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Salvianolic acid B protects cardiomyocytes from angiotensin II-induced hypertrophy via inhibition of PARP-1

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ABSTRACT

Salvianolic acid B (SalB), one of the major bioactive components in *Salvia miltiorrhiza*, has plenty of cardioprotective effects. The present study was designed to investigate the effect of SalB on angiotensin II (AngII)-induced hypertrophy in neonatal rat cardiomyocytes, and to find out whether or not this effect is attributed to inhibition of poly (ADP-ribose) polymerase-1 (PARP-1), which plays a key role in cardiac hypertrophy. Our results showed that SalB prevented the cardiomyocytes from AngII-induced hypertrophy, associated with attenuation of the mRNA expressions of atrial natriuretic factor and brain natriuretic peptide, and reduction in the cell surface area. SalB inhibited the activity of PARP-1. The inhibitory effect was comparable to that of the PARP-1 inhibitor 3-Aminobenzamide (3-AB). In addition, SalB reversed the depletion of cellular NAD^+ induced by AngII. Moreover, overexpression of PARP-1 attenuated the anti-hypertrophic effect of SalB. These observations suggested that SalB prevented the cardiomyocytes from AngII-induced hypertrophy, at least partially through inhibition of PARP-1. Moreover, SalB attenuated the generation of oxidative stress via suppression of NADPH oxidase 2 and 4, which might probably contribute to the inhibition of PARP-1. These present findings may shed new light on the understanding of the cardioprotective effect of SalB.

1. Introduction

Cardiac hypertrophy, especially pathological hypertrophy, is a key risk factor in the pathogenesis of heart diseases [1], but the molecular mechanisms remain to be elucidated. Recently, accumulating evidences have revealed that poly (ADP-ribose) polymerase-1 (PARP-1) plays an important role in the development and progression of cardiac hypertrophy and heart failure [2–5]. PARP-1 catalyzes the conversion of nicotinamide adenine dinucleotide (NAD^+) into poly (ADP-ribose) (PAR) chains [6–8]. In response to oxidative stress or other stimulations which lead to DNA damage, PARP-1 recognizes single- and double-stranded DNA breaks and catalyzes the formation of long PAR chains, which subsequently causes modification of intracellular target proteins and produces biological effects including DNA repair, gene transcription, chromatin remodeling and cell survival [7,9,10]. However, overactivation of PARP-1 depletes its substrate NAD^+, which is important for intracellular glycolysis rate, Ca^{2+} regulation and energy metabolism, eventually resulting in cell death [5]. In response to various pro-hypertrophic agents, such as angiotensin II (AngII), phenylephrine and isoproterenol, the activity of PARP-1 is remarkably increased, suggesting that PARP-1 activation is a common downstream mechanism of hypertrophy induced by different stimuli [3]. PARP-1 may impair the functions of the NAD^+-dependent enzymes during cardiac hypertrophy [2,4,5]. In contrast, PARP-1 inhibitors or PARP-1 gene deficiency can alleviate cardiac hypertrophy [2–4].

Salvianolic acid B (SalB) is a polyphenolic acid derived from the dried roots and rhizomes of *Salvia miltiorrhiza*, which is a commonly used traditional Chinese herbal medicine for the treatment of cardiovascular diseases [11]. It has been proved that SalB can protect the heart from ischemia-reperfusion injury, myocardial infarction and cardiac remodeling [12–15]. The present study is aimed to investigate the effect of SalB on AngII-induced hypertrophy in neonatal rat cardiomyocytes, and to clarify whether or not the underlying mechanisms are due to the inhibition of PARP-1. Our results may shed new light on the understanding of the cardioprotective effect of SalB, and support the application of SalB for the precaution and treatment of cardiac hypertrophy.

Abbreviations: SalB, Salvianolic acid B; 3-AB, 3-Aminobenzamide; ANF, atrial natriuretic factor; AngII, Angiotensin II; BNP, brain natriuretic polypeptide; DHE, dihydroethidium; MTT, 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide; NAD, nicotinamide adenine dinucleotide; NOX, NADPH oxidase; PARP-1, poly (ADP-ribose) polymerase-1; ROS, reactive oxygen species.

