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Cardiovascular effects in vitro of a polysaccharide from *Salvia miltiorrhiza*

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ABSTRACT

A polysaccharide (SMP1) was isolated from the roots of *Salvia miltiorrhiza*. This study is designed to investigate whether SMP1 prevents H9c2 cells from hydrogen peroxide (H_2O_2)-induced apoptosis. The present study showed that exposure of H9c2 cells to 100 mM H_2O_2 for 24 h caused a significant increase in cell death and apoptosis, but pretreatment with SMP1 eliminated H_2O_2-induced apoptotic cell death. Furthermore, pretreatment with SMP1 significantly prevented the mitochondria disruption, cytochrome *c* release, the rise of the ratio between proapoptotic Bax and antiapoptotic Bcl-2 protein expression, and caspase-3 activation in H9c2 cells upon H_2O_2 stimulation. Moreover, the decline of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities together with the elevation of malondialdehyde (MDA) in PC12 cells exposed to H_2O_2 were remarkably reversed to normal levels by pretreatment with SMP1. These results suggest that SMP1 protects H9c2 cells from H_2O_2-induced apoptosis through inhibition of mitochondrial dysfunction, inactivation of caspase-3 cascade and enhancement of antioxidant capacity.

1. Introduction

Cardiovascular diseases remain a leading contributor to the high cause of death in many developed countries (Shaik et al., 2012). Emerging evidences suggest that oxidative stress is a pivotal factor for promoting apoptosis (Crow, Mani, Nam, & Kitsis, 2004), and reactive oxygen species (ROS)-induced apoptosis is intimately involved in the pathogenesis of various forms of cardiovascular diseases, including myocardial ischemia, arteriosclerosis, cardiomyopathy and transplant rejection (Chenand & Keaney, 2012; Murdoch, Zhang, Cave, & Shah, 2006; Olivetti et al., 1997). Despite these evidences, the mechanisms involved in oxidative stress-induced cardiomyocyte apoptosis are largely unknown. In support to these notions, it is very important to inhibit cardiomyocyte apoptosis for preserving myocardial function after injury.

At present, natural products and medicinal herbs have drawn much attention as potential source of antioxidants due to their excellent effectiveness against reactive oxygen species (ROS)-induced pathologies (Ren, Qiao, Yang, & Zhou, 2010). In our previous study, we purified and characterized one homogeneous polysaccharide from the roots of *Salvia miltiorrhiza*. SMP1 contained 91.3% of carbohydrate, 4.34% of protein and 2.81% of uronic acid. As determined by high-performance gel permeation chromatography (HPGPC), SMP1 showed a single and symmetrically sharp peak and its average molecular weight was about 5.5×10^5 Da. Gas chromatography (GC) analysis showed that SMP1 was composed of galactose, glucose, fucose, rhamnose, arabinose and mannose with the molar ratio of 1.0:1.2:0.3:1.5:1.3:1.9. The cardio-protective potential of SMP1 in a rat ischemia–reperfusion (I/R) model was studied. The results showed that administration of SMP1 at 400 and 800 mg/kg had a protective effect against myocardial I/R injury in rats by ameliorating oxidative stress and inhibiting myocardial apoptosis (Song, Huang, Zhao, & Song, 2013). However, the effects of SMP1 on ROS-induced cardiomyocyte apoptosis have yet to be fully elucidated. In this study, hydrogen peroxide (H_2O_2) was used to induce apoptosis in H9c2 cells, as it can damage cells by inducing apoptosis and has been used as a well-established model in many studies on cardiovascular disease (Pechtelidou, Beis, & Gaitanaki, 2008; Qi et al., 2010; Ren et al., 2009). The H9c2 rat cardiomyocyte cell line was chosen in the present study, since this cell line retains the characteristics of isolated primary cardiomyocytes and has been considered as a proper model to study molecular responses of the cardiomyocyte to oxidative damage (Silva, Sardão, Coutinho, & Olveira, 2010; Watkins, Borthwick, & Arthur, 2011).

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Herein we examine whether SMP1 protects H9c2 cells from H_2O_2-induced cell death for the first time and try to find out the possible mechanisms.

