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North American ginseng protects the heart from ischemia and reperfusion injury via upregulation of endothelial nitric oxide synthase

Yan Wu[a,d], Xiangru Lu[c], Fu-Li Xiang[a], Edmund M.K. Lui[a], Qingping Feng[a,b,c,*]

[a] Department of Physiology and Pharmacology, University of Western Ontario, London, Ontario, Canada
[b] Department of Medicine, University of Western Ontario, London, Ontario, Canada
[c] Lawson Health Research Institute, London Health Sciences Centre, London, Ontario, Canada
[d] Department of Pharmacology, School of Pharmaceutical Sciences, Central South University, Changsha, China

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ABSTRACT

Emerging evidence suggests ginseng has therapeutic potential in cardiovascular disease. The aim of this study was to investigate the role of endothelial nitric oxide synthase (eNOS) in the cardioprotective effects of ginseng during myocardial ischemia and reperfusion (I/R). Treatment with ginseng extract significantly increased Akt phosphorylation and eNOS protein levels in cultured neonatal cardiomyocytes. Upregulation of eNOS was blocked by LY294002, a PI3-kinase inhibitor, suggesting a PI3-kinase/Akt-dependent mechanism. To simulate I/R, cultured neonatal cardiomyocytes from eNOS$^{-/-}$ and wild-type (WT) mice were subjected to anoxia and reoxygenation (A/R). Ginseng treatment inhibited A/R-induced apoptosis in WT, but not in either eNOS$^{-/-}$ cardiomyocytes or WT cardiomyocytes treated with LY294002. To further study the cardioprotective effects of ginseng *in vivo*, WT and eNOS$^{-/-}$ mice were pretreated with ginseng extract (50 mg/kg/day, oral gavage) for 7 days before they were subjected to myocardial I/R. Treatment with ginseng significantly increased Akt phosphorylation and eNOS protein levels in the myocardium. Furthermore, ginseng-induced myocardial eNOS expression was inhibited by LY294002. Strikingly, ginseng treatment significantly decreased infarct size and myocardial apoptosis following I/R in WT mice, but not in either eNOS$^{-/-}$ mice or WT mice treated with LY294002. We conclude that ginseng treatment protects the heart from I/R injury via upregulation of eNOS expression. Our study suggests that ginseng may serve as a potential therapeutic agent to limit myocardial I/R injury.

1. Introduction

Ginseng has been used as an herbal medicine for more than 2000 years in China, Korea and Japan, and in the last two decades has gained popularity in the United States, Canada and Europe [1,2]. Its major active components are ginsenosides and about 40 distinct ginsenosides have been identified [3]. Ginseng possesses a wide range of pharmacological effects including immune modulation, anti-diabetic and anti-cancer effects [1,4–6]. In the cardiovascular system, ginseng has been shown to stimulate nitric oxide release, vasorelaxation, and to improve lipid profiles, and has been used to treat hypertension and heart failure [7–11]. Furthermore, ginseng treatment also decreases the frequency and severity of angina attacks in patients with coronary heart disease [12]. Recent studies have shown that ginsenosides protects the heart from ischemia and reperfusion (I/R) injury in rats [13]. However, the mechanisms by which ginseng mediates its cardioprotection are still not fully understood.

Endothelial nitric oxide synthase (eNOS) is constitutively expressed in the heart and produces low levels of nitric oxide (NO) that mediate cellular signaling [14,15]. NO production from eNOS protects the heart from I/R injury via inhibition of myocardial apoptosis [16,17]. The anti-apoptotic effects of NO produced from eNOS are mediated by reduction of oxidative stress and inhibition of caspase activation through S-nitrosylation [18]. We have demonstrated that activation of PI3-kinase/Akt (protein kinase B, PKB) signaling upregulates eNOS expression leading to increased eNOS activity in cardiomyocytes [16,19]. Recent studies have shown that ginsenosides activate PI3-kinase/Akt/eNOS pathway and increase coronary artery perfusion in an isolated rat heart preparation [20]. However, whether increased eNOS expression mediates cardioprotection after ginseng treatment remains to be determined.

In the present study, we hypothesized that ginseng upregulates eNOS expression via PI3-kinase/Akt signaling. We further hypothesized that increased eNOS expression contributes to the cardioprotective effects of ginseng during myocardial I/R. To test

* Corresponding author at: Department of Physiology and Pharmacology, University of Western Ontario, London, Ontario, N6A 5C1, Canada. Tel.: +1 519 850 2989; fax: +1 519 661 4051.
E-mail address: qfeng@uwo.ca (Q. Feng).