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2. Materials and methods

2.1. Chemicals and materials

SalB was obtained from GreenValley Pharmaceutical (Shanghai, China); NAD^+ was purchased from Roche (Cat. 004626); High-specific-activity PARP-1 and activated DNA were purchased from Trevigen (Gaithersburg, MD, USA); AngII, 3-Aminobenzamide (3-AB) and MTT were obtained from Sigma (Louis, MO, USA). Rabbit anti-NOX2 polyclonal antibody was obtained from Upstate (Billerica, MA, USA); rabbit anti-NOX4 polyclonal antibody was purchased from Norvus Biologicals (Littleton, CO, USA); rabbit anti-PARP-1 polyclonal antibody was purchased from Sangon (Shanghai, China); Flag-PARP-1 plasmid was obtained from Genechem (Shanghai, China); Lipofectamine 2000 reagent was purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Cell culture

Neonatal rat cardiomyocytes were isolated from the hearts of one to two-day-old Sprague-Dawley (SD) rats by a previously validated method [16] and cultured in Dulbecco's modified Eagle's medium (DMEM) supplied with 10% fetal bovine serum (FBS) and 0.1 mM 5-bromodeoxyuridine. After 48 h, the culture medium was replaced by DMEM containing 0.1% FBS, and the cells were further incubated for 24 h. Subsequently, the cardiomyocytes were treated with SalB, 3-AB or NAD^+ for 24 h, followed by stimulation with 1 μM AngII as previously described [3,10,17]. For PARP-1 overexpression, cardiomyocytes were transiently transfected with FLAG-PARP-1 plasmid using Lipofectamine 2000 according to the manufacturer's instructions. The protein level and activity of PARP-1 in cells were showed in Supplementary Fig. S1.

2.3. Cell viability assay

MTT assay was performed to test the viability of cardiomyocytes as described previously [18]. MTT was added to the culture medium at a final concentration of 0.5 mg/mL and was incubated for 4 h at 37 °C. After that, the culture medium was removed and DMSO was added to dissolve the resulting formazan crystals. After slightly shaking for 10 min at 37 °C, the absorbance was measured at a wavelength of 570 nm using a microplate reader (Bio-Tek, Elx800, USA). The cell viability was expressed as the relative absorbance of the treated cells versus the control.

2.4. Enzymatic assay for measurement of PARP-1 activity

A chemical quantitation method for PARP activity was performed as reported by Putt and Hergenrother [19], which relies on the conversion of NAD^+ into a highly fluorescent agent by commercial PARP-1. The fluorescence intensity was determined by Multimode Microplate Reader (Tecan, Infinite M1000, Switzerland) at the wavelength of 360 nm for excitation and 445 nm for emission. The concentration–response curves were plotted and the IC_{50} values were calculated. For the measurement of cellular PARP-1 activity, the commercial PARP-1 was replaced by nuclear extracts from cardiomyocytes [20].

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from ventricular tissue using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instruction and subjected to a quantitative reverse transcription and polymerase chain reaction (RT-PCR) analysis. β-Actin was served as an endogenous control. Rat-specific primers for PARP-1, atrial natriuretic factor (ANF), brain natriuretic polypeptide (BNP) were synthesized by Invitrogen. The sequences of the primers are listed in Supplementary Table S1.

2.6. Measurement of cell surface area

Rhodamine-phalloidin (Invitrogen) was employed to visualize actin fragment by fluorescence microscopy [7]. Cardiomyocytes grown on 48-well plates were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, and further incubated with 0.1‰ Triton-X 100 for 30 min. After blocking with Normal Goat Serum, the plate was incubated with 0.1% rhodamine-phalloidin for 1 h. After washed with phosphate buffer saline (PBS, pH 7.4), the plates were mounted in prolong Gold anti-fade reagent with DAPI (Invitrogen) and detected by high Content Analysis System (Thermo, ArrayScanVTI, USA).

2.7. Determination of NAD^+

The NAD^+ levels were measured as described previously [21]. The absorbance of the reaction mixture was read at a wavelength of 570 nm. The NAD^+ content was calculated from the standard curve and normalized to the protein content of the sample.

2.8. Dihydroethidium (DHE) fluorescence

The cardiomyocytes were seeded onto 48-well plates. After the treatments, the cells were washed with PBS, and were then stained with 10 μM DHE in serum-free medium for 30 min at 37 °C in the dark. After that, the cells were washed with PBS and maintained in 200 μl serum-free medium. The DHE fluorescence of the cells was imaged with a fluorescence microscope (Leica, Germany). Cellular fluorescence intensity was determined by High Content Analysis System (Thermo, ArrayScanVTI, USA). Values were normalized as percentage of the fluorescence intensity of the control cells.