2. Materials and methods

2.1. Materials and chemicals

The roots of *S. miltiorrhiza* were purchased from a local herb shop in July 2012 (Chongqing City, China). Hydrogen peroxide (H_2O_2), rhodamine 123 (Rh123) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma–Aldrich Co. (St Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and other tissue culture reagents were obtained from Life Technologies Inc (Gaithersburg, MD, USA). Rabbit anti-Bax, anti-Bcl-2 and anti-caspase-3 polyclonal antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA), while β-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). BCA protein assay kit, Annexin V-FITC kit, Cytochrome *c* ELISA kit and caspase-3/CPP32 colorimetric assay kit were provided by Pierce Chemical (Rockford, IL), Invitrogen (CA, USA), Roche (Mannheim, Germany) and Biovision (Mountain View, CA), respectively. SOD, GSH-Px, and MDA assay kits was purchased from Institute of Biological Engineering of Nanjing Jian-cheng (Nanjing, China). All other chemical reagents were analytical grade.

2.2. Isolation and purification of polysaccharide SMP1

Isolation and characterization of a polysaccharide (SMP1) from the roots of *S. miltiorrhiza* have been reported in our previous work (Song et al., 2013).

2.3. GC–MS analysis

The vacuum-dried SMP1 (10 mg) was methylated three times according to the Needs' method (Needs & Selvendran, 1993). After being methylated completely, the resulting partially methylated products were hydrolyzed, reduced, acetylated using the method described by Sweet, Shapiro, and Albersheim (1975) and analyzed by gas chromatography–mass spectrometry (GC–MS) on a Shimadzu QP-5050A apparatus equipped with a DB-1 capillary column (30 m × 0.25 mm i.d.) under a temperature program starting at 160 °C followed by 2 °C/min to 210 °C, then 5 °C/min to 240 °C.

2.4. Cell culture and treatment

H9c2 rat cardiomyocyte cells from the American Type Culture Collection (ATCC, Cedarlane Laboratories) were cultured in 100 mm dishes and grown in DMEM supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C with 5% CO_2. When cell confluency reached 70–80%, H9c2 cells were used for experiments. H9c2 cells were plated at a density of 1×10^6 cells/100 mm dish with media refreshed every 3 days. To determine the effect of SMP1 the cell growth of H9c2 cells, cells were incubated with increasing concentrations of SMP1 (25, 50 and 100 μg/mL) for 24 h. To determine the effect of H_2O_2 one the cell growth of H9c2 cells, cells were incubated with increasing concentrations of H_2O_2 (25, 50 and 100 μM) for 24 h (Cheng et al., 2007). To determine the protective effect of SMP1 against H_2O_2 induced cell loss in H9c2 cells, cells were incubated with increasing concentrations of SMP1 (25, 50 and 100 μg/mL) for 24 h and then treated with 100 μM H_2O_2 for another 24 h. Cell viability was determined colorimetrically using MTT assay.

2.5. MTT assay

Cell viability was measured quantitatively by using MTT, showed the activity of living cells (Ren, Zhao, Yang, & Fu, 2008). Briefly, The H9c2 cells (1×10^5 cells/well) were seeded in 24-well plates. After different treatment, 20 μl of 5 mg/ml MTT solution was added to each well, and wells were incubated at 37 °C for 4 h. 200 μl DMSO was added to each well to dissolve the formazan crystals after supernatants were aspirated. The absorbance was measured at 490 nm by an ELISA reader (Tecan, Maennendorf, Switzerland) and used to calculate the relative ratio of cell viability. Cell viabilities were presented as percentages of the control (Xie et al., 2010).

2.6. Detection of apoptosis by Annexin V and PI binding assay

Apoptosis was quantitated using an Annexin V-FITC kit according to the manufacturer's recommendations on flow cytometric analysis as performed by Verma et al. (2008), with some modification. After treatment with H_2O_2, a single-cell suspension was prepared and cultured in a six-well plate at a density of 1×10^5/well and then double stained with 5 μl of Annexin-V-FITC (fluorescein isothiocyanite) and 5 μl of propidium iodide (PI, 50 μg/ml) for 15 min at room temperature. After washing out the unbound dye, the cells were subjected to flow cytometric analysis. Data acquisition and analysis were performed on a FACScan flow cytometry (Beckman, San Diego, CA, USA) using CellQuest software to differentiate apoptotic cells from necrotic cells by quantitatively estimating the relative amounts of the Annexin V/PI-stained cells in the cell population. Apoptotic rate was calculated as the relative number of apoptotic cells (Annexin V-positive) compared to the total number. A minimum of 10,000 cells were collected per sample for each data file analysis.

