quantitative analysis of caspase-3 and caspase-9 activities. Eight independent experiments were done and all gave similar results. $^{\#\#}P < 0.01$ versus control group; $^{**}P < 0.01$ versus A/R group.

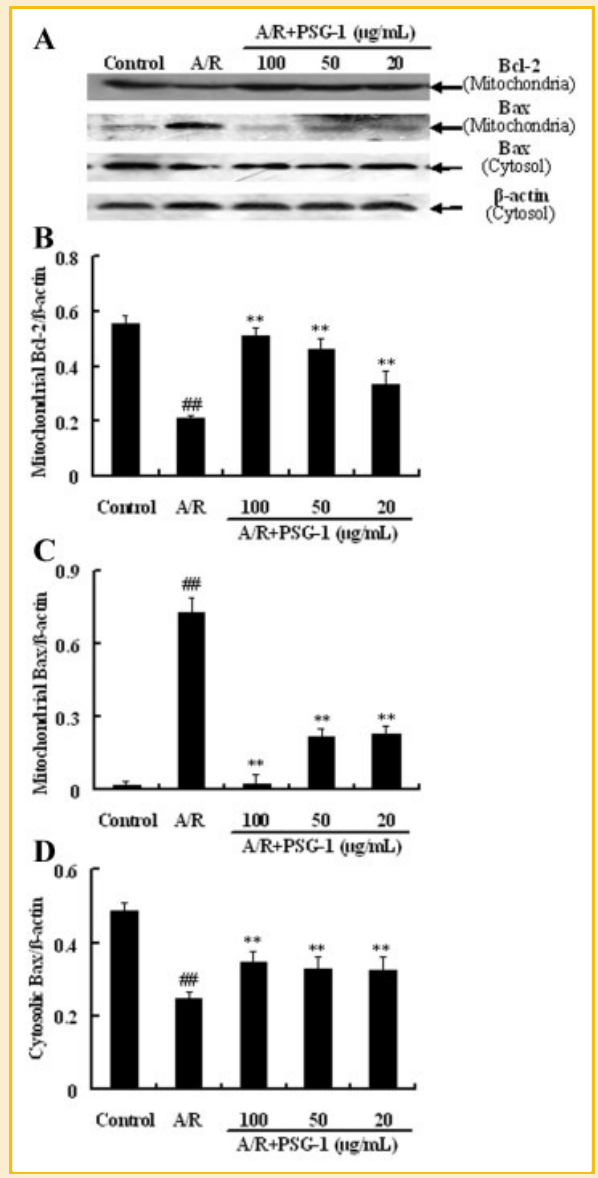

Fig. 6. Effect of PSG-1 on the expression of Bcl-2 family in cardiomyocytes exposed to A/R by Western blot analysis. A: The cytosolic and mitochondrial proteins were analyzed by Western blot with anti-β-actin, Bax, and Bcl-2 antibodies. B,C,D: Blots were scanned and expression of mitochondrial Bcl-2, cytosolic Bax, mitochondrial Bax, and β-actin was quantified by densitometric analysis. The ratios for these proteins are shown. Eight independent experiments were done and all gave similar results. $^{\#\#}P < 0.01$ versus control group; $^{**}P < 0.01$ versus A/R group.

apoptosis by disrupting mitochondrial function [Gross et al., 1999]. Western blot analysis revealed that mitochondrial Bax protein increases after the exposure of cardiomyocytes to A/R, followed by a decrease in cytosolic Bax. PSG-1 pretreatment blocked the translocation of Bax from cytosol to mitochondria. These data indicate that PSG-1 preserves A/R-induced loss of Bcl-2 from mitochondria and Bax translocation in cardiomyocytes.

EFFECTS OF PSG-1 ON THE EXPRESSION OF MANGANESE SUPEROXIDE DISMUTASE (MnSOD) IN CARDIOMYOCYTES UNDERGOING A/R

MnSOD, the primary antioxidant enzyme that scavenges superoxide radicals in mitochondria, is essential for the survival of aerobic life. The expression of MnSOD in cardiomyocytes was elucidated by Western blot analysis. As shown in Figure 7, the data indicated that the expression of MnSOD significantly increased in PSG-1 (20, 50, or 100 μg/ml) pretreatment group, compared with control and A/R group in a dose-dependent manner. Interestingly, when PSG-1 was alone applied under the normoxic conditions without A/R injury, it also showed a significant increase of the expression of MnSOD protein, compared with control group in a dose-dependent manner. These findings indicate that upregulation of MnSOD may contribute to the protective effect of PSG-1 in cardiomyocytes undergoing A/R,