2.9. Western blotting

Cardiomyocytes were lysated on ice for 30 min with RIPA lysis buffer. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Western blot analysis was performed according to standard procedures. Rabbit anti-PARP-1 polyclonal antibody (diluted 1:500), Rabbit anti-NOX2 polyclonal antibody (diluted 1:500) and rabbit anti-NOX4 polyclonal antibody were used as primary antibodies. Mouse anti-α-tubulin monoclonal antibody (Sigma, diluted 1:10,000) was served as a loading control. Immunoreactive bands were detected with the Super-Signal WestPico Chemiluminescent Substrate (Pierce). The intensity of protein bands was analyzed by Lab Works software (Bio-Rad).

2.10. Statistical analysis

Data were presented as mean ± SD. Statistical analyses were performed by unpaired Student's *t*-test between two groups or by one-way analysis of variance (ANOVA) with Tukey's *post hoc* test for multiple comparisons. *P*-values equal to or less than 0.05 were considered to indicate statistically significant differences.

3. Results

3.1. The effects of SalB on AngII-induced hypertrophic responses in cardiomyocytes

MTT assay was applied to examine the effect of SalB on the viability of the cardiomyocytes. The results showed that the

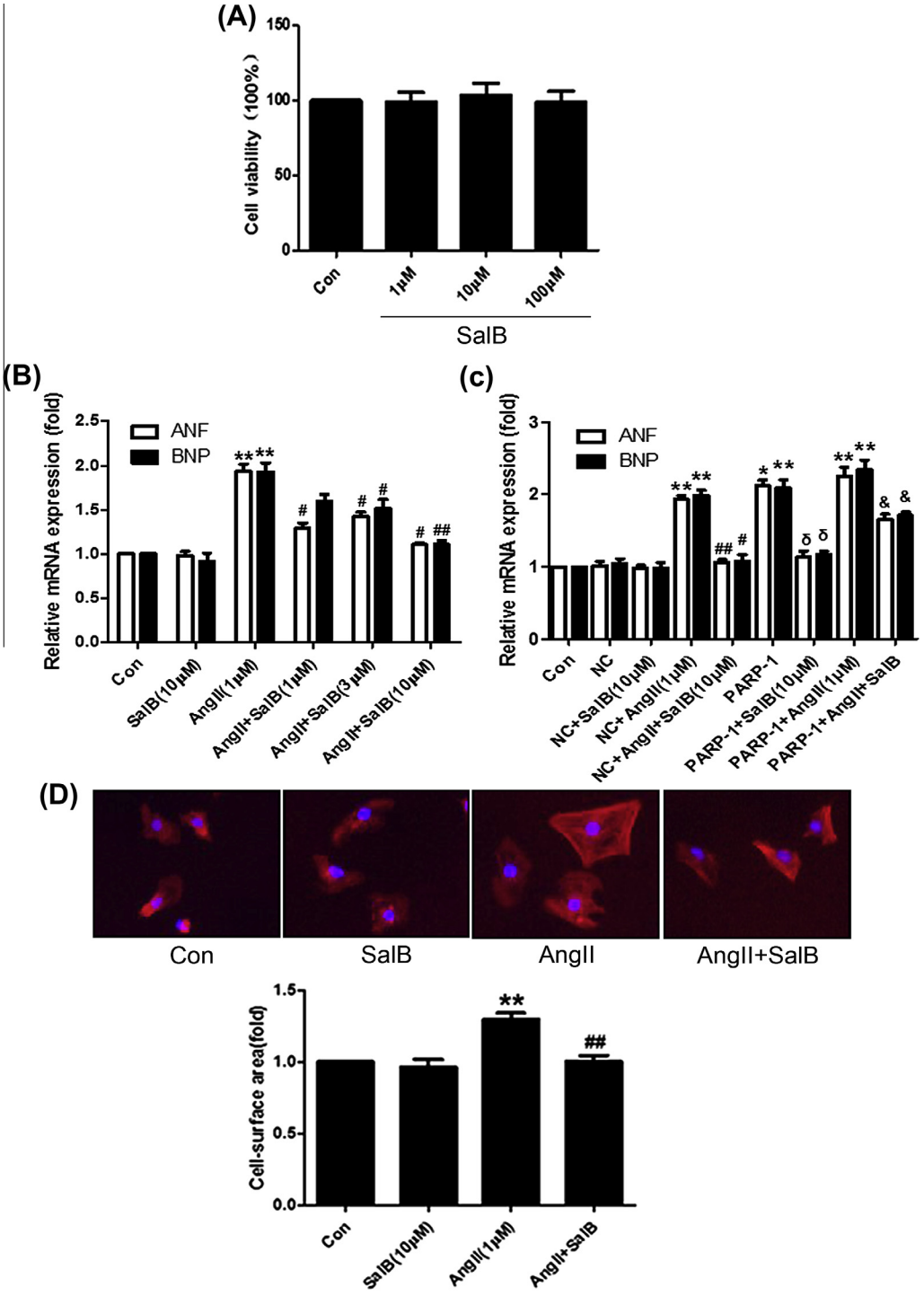

Fig. 1. Effect of SalB on AngII-induced hypertrophy in cardiomyocytes. (A) Cell viability was measured by MTT assay, and data were presented as percentage of the control, $n = 6$. (B) The mRNA levels of ANF and BNP were determined by qRT-PCR with bar graphs showing the relative expression to β-actin. Data were presented as fold of the control, $n = 5$. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared to control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ as compared to AngII group. (C) The mRNA levels of ANF and BNP in cardiomyocytes transfected with PARP-1 plasmid (PARP-1) or empty vector (NC) were determined by qRT-PCR. Data were presented as fold of NC, $n = 5$. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared to NC group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ as compared to NC + AngII group; $^{\&}P < 0.05$ as compared to NC + AngII (1 μM) + SalB (10 μM) group; $^{\delta}P < 0.05$ as compared to PARP-1 group. (D) Cell surface area was measured by rhodamine-phalloidin staining (red color indicating the stained actin, blue color indicating the DAPI-stained nucleus), and bar graphs showed the cell surface area presented as fold of the control, $n = 5$. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared to the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ as compared to the AngII group; data were presented as mean ± SD. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