2.7. Measurement of mitochondrial membrane potential ($\Delta\Psi m$)

To observe the change in $\Delta\Psi$m, flow cytometry was performed with the fluorescent cationic dye rhodamine 123 (Rh123) as previously described (Fang et al., 2011; Jadeja, Thounaojam, Devkar, & Ramachandran, 2011), with minor modifications. H9c2 cells (1×10^5 cells per well) cultured under various experimental conditions in 6 well culture plates were incubated with Rh123 (1 mg/mL in DMSO) at 37 °C for 30 min, then washed twice with PBS and harvested through trypsinization followed by washing with PBS again. The fluorescence intensity was observed with a fluorescence spectrophotometer at an excitation of 485 nm and an emission wavelength of 530 nm, respectively. The change of $\Delta\Psi$m in cells was analyzed a Beckman FACSCalibur flow cytometer using CellQuest software and calculated as the percentage of the vehicle control, which was set as 100%.

2.8. Caspase-3 activity assay

The caspase-3 activity was measured with a caspase-3/CPP32 colorimetric assay kit according to the manufacturer's instructions. To measure the caspase activity, the whole cell lysate was prepared in 500 μl of cell lysis buffer (1% Triton X-100, 0.32 M sucrose, 4 mM EDTA, 1 μg/mL aprotinin, 1 mM PMSF, 2 mM DTT, 1 μg/mL leupeptin, 10 mM Tris/HCl, pH 8.0) on ice for 30 min. After centrifugation at 20,000 × *g* for 15 min, the supernatant were mixed with 50 μl of reaction buffer (containing 10 mM dithiothreitol) and 200 μM DEVD-pNA substrate at 37 °C for 1 h and caspase-3 activity was measured by a spectrofluorometer with a wavelength at 400 nm. Data were expressed as the relative activity over control.

2.9. Quantification of cytochrome c level

The cytosolic and mitochondrial fractions were prepared using mitochondrial fraction kit, according to the manufacturer's instruction. Each fraction was then frozen in aliquots at −70 °C until required. The protein concentration in the cytosolic and mitochondrial fraction was determined using the Bradford protein assay (Bradford, 1976). Cytochrome *c* was measured using a commercially available cytochrome *c* ELISA kit, following the manufacturer's manual with minor modifications. Briefly, 100 μL of rat/mouse cytochrome *c* conjugate was mixed with 100 μL of cytosol or mitochondria lysates for 2 h at room temperature. Then the wells were washed twice with PBS and incubated with 100 μL of substrate solution for 30 min at room temperature in the darkness before termination. Subsequently, absorbance was measured at 450 nm using a microplate reader (Tecan, Maennendorf, Switzerland). The amount of cytochrome *c* was expressed as ng/mg for the cytosol and mitochondria fraction.

2.10. Western blot analysis

H9c2 cells growing on six-well plates were pretreated with or without SMP1 (200) for 24 h prior to exposing to 400 μM H_2O_2, as indicated. Cells were harvested and lysed using RIPA lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% sodium deoxycholate, 1% Nonidet P (NP)-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, and protease inhibitors. The protein concentration of each sample was determined using a BCA protein assay kit (Pierce Chemical, Rockford, IL). 40 μg of the total protein was electrophoresed on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. After being washed three times with PBS buffer, the PVDF membranes were blocked in 5% bovine serum albumin (BSA) for 2 h and incubated overnight at 4 °C with the primary antibodies against Bax, Bcl-2, capase-3, or β-actin. Subsequently, the membranes were washed in Tris-buffered saline-Tween 20 (TBST) containing 1% BSA, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature, and developed using the ECL chemiluminescence detection system (Amersham).

2.11. Assessment of SOD, GSH-Px and MDA Levels

The intracellular SOD, GSH-Px and MDA levels were detected using commercial kits based on colorimetric methods. After treatment, PC12 cells were harvested by centrifugation and the supernatants were removed. The remaining cells were washed with PBS twice, dissolved in physiological saline solution, and then lysed for 30 min at 4 °C. Following centrifugation, the resulting suspension was collected to analyze the levels of SOD, GSH-Px and MDA according to the manufacturer's instructions.