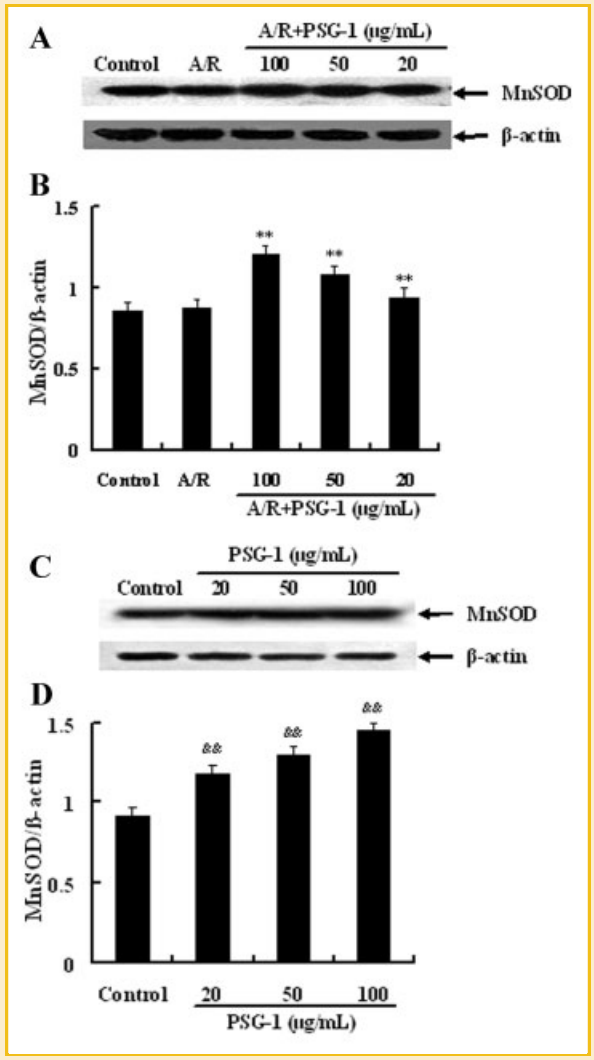

Fig. 7. Effect of PSG-1 on the expression of MnSOD in cardiomyocytes treated with or without A/R injury. A,C: Cardiomyocytes were lysed and expression of MnSOD and β-actin was analyzed by Western blot. B,D: Blots were scanned and expression of MnSOD and β-actin was quantified by densitometric analysis. The ratios for these proteins are shown. Eight independent experiments were done and all gave similar results. $^{\&\&}P < 0.01$ versus control group; $^{**}P < 0.01$ versus A/R group.

and further lead us to speculate that antioxidant effects of PSG-1 may be the basis for its cardioprotection.

DISCUSSION

Cellular defenses that protect the cardiomyocytes against oxidative stress have been proposed to be an important way to reduce the ischemia/reperfusion injury [Yuan et al., 2005; Fan et al., 2008; Sasaki et al., 2009]. Previous studies have demonstrated that A/R-induced injury in cardiomyocytes is a useful model for studying ischemia/reperfusion injury [Rui et al., 2005; Chen et al., 2007a]. In this study, PSG-1 markedly suppressed the decrease of cell viability resulting from A/R in a dose-dependent manner up to 100 μg/ml, and the cell viability showed no significant difference between PSG-1-treated cells and media-tread cells. Moreover, flow cytometry analysis using Annexin V and PI showed that A/R induced apoptosis in cardiomyocytes whereas PSG-1 significantly reduced the apoptosis in A/R-treated cells. These results indicate that PSG-1 exerts a protective effect by inhibiting A/R-induced cell death.

Oxidative stress can disrupt the balance between ROS production and the radical scavenging effect and lead to apoptotic cell death through the mitochondrial apoptosis pathway. Previous studies have shown mitochondrial protection to be important in A/R injury. The collapse of the $\Delta\psi_m$ results in the rapid release of cytochrome *c* into the cytoplasm [Chen and Burger, 2004; Kumar and Sitasawad, 2009; Wang et al., 2009]. Consistent with these findings, A/R-treated cardiomyocytes showed an increased generation of intracellular ROS, a loss of $\Delta\psi_m$, and an increased release of mitochondrial cytochrome *c* into the cytoplasm. However, pretreatment with PSG-1 attenuated the increase in ROS level and prevented the loss of $\Delta\psi_m$ and the release of cytochrome *c*. These results indicate that A/R is an oxidative stress, which stimulates the generation of ROS and triggers the mitochondria-mediated apoptosis pathway, and these effects can be suppressed by PSG-1.