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these hypotheses, cultured neonatal cardiomyocytes from eNOS$^{-/-}$ and WT mice were pretreated with ginseng aqueous extract from North American ginseng (*Panax quinquefolius*) and subjected to anoxia and reoxygenation (A/R) to simulate I/R. Furthermore, eNOS$^{-/-}$ and corresponding wild-type (WT) mice were pretreated with ginseng for 7 days before induction of myocardial I/R. Our results showed an important role of eNOS in the cardioprotective effects of ginseng.

2. Materials and methods

2.1. Animals

WT and eNOS$^{-/-}$ mice of C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, Maine), and a breeding program was carried out at the University of Western Ontario animal care facilities. All animals have access to food and water *ad libitum* and were housed in a temperature and humidity controlled facility with 12-h light and dark cycles. The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH, publication No. 85–23). Animal manipulation was approved by the Animal Use Subcommittee at the University of Western Ontario.

2.2. Isolation and cultures of neonatal mouse cardiomyocytes

Neonatal cardiomyocytes were prepared from mice born within 24 h as in our previous reports [21,22]. Non-cardiomyocytes were removed through 1 h of preplating, after which cardiomyocytes were plated on culture plates precoated with 1% gelatin (Sigma) in M199 medium containing 10% fetal bovine serum (FBS). The cells were incubated in a tissue incubator containing 5% CO_2 at 37 °C. After 48 h of cell culture, cardiomyocytes were subjected to various treatments and subsequent experimental protocols.

2.3. Anoxia and reoxygenation

After cardiomyocytes were pretreated with ginseng (2 μg/ml) and LY294002 (10 μM, Sigma) for 24 h, the cells were subjected to A/R as previously described with modifications [23,24]. Briefly, cardiomyocytes were exposed to serum-free M199 bubbled with 100% N_2 to remove soluble oxygen and then incubated in an anoxia chamber (BioSpherix, Redfield, NY) with 95% N_2 and 5% CO_2 for 2 h to induce anoxia. The oxygen level was monitored and controlled below 0.1%. After 2 h of anoxia, the medium was replaced by fresh M199 containing 10% FSB and cardiomyocytes were maintained in a regular tissue culture incubator with 5% CO_2 for 2 h to allow reoxygenation (anoxia/reoxygenation, A/R). Cardiomyocytes exposed to serum-free M199 without anoxia served as a control (normoxia/normoxia, N/N).

2.4. Myocardial ischemia and reperfusion

Myocardial I/R was performed on adult male WT and eNOS$^{-/-}$ mice as previously described [16,25]. Briefly, mice were anesthetized with ketamine (50 mg/kg) and xylazine (12.5 mg/kg), intubated and ventilated with a respirator. The left side of the heart was exposed through a left intercostal thoracotomy and the pericardium was opened. The left anterior descending coronary artery was occluded by placing an 8–0 suture with a PE-50 plastic tubing for 45 min to induce myocardial ischemia. The coronary artery was loosened by removing the tubing to allow reperfusion for 3 h.

2.5. Measurement of infarct size

At the end of the 3 h of reperfusion, the coronary artery was religated and 2% Evans blue solution was perfused into the coronary artery through a cannulated right carotid artery. Hearts were excised and cut into four transverse slices from the apex to base. The slices were then incubated with 1.5% triphenyltetrazolium chloride (TTC) solution for 30 min at 37 °C. All sections were then photographed and weighed. For each side of sections, the nonischemic area (blue), area at risk (red), and infract area (pale) were analyzed by using SigmaScan Pro. Infarct size was presented as a percentage of the weight of the infarct area to the area at risk.

2.6. Caspase-3 activity assay

Caspase-3 activity was measured by using the caspase-3 assay kit (BIOMOL, Plymouth Meeting, PA) as previously reported [16]. Briefly, cultured cardiomyocytes or myocardial tissues from the peri-infarcted area were lysed in lysis buffer and the total protein concentration was determined by Lowry assay. Samples (50 μg proteins from cardiomyocytes or 200 μg proteins from the myocardial tissues) were incubated in the presence of caspase-3 substrate Ac-DEVE-AMC with or without the caspase-3 inhibitor Ac-DEVE-CHO for 16 h at 37 °C. Fluorescence intensity (excitation at 355 nm, emission at 405 nm) was measured using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA). Data are expressed as the amount of cleaved AMC substrate per μg protein.