treatments with SalB at the concentrations of 1–100 μM for 24 h did not alter the viability of the cardiomyocytes as compared to the control (Fig. 1A). Atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) are used as common diagnostic and prognostic markers for hypertrophy [22]. To investigate the potential effect of SalB on AngII-induced hypertrophy in cardiomyocytes, the mRNA expression of ANF and BNP, as well as the cardiomyocyte surface area were determined. As shown in Fig. 1B, ANF and BNP mRNA levels in cultured neonatal rat cardiomyocytes were significantly elevated following AngII treatment, which could be reduced by SalB (3–10 μM). To further determine the involvement of PARP-1 in the anti-hypertrophic effect of SalB, cells were

transfected with PARP-1 plasmid (Fig. 1C). Transfection with empty vector did not influence the mRNA levels of ANF and BNP in cardiomyocytes. In contrast, overexpression of PARP-1 remarkably increased the expression of these hypertrophic markers, which could be reversed by 10 μM SalB. Moreover, overexpression of PARP-1 in plasmid transfected cardiomyocytes significantly attenuated the anti-hypertrophic effect of SalB. Furthermore, SalB also inhibited the increase in cell surface area induced by AngII (Fig. 1D).

3.2. Inhibition of PARP-1 activity by SalB

The effects of SalB and 3-AB on PARP-1 activity were assessed by using a rapid and highly sensitive enzyme assay [19]. Both SalB and 3-AB, a well-established PARP-1 inhibitor, showed significant inhibitory effects on PARP-1 activity at the concentrations from 10^{-6} to 10^{-4} M (Fig. 2A and B). The IC_{50} value calculated from the concentration–response curves for SalB and 3-AB were 3.55 ± 0.96 and 9.08 ± 3.28 μM, respectively.

AngII stimulation led to activation of PARP-1 in a dose- and time-dependent manner in cultured neonatal rat cardiomyocytes (Supplementary Fig. S2A and B), while it did not influence the mRNA and protein expressions of PARP-1 as revealed by qRT-PCR and Western blotting assay (Fig. 2C and D). Although SalB alone did not affect the expression and activity of PARP-1, pre-treatment with SalB (3 μM or 10 μM) could obviously inhibited PARP-1 activation in response to AngII stimulation (Fig. 2E). Similar results could be obtained by 3-AB (20 μM) (Fig. 2E).

3.3. The effect of SalB on cellular NAD^+ level

Since it has been well documented that excessive PARP-1 activation leads to depletion of cellular NAD^+ [6–8], the effect of SalB on NAD^+ level in neonatal rat cardiomyocytes was further