2.12. Statistical analysis

All the experiments were performed at least three times. The data are expressed as the mean ± SEM. Statistical significance was analyzed by one-way analysis of variance (ANOVA). Values with $P < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Sugar linkage of SMP1

Methylation linkage analysis of SMP1 was summarized in Table 1. The GC–MS results indicated that the backbone chain of SMP1 are mainly (1→3,6)-β-D-mannopyranosyl (Residue-A), (1→6)-β-D-glucopyranosyl (Residue-B), and (1→3,6)-β-D-galactopyranosyl (Residue-C) residues. The side chains in the native polysaccharide attached to the O-3 position of Residue-A and Residue-C contained three terminals, namely (1→)-β-L-fucopyranosyl (Residue-D), (1→)-β-L-arabinofuranosyl (Residue-E) and (1→)-α-L-rhanopyranosyl (Residue-F) units. According to the peak areas, the proportion of six types of residues was in the ratio of 7.8:4.9:4.1:1.1:5.1:6.0, respectively.

Table 1
The results of methylation analysis of SMP1.

Peak no.	Methylated sugar	Molar ratio	Linkage type
1 (Residue-A)	2,3-Me_2-Man	7.8	→3,6)-β-D-Man*p*-(1→
2 (Residue-B)	2,3,4-Me_3-Glc	4.9	→6)-β-D-Glc*p*-(1→
3 (Residue-C)	2,4-Me_2-Gal	4.1	→3,6)-β-D-Gal*p*-(1→
4 (Residue-D)	2,3,4,6-Me_4-Fuc	1.1	β-L-Fuc*p*-(1→
5 (Residue-E)	2,3,5-Me_3-Ara	5.1	β-L-Ara*f*-(1→
5 (Residue-F)	2,3,4,6-Me_4-Rha*p*	6.0	α-L-Rha*p*-(1→

3.2. SMP1 pretreatment decreased cell death induced by H_2O_2 stimulation in H9c2 cells

First, to evaluate whether SMP1 alone was cytotoxic to H9c2 cells, we determined the viability of cells treated with SMP1 (25–100 μg/mL) for 24 h using the MTT reduction assay. As shown in Fig. 1A, SMP1 did not influence cell viability in cultured H9c2

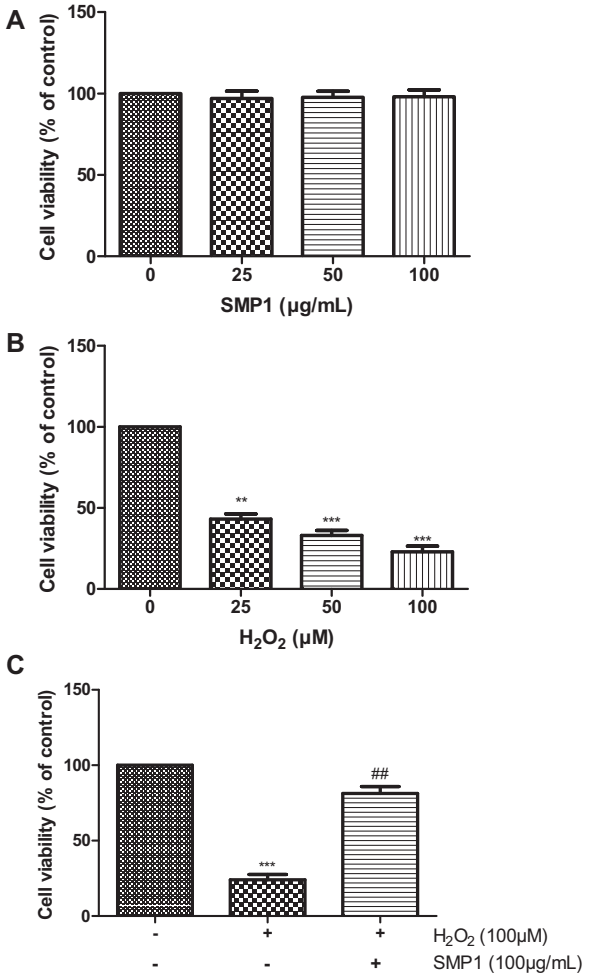

Fig. 1. Preventive effects of SMP1 on cell viability against H_2O_2-induced injury determined by MTT assay. (A) The toxic effect of SMP1 in H9c2 cells after 24 h incubation. (B) The toxic effect of H_2O_2 in H9c2 cells after 24 h incubation. (C) SMP1 protects H9c2 from H_2O_2-induced cytotoxicity.