2.7. Cell death detection ELISA

Cytoplasmic histone-associated DNA fragments were measured using the In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. A total of 40 μg cytoplasmic proteins from the peri-infarcted myocardium or cultured cardiomyocytes was added to 96 well plates coated with anti-histone antibody, followed by anti-DNA-peroxidase (POD) antibody. The amount of peroxidase retained in the immunocomplex was determined with ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulfonate(6)]) as a substrate at 405 nm wavelength using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA).

2.8. Analysis of cell necrosis

Cell necrosis in cultured cardiomyocytes was analyzed using a live/dead viability/cytotoxicity assay kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Neonatal cardiomyocytes were cultured in 96-well plates (100,000 cells per well) for 48 h. Cells were then treated with vehicle or ginseng extract (2 μg/ml) for 24 h followed by N/N or A/R as described above. Cell necrosis was determined by measuring the red fluorescence (excitation/emission, 528/617 nm) of ethidium homodimer, which enters the cell through the damaged cell membranes. The detection limit of this assay was 3000 cells per well (3%).

2.9. Western blot analysis

A total of 50 μg protein from the myocardium or cultured cardiomyocytes was loaded and separated by 10% SDS-PAGE, followed by electrotransfer to a nitrocellulose membrane. The blots were probed with specific primary antibodies against mouse total-Akt (Cell Signaling), phospho-Akt (Cell Signaling), eNOS (BD Transduction Laboratories) and α-actinin (Sigma), respectively. Each band was visualized by utilizing an enhanced chemiluminescence detection and exposure to X-ray film. Optical density for individual band