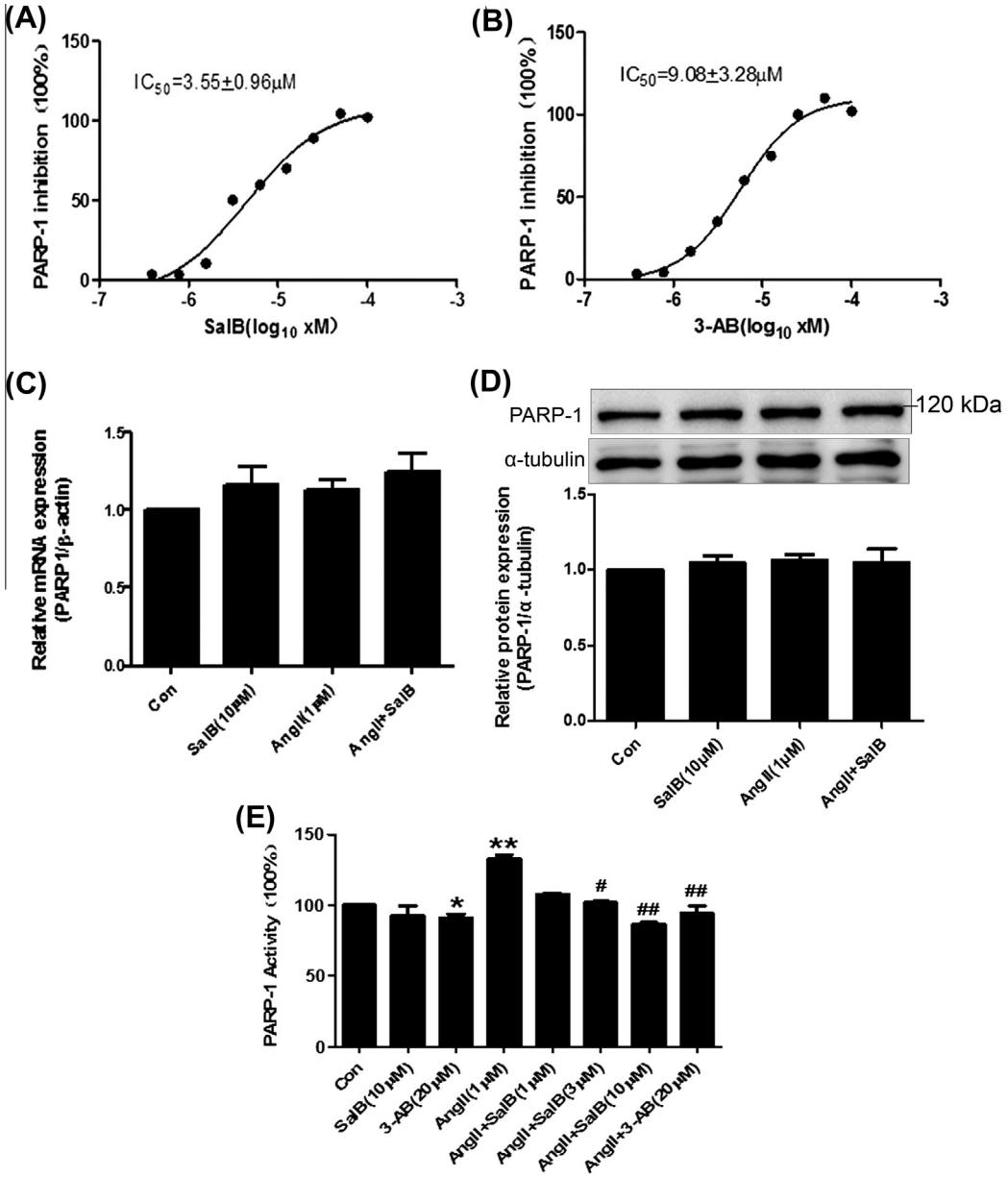

Fig. 2. Effect of SalB on PARP-1 activity. Concentration–response curves showed the inhibition of PARP-1 activity by SalB (A) and 3-AB (B) measured by PARP-1 enzymatic assay; the mRNA levels (C) and protein expression (D) of PARP-1 were detected in the cardiomyocytes treated with SalB or AngII for 24 h; (E) effects of SalB and 3-AB on PARP-1 enzyme activity were determined in cardiomyocytes treated with AngII for 24 h. Data were presented as percentage of the control and shown as mean ± SD. $^{**}P < 0.05$, $^{*}P < 0.01$ as compared to the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ as compared to the AngII group. $n = 5$.

investigated. As shown in Fig. 3A, SalB (10 μM) alone did not influence NAD^+ level, but could significantly reversed AngII-induced decline of NAD^+ content. Exogenous addition of NAD^+ was capable of blocking the agonist-induced cardiac hypertrophic response [10]. In present study, supplement of NAD^+ in culture medium reversed AngII-induced expression of ANF and BNP (Fig. 3B and C), as well as the increase in cell surface area (Fig. 3D).