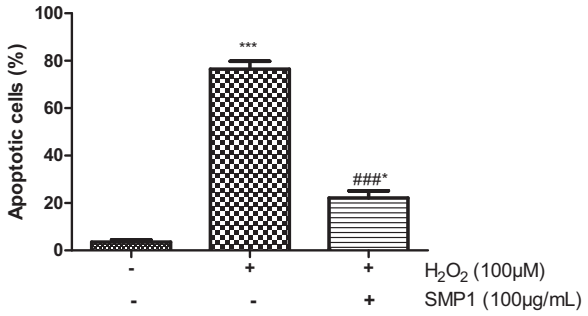

Fig. 2. SMP1 blocked H_2O_2-triggered apoptosis in H9c2 cells. Apoptotic cells were detected by Annexin V and propidium iodide double staining.

cells up to 100 μg/mL (Fig. 1A). Next, to examine the direct cytotoxic effect of oxidative stress, an MTT assay was carried out to measure the cell viability of H9c2 cells upon exposure with various concentrations of H_2O_2 for 24 h. As shown in Fig. 1B, H_2O_2 significantly ($P < 0.01$) reduced the viability in a concentration-dependent manner over the tested concentration range (25–100 μM). A maximum reduction of 51 ± 8% was observed in H9c2 cells treated with 100 μM of H_2O_2. Therefore, in order to determine the effects of SMP1 on H_2O_2-stimulated cell death, the H9c2 cells were pretreated with SMP1 (100 μg/mL) for 24 h, and then co-incubated with 100 μM of H_2O_2 for additional 24 h. The decrease in H9c2 cell viability induced by H_2O_2 insult was improved significantly by SMP1 compared with the control ($P < 0.01$).

3.3. SMP1 pretreatment inhibits apoptosis induced by H_2O_2 stimulation in H9c2 cells

As a further test to quantify the effects of SMP1 on H_2O_2-induced H9c2 cells apoptosis, the percentage of apoptotic cells was detected by Annexin V-FITC and PI double staining using flow cytometry. As shown in Fig. 2, compared with the control, the number of apoptotic cells was increased significantly when cells were treated with 100 μM H_2O_2 ($P < 0.001$, 76.46% vs 3.56%), whereas preincubation in H9c2 cells with SMP1 prior to H_2O_2 exposure for another 24 h, significantly attenuated the apoptosis induced by H_2O_2, the percentages of apoptotic cells was 22.12%.

3.4. SMP1 pretreatment restores the change of ΔΨm induced by H_2O_2 stimulation in H9c2 cells

Mitochondria are common integrators and transducers of various proapoptotic signals, and the loss of ΔΨm is an important event of mitochondrial cell death associated with apoptosis (Miao et al., 2013; Oliveira, Goncalves, Monteiro, Providencia, & Moreno, 2005; Park et al., 2003). To determine whether mitochondria participated in H_2O_2-induced apoptosis in H9c2 cells, the cultured H9c2 cells were stained with Rh 123. As shown in Fig. 3A and B, exposure of H9C2 cells to H_2O_2 (100 μM) resulted in significantly low levels of ΔΨm as evidenced by Rho 123 staining. But, SMP1 pretreatments were able to restore the loss of ΔΨm induced by H_2O_2.

3.5. SMP1 pretreatment alters cytochrome c release, Bcl-2/Bax expression, as well as caspase activation induced by H_2O_2 stimulation in H9c2 cells

Disruption of ΔΨm usually allows the release of cytochrome *c* from mitochondria to cytosol, which was a hallmark event of cells undergoing apoptosis (Von Ahsen, Waterhouse, Kuwana, Newmeyer, & Green, 2000). To further examine the effect of SMP1 on the H_2O_2-induced apoptosis, the level of released cytochrome *c* in mitochondrial and cytosolic parts was determined. As shown in Fig. 4, the amount of cytochrome *c* released into the cytosol was markedly increased at 24 h after H_2O_2 treatment, while cytochrome *c* in mitochondria fraction was dramatically reduced, demonstrating a self-complementary trend of cytochrome *c* in these two fractions. SMP1 pretreatment significantly reduced cytochrome *c* release into the cytosol, while that of the mitochondria fraction showed inverse results, demonstrating inhibitory effect of SMP1 on cytochrome *c* translocation.