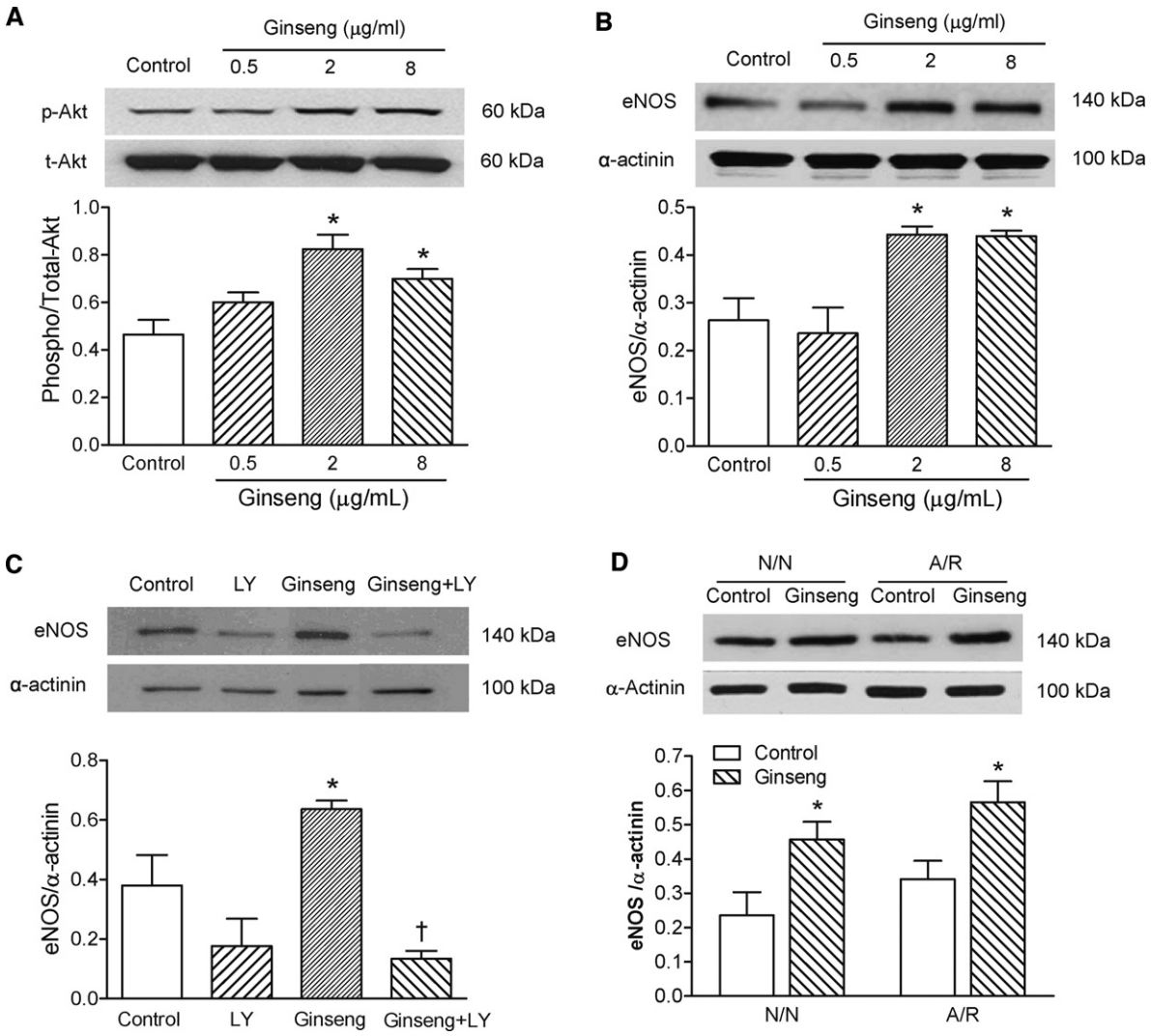

Fig. 1. Ginseng upregulates Akt phosphorylation and eNOS protein expression in cultured neonatal cardiomyocytes. Cultured neonatal cardiomyocytes from WT mice were treated with ginseng (0.5, 2 and 8 μg/ml) for 24 h followed by Western blot analysis. Phosphorylated Akt (p-Akt; total Akt, t-Akt) (A) and eNOS protein levels (B) were examined. (C) Effects of a PI3-kinase inhibitor LY294002 (LY) on ginseng-induced eNOS expression in WT cardiomyocytes. Cells were treated with ginseng (8 μg/ml) with and without LY294002 (10 μM) for 24 h. The ratio of eNOS to α-actinin protein levels was assessed. (D) Effects of ginseng (8 μg/ml) on eNOS protein expression in cultured cardiomyocytes during normoxia/normoxia (N/N) and anoxia/reoxygenation (A/R). Data are mean ± SEM from 3 independent experiments. Half a million cardiomyocytes were used for each measurement. *$P < 0.05$ vs. control; †$P < 0.05$ vs. ginseng.

was examined using FluorChem 8000 (Alpha Innotech, San Leandro, CA). The densitometry ratios of phosphorylated Akt to total Akt or eNOS to α-actinin were presented.

2.10. Ginseng extraction procedures

Four-year old North American ginseng (*P. quinquefolius*) roots from 5 farms in Ontario, Canada were collected and processed at Naturex (South Hackensack, NJ) using an aqueous extraction procedure. Briefly, ground ginseng roots were soaked in water at 40 °C over 5 h followed by filtering and the filtrate was vacuum dried at 45 °C. The extracts were further concentrated to approximately 60% solids, which was then lyophilized in the Ontario Ginseng Innovation and Research Consortium Central Laboratory at the University of Western Ontario. The concentration of ginsenosides, the major active ingredients was measured by high performance liquid chromatography (HPLC) [26]. The aqueous extracts of ginseng were used in the present study.

2.11. Statistical analysis

All data were given as mean ± SEM from at least 3 independent experiments. Statistical analysis was performed using one- or two-way ANOVA followed by Newman–Keuls or Bonferroni post hoc test. Differences were considered significant at the level of $P < 0.05$.

3. Results

3.1. Characterization of the aqueous extract of North American ginseng

Ginsenoside content of the aqueous extract of North American ginseng from 5 Ontario farms was analyzed by HPLC. The aqueous extract contained 23.8 ± 3.2, 21.3 ± 3.6, 5.4 ± 0.3, 3.5 ± 0.2, 1.9 ± 0.3 and 1.4 ± 0.2 (mg/g crude ginseng dry weight, mean ± SEM) for Re, Rb1, Rc, Rd, Rg1 and Rb2, respectively. The results showed that the predominant ginsenosides are Re and Rb1.