3.4. The effect of SalB on reactive oxygen species (ROS) generation and NADPH oxidases expression

According to the previous studies, SalB acts as reactive oxygen species scavengers [23]. The antioxidant effect of SalB was evaluated by using the ROS staining dye DHE. The data demonstrated that SalB inhibited the generation of ROS induced by AngII (Fig. 4A). The effect of SalB on the expression of the two main ROS-generating NADPH oxidases, NOX2 and NOX4, was detected by Western blotting. As shown in Fig. 4B and C, AngII markedly augmented the protein levels of NOX2 and NOX4, which could be ameliorated by SalB pre-treatment.

4. Discussion

AngII is a major neurohumoral factor that induces cardiac hypertrophy [24]. The present study reveals that SalB can

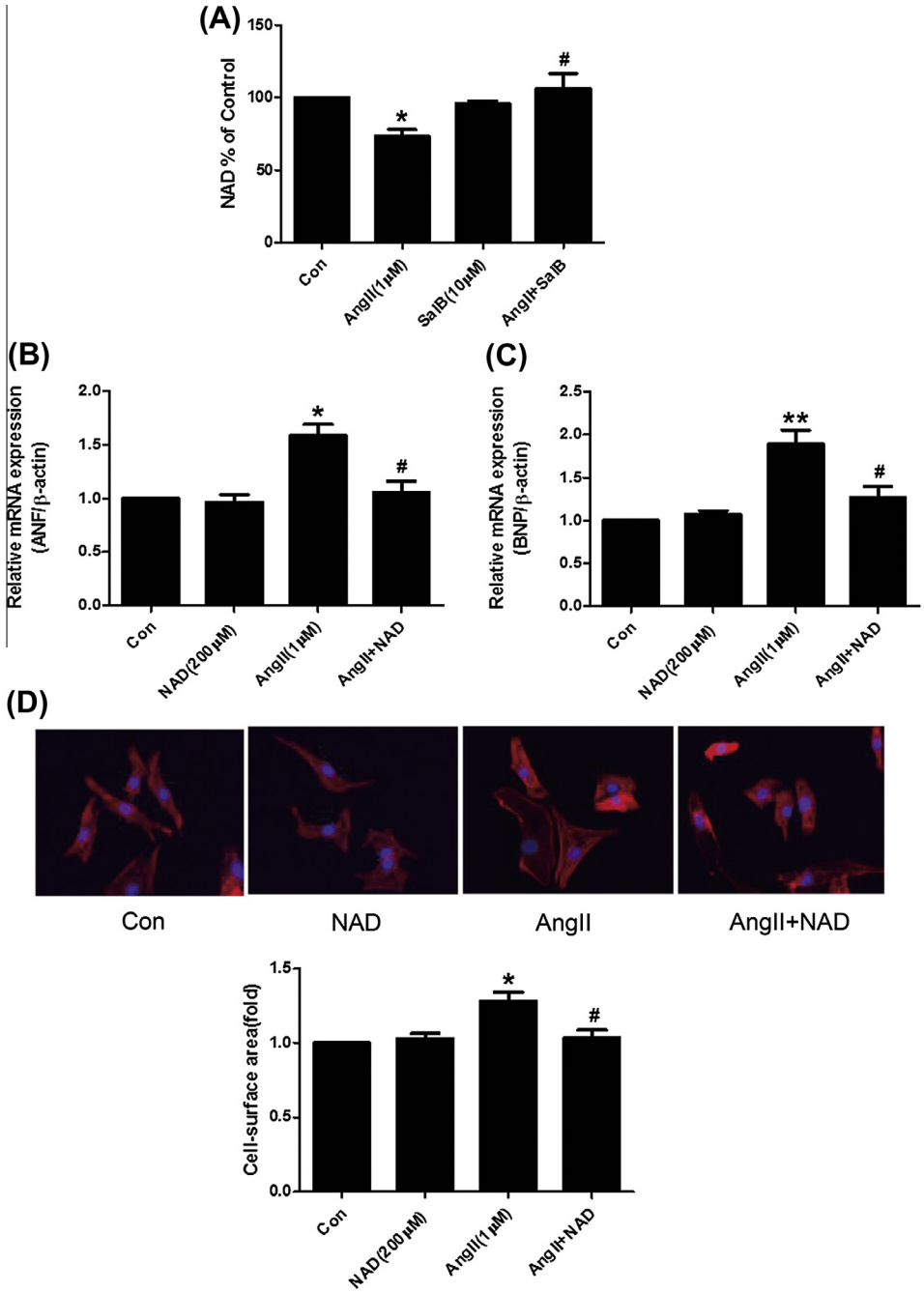

Fig. 3. Effect of SalB on the NAD^+ level. (A) NAD^+ content in the cardiomyocytes treated with AngII for 24 h was determined; the mRNA levels of ANF (B) and BNP (C) were detected by qRT-PCR. Bar graphs showed the relative expression level to β-actin, presented as fold of the control; (D) cell surface area was measured by rhodamine-phalloidin staining. Images showed the cells detected under high Content Analysis System (red color indicating the stained actin, blue color indicating the DAPI-stained nucleus), and bar graphs showed the cell surface area presented as fold of the control. Data were presented as mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$ as compared to the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ as compared to the AngII group. $n = 5$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