Mitochondrial-mediated apoptosis is controlled by multiple layers of regulation in response to various stimuli, the most prominent players being members of Bcl-2 family (Ralph, Low, Dong, Lawen, & Neuzil, 2006). The Bcl-2 family, composed of both anti-apoptotic

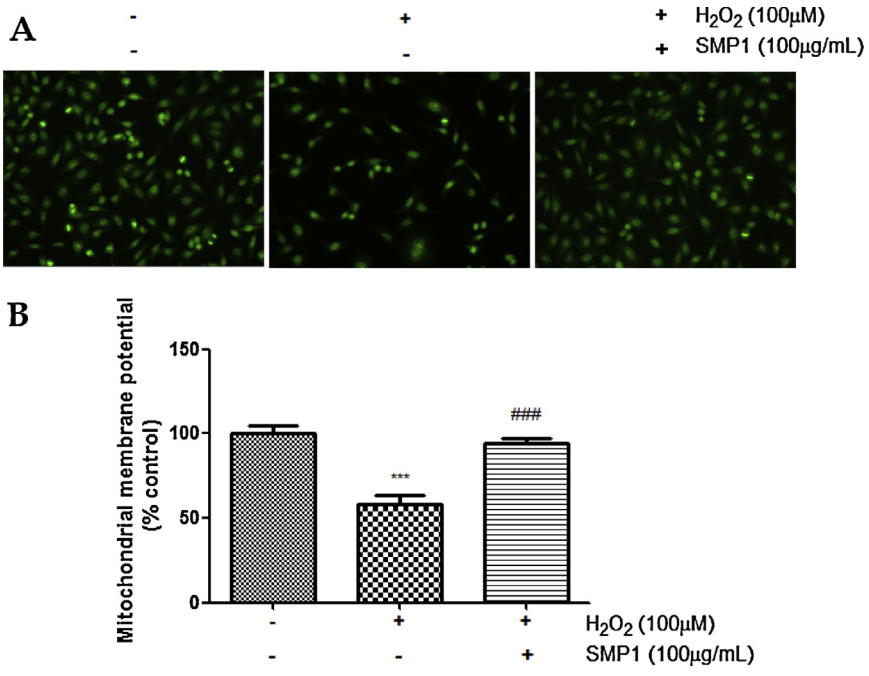

Fig. 3. Effect of SMP1 on mitochondrial membrane potential of H_2O_2-treated H9C2 cells.

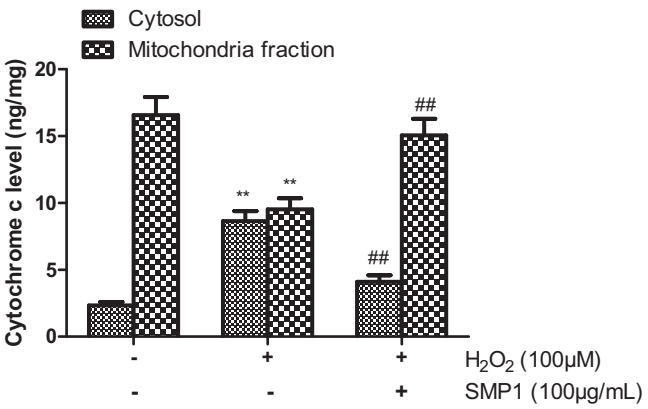

Fig. 4. Effect of SMP1 on cytochrome *c* release in H_2O_2-treated H9C2 cells.

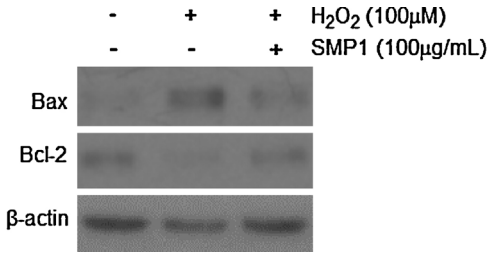

Fig. 5. Effect of SMP1 on the expression of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 in H_2O_2-treated H9c2 cells.