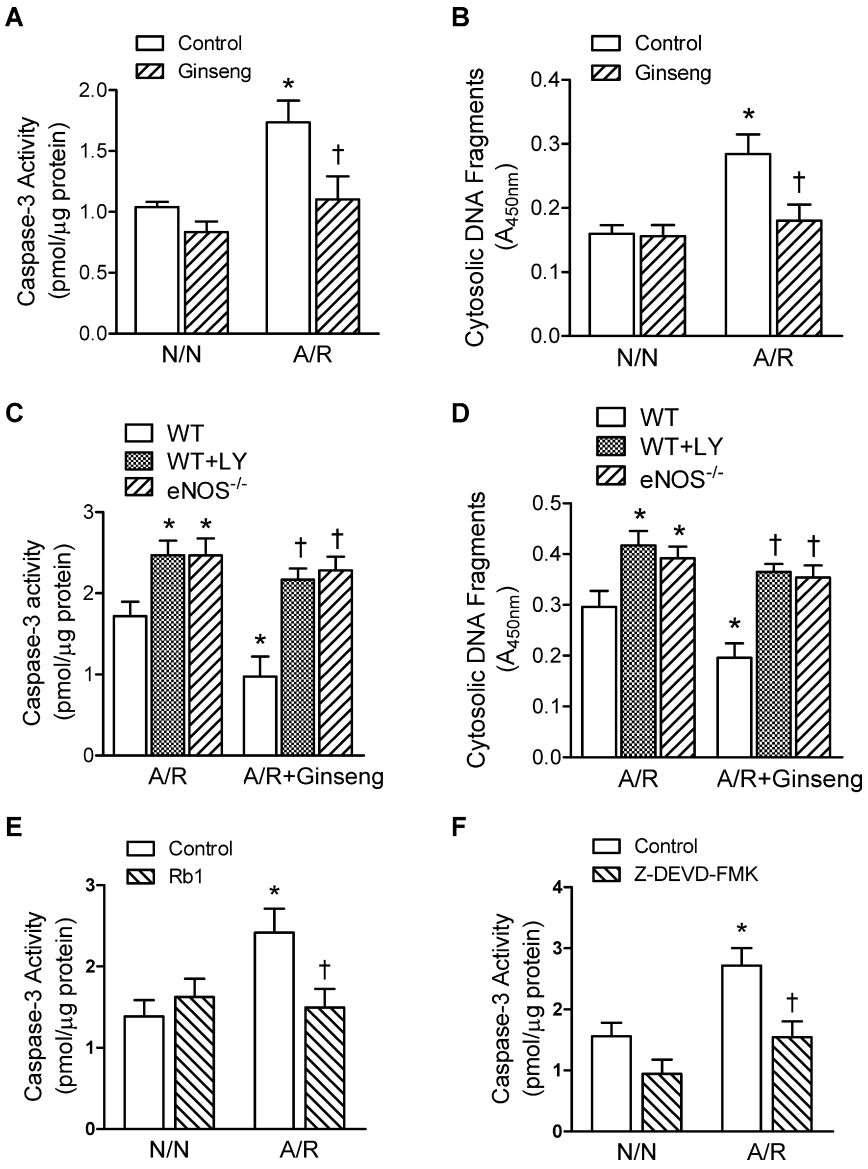

Fig. 2. Effects of ginseng on A/R-induced cardiomyocyte apoptosis. (A) and (B) Cultured neonatal cardiomyocytes from WT mice were treated with ginseng extract (2 μg/ml) for 24 h. A/R-induced apoptosis was assessed by caspase-3 activity (A) and cytosolic DNA fragments (B), respectively. (C) and (D) Cultured neonatal cardiomyocytes from WT and eNOS$^{-/-}$ mice were treated with ginseng extract (2 μg/ml) for 24 h with or without LY294002 (10 μM) followed by an anoxia/reoxygenation challenge. Apoptosis was examined by caspase-3 activity (C) and cytosolic DNA fragments (D). (E) Effects of ginsenoside Rb1 (10 μM) on caspase-3 activity in cultured cardiomyocytes during N/N and A/R. (F) Effects of caspase-3 inhibitor Z-DEVD-FMK (2 μM) on cardiomyocyte apoptosis induced by A/R. Data are mean ± SEM of 5 independent experiments. One million cardiomyocytes were used for each measurement. *$P < 0.05$ vs. N/N control or WT A/R; †$P < 0.05$ vs. A/R control or WT A/R + ginseng.