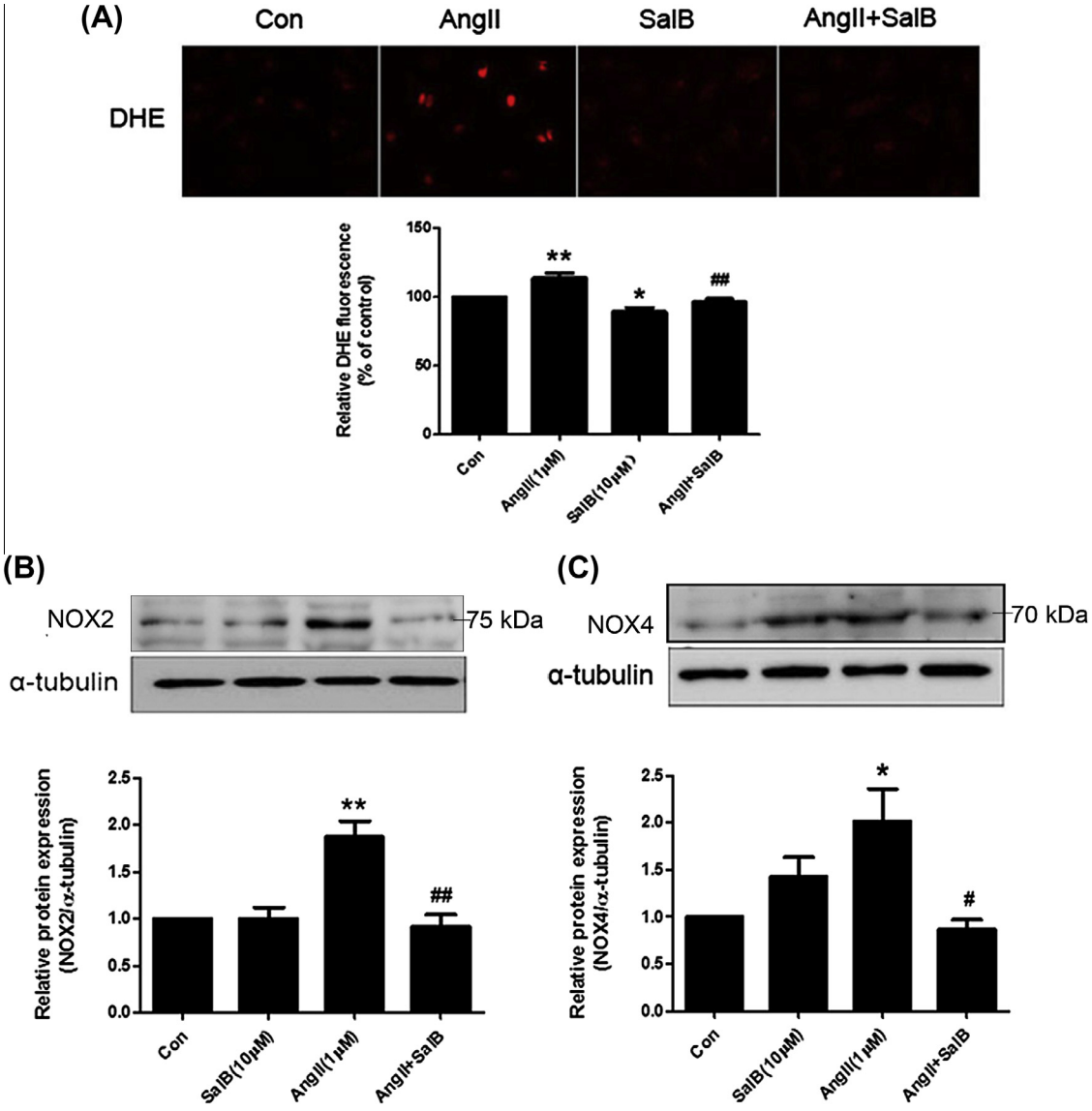

Fig. 4. Effect of SalB on ROS production and the expression of NOX2 and NOX4. (A) ROS production was measured by DHE fluorescence. Images showed DHE-stained cells (red), and bar graphs showed DHE fluorescence intensity. Data were presented as percentage of the control. (B and C) The protein expression of NOX2 and NOX4 were detected by Western blotting. Bar graphs showed the relative expression level to tubulin, presented as fold of the control. Data were presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared to the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ as compared to the AngII group. $n = 5$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

obviously block the hypertrophic responses in cultured neonatal rat cardiomyocytes stimulated with AngII. These observations are in line with the previous findings that SalB attenuates heart hypertrophy in animal models with myocardial infarction [25].