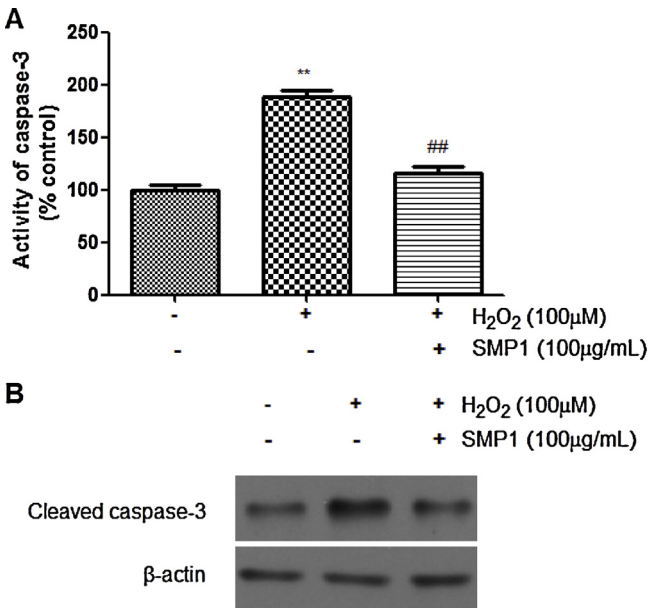

Fig. 6. (A) Effect of SMP1 on caspase-3 activity in H_2O_2-treated H9c2 cells. (B) Effect of SMP1 on the expression of caspase-3 protein in H_2O_2-treated H9c2 cells.

and pro-apoptotic proteins, regulates cell death by controlling mitochondrial membrane permeability during apoptosis (Adams & Cory, 1998; Scorrano & Korsmeyer, 2003; Yang et al., 1997). Anti-apoptotic protein Bcl-2 inhibits apoptosis by preventing cytochrome *c* release from the mitochondria and inhibiting caspase activation (Ow, Green, Hao, & Mak, 2008). The pro-apoptotic protein Bax induces mitochondrial membrane permeabilization to cause the release of cytochrome *c* into the cytoplasm, which in turn activates the proteolytic caspases (Dan & Yamori, 2001). In view of that, we next investigated the effect of SMP1 on the expression of Bax and Bcl-2 in H_2O_2-exposed H9c2 cells. As expected in Fig. 5, when H9c2 cells were exposed to H_2O_2 for 24 h, we found that the Bcl-2 expression was down-regulated and the Bax expression was up-regulated. However, we found that SMP1 (100 μg/mL) prevented H_2O_2-induced downregulation of Bcl-2 and upregulation of Bax, thus decreasing the Bax/Bcl-2 expression ratio. Our results suggest that SMP1 could inhibit apoptosis through changing the Bax/Bcl-2 ratio and, therefore, decreasing the mitochondrial membrane permeability to block the whole cascade of apoptotic reactions.

Caspases, a family of cysteine proteases, play a central role in the initiation and execution of apoptosis (Danial & Korsmeyer, 2004). As the levels of cytochrome *c* increase in cytosol, it can bind to apoptosis-inducing factor (AIF)-1 and further activate caspase-9, which in turn cleaves and activates executioner caspase-3 (Zou, Li, Liu, & Wang, 1999). Caspase-3 is a prevalent caspase that is ultimately responsible for the majority of apoptotic processes, especially including cardiovascular cell death (Debunne et al., 2009; Miao et al., 2013). As shown in Fig. 6A, an increase in caspase-3 activity was evident in cells incubated with 100 mM H_2O_2 for 6 h compared with the control. However, when the cells were pretreated with 100 μg/mL SMP1 for 24 h prior to addition of 100 mM H_2O_2, the caspase-3 activation decreased. Consistently, Western blot also proved that H_2O_2-induced elevation of caspase-3 protein was inhibited by SMP1 pretreatment (Fig. 6B). There results have proven that SMP1 can significantly protect H9c2 cells from H_2O_2 induced apoptosis by eliminating caspase-3 activation under oxidative stress.