3.2. *Effects of ginseng on Akt phosphorylation and eNOS expression in cultured neonatal cardiomyocytes*

Cardiomyocytes express eNOS under basal physiological conditions and its expression is regulated by PI3-kinase/Akt signaling [16,27]. To study the effects of ginseng on eNOS expression, cultured neonatal mouse cardiomyocytes were treated with ginseng extract for 24 h. Akt phosphorylation and eNOS protein levels were determined by Western blot analysis. As shown in Fig. 1A and B, Akt phosphorylation and eNOS protein levels were significantly increased in cardiomyocytes with either 2 or 8 μg/ml of ginseng extract treatment ($P < 0.05$). The data showed that ginseng increases Akt phosphorylation and eNOS expression in cardiomyocytes.

To further determine if enhanced Akt phosphorylation is responsible for ginseng-induced eNOS expression, cultured cardiomyocytes were treated with ginseng (8 μg/ml) in the presence or absence of LY294002 (10 μM) to inhibit PI3-kinase/Akt activity. Our data showed that inhibition of PI3-kinase/Akt activity completely blocked the increase in cardiomyocyte eNOS expression induced by ginseng ($P < 0.05$, Fig. 1 C). These results suggest that ginseng upregulates eNOS expression via PI3-kinase/Akt signaling in cardiomyocytes. Additionally, ginseng treatment significantly increased eNOS expression in cardiomyocytes during both normoxia/normoxia (N/N) and A/R conditions ($P < 0.05$, Fig. 1D).

3.3. *Effects of ginseng on A/R-induced apoptosis in WT and eNOS$^{-/-}$ cardiomyocytes*

To study the protective effects of ginseng, cultured neonatal cardiomyocytes from WT mice were subjected to A/R challenge to induce apoptosis, with N/N as a control. Our data showed that A/R significantly induced cardiomyocyte apoptosis as measured by caspase-3 activity and cytosolic DNA fragments (Fig. 2A and B).