To further investigate the mechanisms underlying the inhibitory effect of SalB on cardiac hypertrophy, its potential effect on PARP-1 was studied. As an enzyme which plays a critical role in repairment of DNA damage, PARP-1 participates in tumor formation, diabetes and various cardiovascular diseases [26]. Recently, the activation of PARP-1 has been observed in animal models with physiological hypertrophy and pathological hypertrophy [5]. In contrast, PARP-1 inhibitors can ameliorate cardiac hypertrophy induced by AngII, indicating that PARP-1 may be a mediator of cardiac hypertrophy [3]. Here, our experiments proved that SalB could significantly inhibit the activity of PARP-1. According to the concentration–response curve, SalB showed an IC_{50} value lower than that of the known PARP-1 inhibitor 3-AB. Moreover, in cultured neonatal rat cardiomyocytes, SalB blocked AngII-induced activation of PARP-1 and hypertrophic responses. Furthermore, anti-hypertrophic response of SalB was significantly attenuated in cultured neonatal rat cardiomyocytes overexpressed with PARP-1. These results suggested that the protective effect of SalB against cardiac hypertrophy was at least partly attributed to inhibition of PARP-1.

PARP-1 is one of the primary metabolic enzymes of NAD^+ [6,8]. Overactivation of PARP-1 by AngII may deplete the stores of cellular NAD^+ and induce a progressive ATP depletion, thereby altering the functions and activities of NAD^+-dependent enzymes including the sirtuin family [27]. Exogenous addition of NAD^+ could improve the agonist-induced cardiac hypertrophic response [10]. To further explore the mechanisms underlying the inhibitory effect of SalB on PARP-1, the cellular content of NAD^+ was determined. Treatment with SalB in the cardiomyocytes prevented the NAD^+ depletion induced by AngII. Moreover, supplement of NAD^+ in AngII-stimulated cardiomyocytes could inhibit the augmented expressions of ANF and BNP, as well as the increase of cell surface area, further

suggesting that the rescue of cellular NAD^+ by SalB through inhibition of PARP-1 may help to prevent cardiac hypertrophy.

SalB has been proved to be a potent scavenger of ROS [23,28]. ROS could cause DNA damage, which induces PARP-1 activation [3]. Based on the observations in the DHE staining experiments, SalB significantly reduced AngII-induced generation of ROS in the cardiomyocytes. In addition, SalB prevented AngII-induced up-regulation of NOX2 and NOX4, the major NADPH oxidases in the heart which play important roles in the pathogenesis of cardiac hypertrophy [29,30]. These observations suggest that the antioxidant effect of SalB is probably attributed to the suppression of NOX2 and NOX4. Recently, Manea et al. reported that activation of NF-κB pathway significantly increased NOX4 expression in human aortic smooth muscle cells, and cotransfection experiments revealed the presence of functionally NF-κB binding sites on the promoter of NOX4 gene [31]. Additionally, it has been proved that SalB inhibited nuclear translocation of NF-κB in human umbilical vein endothelial cells [32], and decreased NF-κB p65 protein levels in the nuclei of human aortic endothelial cells [33]. Thus, SalB may suppress expression of NOX2 and NOX4 by inhibiting NF-κB pathway. It's now known that the activation of PARP-1 by AngII is associated with the activation of the membrane-bound NADPH oxidases, which leads to an increased production of reactive oxygen and nitrogen species [34,35] and causes DNA damage [3]. The antioxidant effect of SalB via suppressing NOX2 and NOX4 might partially contribute to its inhibitory effect on PARP-1, besides its directly inhibition of PAPR-1 activation.

In summary, the key findings of the present study are that SalB is a potent inhibitor of PARP-1, and SalB can protect cardiomyocytes from AngII-induced hypertrophy partially through inhibition of PARP-1. SalB can inhibit the activity of PARP-1 and prevent the depletion of NAD^+, finally contributing to cardiac protection. Additionally, the antioxidant effect of SalB via suppression of NOX2 and NOX4 probably also contributes to the inhibition of PARP-1. Understanding of this novel role of SalB might provide clues to the rational development and exploitation of PARP-1 inhibitors in clinical setting including cardiac hypertrophy.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.045.

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