3.6. SMP1 pretreatment reverses SOD, GSH-Px and MDA levels induced by H_2O_2 stimulation in H9c2 cells

Oxidative stress generated as a result of environmental and physiological factors is usually associated with the production of various reactive oxygen species (ROS), which possess highly reactive and toxic properties. A growing body of evidence indicates ROS causes oxidative damage of plasma membrane and results in lipid peroxidation, thus contributes to the progression of cardiovascular diseases (Griendling & FitzGerald, 2003; Taniyama & Griendling, 2003). In response to this, organism has developed a natural antioxidant system to fight against tissue and cellular damage caused by ROS. Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) are two critical antioxidant enzymes to scavenge ROS to prevent cell damage (Wang, Sun, Bao, Liu, & An, 2010). Malondialdehyde (MDA), the degradation product of lipid peroxidation,

Table 2
Effect of SMP1 and or H_2O_2 on the levels of SOD, GSH-Px and MDA in H9c2 cells.

Groups	SOD (U/mg pro)	GSH-Px (nmol/mg pro)	MDA (nmol/mg pro)
Control	35.12 ± 3.58	87.11 ± 8.21	1.01 ± 0.15
H_2O_2 (100 mM)	6.54 ± 5.87[a]	40.82 ± 5.62[a]	3.25 ± 0.32[a]
SMP1 (100 μg/mL) + H_2O_2 (100 mM)	30.11 ± 3.10[b]	80.27 ± 7.41[b]	1.12 ± 0.17[b]

Data represent means ± SEM of three independent experiments.
[a] Significance compared with control, $P < 0.01$.
[b] Significance compared with H_2O_2, $P < 0.01$.

reflected the damage caused by ROS (Qin et al., 2009). The degree of lipid peroxidation in cells was confirmed by the MDA level. Therefore, elevation of antioxidant enzymes activities and Inhibition of MDA may be beneficial to quench free radicals. As illustrated in Table 2, the antioxidant enzymes (SOD and GSH-Px) levels declined significantly, and the level of MDA obviously increased in H_2O_2-treated group compared with those of the vehicle group ($P < 0.01$). Whereas pretreatment of H9c2 cells with 100 μg/mL of SMP1 markedly reversed these changes as compared with cells treated with H_2O_2 alone ($P < 0.01$), suggesting that the cardioprotective effects of SMP1 were related to its antioxidant ability.

4. Conclusions

Oxidative stress in cardiomyocytes plays an important role in the pathogenesis of cardiovascular disease, whereas the protective effects of SMP1 on oxidative stress-triggered apoptosis of cardiac myocytes have not been elucidated. Herein we investigate the protective effects of SMP1 on H_2O_2-induced apoptosis in H9c2 cells, as well as the possible molecular mechanisms involved. The results assessed by MTT assay and Annexin V–propidium iodide dual staining demonstrated that pretreatment with SMP1 could prevent H9c2 cells from H_2O_2-induced cell death and apoptosis. To investigate the molecular mechanisms by which SMP1 protects H9c2 cells from H_2O_2-induced apoptosis, we examined the change of $\Delta\Psi m$, cytochrome *c* release, Bcl-2, Bax and caspase-3 protein expression, as well as SOD, GSH-Px and MDA levels in H9c2 cells. Pretreatment of H9c2 cells with SMP1 significantly protected the mitochondria from disruption and prevented the release of cytochrome *c* that had been altered by exposure of H9c2 cells to H_2O_2. Western blot results showed that H_2O_2 increased the expression of caspase-3 and proapoptotic Bax protein, but decreased antiapoptotic Bcl-2 protein in H9c2 cell. However, pretreatment with SMP1 antagonized these alternations as evidenced by decreased Bax/Bcl-2 ratio and caspase-3 level. Furthermore, the decline of SOD and GSH-Px activities and increase of MDA level in PC12 cells exposed to H_2O_2 were remarkably reversed by pretreatment with SMP1. In this study, it was demonstrated that SMP1 protected H9c2 cardiomyoblast cells from oxidative stress-induced apoptosis, possibly by suppression of mitochondrial dysfunction, activation of caspase-family proteases and enhancement of antioxidant defense capacity. Taken together, our findings provide novel insights to the mechanisms of physiological benefits of SMP1 in the treatment of oxidative stress-mediated cardiovascular disorders.

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