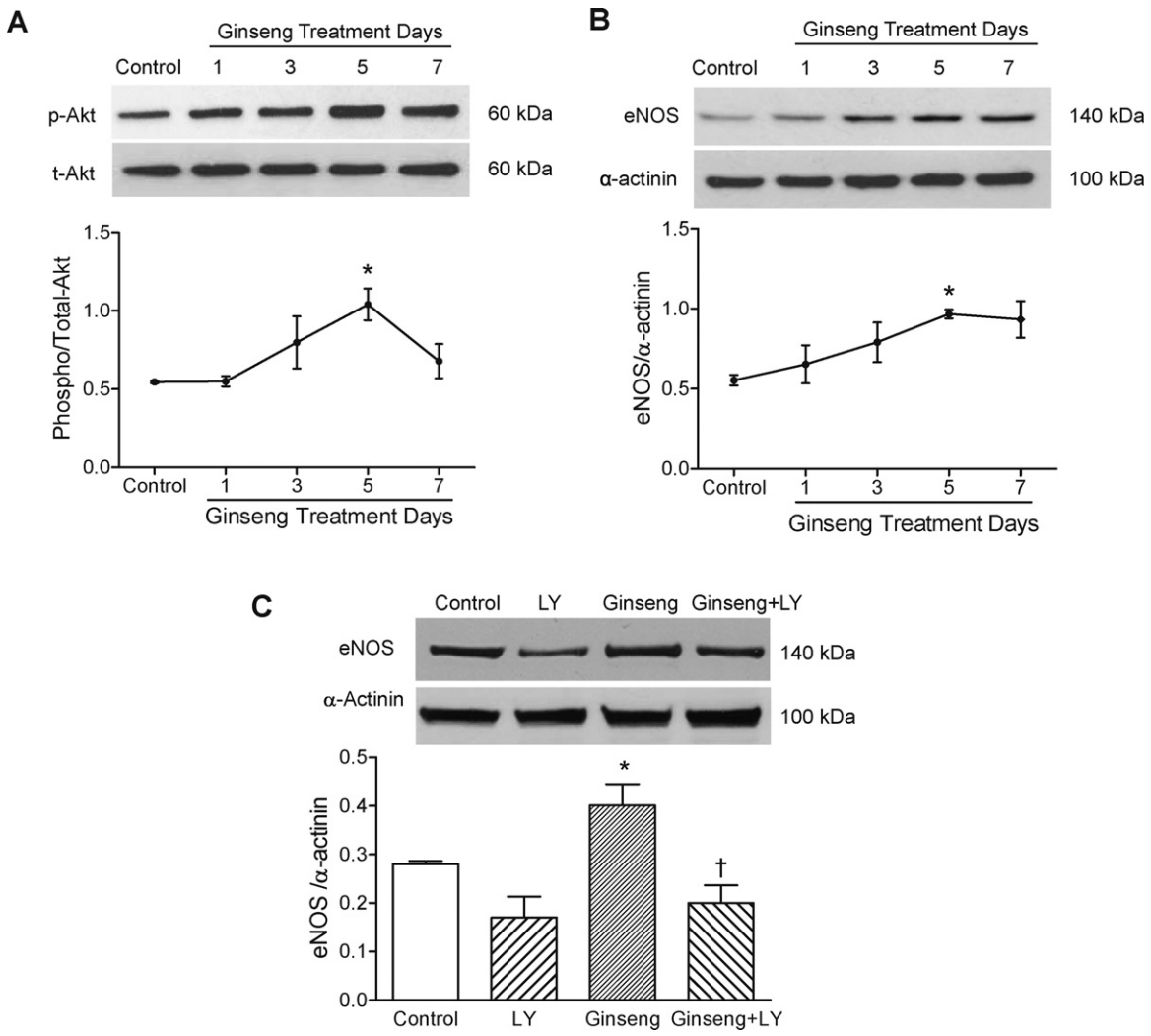

Fig. 3. Ginseng upregulates Akt phosphorylation and eNOS protein expression in the myocardium. WT mice were treated with ginseng aqueous extracts (50 mg/kg, oral gavage) for 1, 3, 5 and 7 days. Myocardial Akt phosphorylation and eNOS protein levels were determined by Western blot analysis. (A) Ratio of phosphorylated Akt (p-Akt) to total Akt (t-Akt) after ginseng treatment. (B) Ratio of eNOS to α-actinin protein after ginseng treatment. (C) Mice were treated with ginseng aqueous extracts (50 mg/kg, oral gavage) for 5 days followed by 3 h of LY294002 (LY, 7.5 mg/kg, i.p.) treatment. Myocardial eNOS protein levels were determined. Data are mean ± SEM, $n = 4$ animals per time point. $^{*}P < 0.05$ vs. control. $^{\dagger}P < 0.05$ vs. ginseng.

Furthermore, pretreatment with ginseng extract (2 μg/ml) for 24 h abrogated cardiomyocyte apoptosis induced by A/R ($P < 0.05$, Fig. 2A and B).

To unravel the role of PI3-kinase/Akt and eNOS in the anti-apoptotic effect of ginseng, WT cardiomyocytes treated with LY294002 and cardiomyocytes isolated from eNOS$^{-/-}$ mice were employed. Our data showed that either inhibition of PI3-kinase or deficiency of eNOS abrogated the anti-apoptotic effects of ginseng in cardiomyocytes during A/R ($P < 0.05$, Fig. 2C and D), demonstrating that activation of PI3-kinase/Akt/eNOS pathway plays an important role in the anti-apoptotic effects of ginseng.

Since one of the major ginsenosides in our ginseng extract is Rb1, the effect of Rb1 on cardiomyocyte apoptosis was studied. Treatment with Rb1 (10 μM) significantly inhibited A/R-induced cardiomyocyte apoptosis ($P < 0.05$, Fig. 2E). To confirm the A/R-induced apoptosis was mediated by caspase-3 activation in the present study, cardiomyocytes were treated with a specific caspase-3 inhibitor, Z-DEVD-FMK (2 μM). Our data showed that A/R-induced apoptosis was significantly blocked by Z-DEVD-FMK ($P < 0.05$, Fig. 2F), indicating caspase-3 dependent cardiomyocyte apoptosis.

To assess necrosis, cultured neonatal cardiomyocytes were subjected to N/N or A/R with and without ginseng (2 μg/ml) treatment. Under these experimental conditions, cardiomyocyte necrosis was not detectable (<3% of cell necrosis) in any of the treatment groups (data not shown), suggesting necrosis is not a major type of cell death in the cultured cardiomyocytes.