Caspases play an important role in regulating cell apoptosis. Caspases transduce the apoptotic signal cascade and engage cellular targets leading to apoptosis [Moorjani et al., 2009]. As one of the key effectors, caspase-3 is initiated by caspase-9 and involved in the mitochondria-mediated pathway. Previous studies have shown that oxidative stress induced the activation of caspase-9 and caspase-3 in cell lines [Park et al., 2007]. Consistent with these results, we demonstrated that the induction of apoptosis induced by A/R was accompanied by an increase of caspase-9 and caspase-3 activities. Furthermore, this effect could be attenuated by a pretreatment with PSG-1. Caspase-9 and caspase-3 might be important effector caspases in A/R-induced apoptosis, and PSG-1 protects against the apoptosis of cardiomyocytes by blocking the activities of caspase-9 and caspase-3.

The Bcl-2 family, which possesses both anti- and pro-apoptotic members, constitutes a decisive checkpoint within the common portion of the cell death pathway. Either the pro-apoptotic or anti-apoptotic Bcl-2 family members can affect the execution of apoptosis. Bcl-2 can prevent ROS production and regulate the mitochondrial transitional pore opening by opposing the effect of Bax thereby blocking cytochrome *c* release and inhibiting caspase activities [Gao et al., 2007]. Moreover, the anti-apoptotic function of Bcl-2 is thought to be primarily derived from Bcl-2 presented in the mitochondria. Normally, Bax exists as a soluble monomer in cytosol or is loosely associated with mitochondria. However, upon apoptotic stimulation, Bax translocates to mitochondria where it forms oligomers that are inserted into the outer mitochondrial membrane and the mitochondria Bcl-2 decreases from mitochondria [Antonsson et al., 2001]. In this study, Western blot analysis revealed that the mitochondrial Bcl-2 was significantly decreased and Bax translocated from cytosol to mitochondria by the treatment with A/R, and these changes were inhibited by the pretreatment with PSG-1. These results indicate that Bcl-2 family proteins may play a critical role in regulating cardiomyocyte apoptosis induced by A/R, and PSG-1 is able to protect against A/R-stimulated apoptosis through a modulation of Bcl-2/Bax expression.

Additionally, cell defense against oxidative stress is provided by antioxidant enzymes. MnSOD, the primary antioxidant enzyme that scavenges superoxide radicals in mitochondria, is essential for the survival of aerobic life [Shen et al., 2006; Bartosh et al., 2008]. Mitochondria are the primary source of ROS production. Under physiological conditions, 1–2% of all electrons passing through the mitochondrial respiratory chain are converted to superoxide anion [Lai et al., 2004; Chaiswing et al., 2005]. Consequently, cardiac tissue is highly susceptible to oxidative damage, because cardiomyocytes contain a large number of mitochondria. Lack of MnSOD expression results in dilated ventricular cardiomyopathy, neonatal lethality, and neurodegeneration [Li et al., 1987; Negoro et al., 2001]. Our data showed that PSG-1 significantly upregulates the expression of MnSOD in cardiomyocytes. These imply that PSG-1 may offer protection against oxidative stress-induced cell death by regulating the expression of MnSOD. On the other hand, these interesting findings further led us to speculate that antioxidant effects of PSG-1 might be related to its cardioprotective effect.

Taken together, the present study suggests A/R to be an oxidative stress which can induce cardiomyocyte apoptosis through mitochondrial pathway. PSG-1, a natural antioxidant, protects cardiomyocytes against apoptosis through mitochondrial survival pathways. Although our findings suggest that the cardioprotective effects of PSG-1 against A/R-induced oxidant injury are likely attributed to its ability to induce expression of endogenous antioxidant (MnSOD), we also found other cardioprotective effects of PSG-1 in cardiomyocytes. We have shown that PSG-1 can directly scavenge intracellular ROS, reduce the loss of $\Delta\psi_m$, decrease the release of cytochrome *c* from the mitochondria into the cytosol, and regulate the activities of caspases. Hence, PSG-1 may be exploited as a potentially useful agent against myocardial ischemia/reperfusion injury.

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