3.4. *Effects of ginseng on myocardial Akt phosphorylation and eNOS expression*

To study the effects of ginseng on myocardial Akt phosphorylation and eNOS expression, WT mice were treated with ginseng aqueous extract (50 mg/kg/day) by oral gavage for 7 days, and myocardial eNOS protein levels were determined at day 1, 3, 5 and 7. Consistent with data in cultured cardiomyocytes, Akt phosphorylation and eNOS protein levels assessed by Western blot analysis were significantly increased 5 days after ginseng treatment compared to untreated controls ($P < 0.05$, Fig. 3A and B). These data showed that oral treatment with ginseng increases Akt phosphorylation and eNOS expression in the myocardium *in vivo*. Consistent with data in cultured cardiomyocytes, treatment with

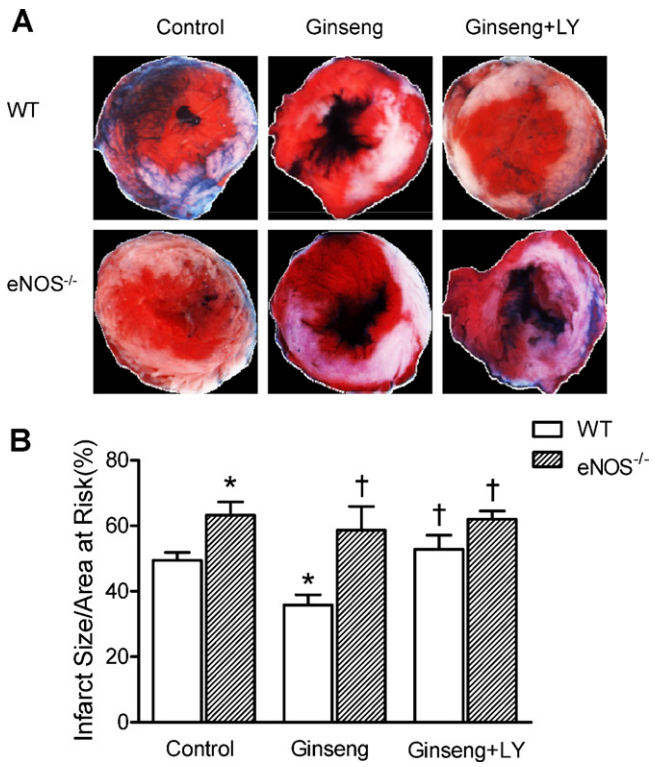

Fig. 4. Effects of ginseng on infarct size following myocardial I/R in WT and eNOS$^{-/-}$ mice. Mice were pretreated with ginseng extract (50 mg/kg/day) or water (control) by oral gavage for 7 days followed by myocardial I/R (45 min of ischemia followed by 3 h of reperfusion). Some animals were treated with LY294002 (LY, 7.5 mg/kg, i.p.) at 15 min before reperfusion. (A) Representative triphenyltetrazolium chloride-stained heart sections from each corresponding group. White, red and blue indicate infarct, area at risk and normal myocardium, respectively. (B) Infarct size expressed as percent of the weight of the infarct to the area at risk. Sham operated mice without myocardial I/R had no infarct. Data are mean ± SEM, $n = 4$–5 per group. *$P < 0.05$ vs. WT control; †$P < 0.05$ vs. WT + ginseng.

LY294002 (7.5 mg/kg, i.p.) in mice *in vivo* significantly blocked ginseng-induced eNOS expression in the myocardium ($P < 0.05$, Fig. 3C), suggesting a PI3K/Akt-dependent mechanism.

3.5. eNOS is pivotal in the cardioprotective effects of ginseng during myocardial I/R

To examine the effects of ginseng on myocardial I/R injury and the role of eNOS in cardioprotection by ginseng, WT and eNOS$^{-/-}$ mice were pretreated with ginseng aqueous extract (50 mg/kg/day) or water by oral gavage for 7 days, after which myocardial I/R was performed and infarct size was measured. Our results showed that pretreatment with ginseng significantly decreased infarct size in WT mice compared to untreated controls ($P < 0.05$, Fig. 4A and B). Consistent with our previous report [16], infarct size was significantly increased in eNOS$^{-/-}$ mice compared to WT mice following myocardial I/R ($P < 0.05$, Fig. 4A and B). Strikingly, the protective effects of ginseng were completely abolished in eNOS$^{-/-}$ mice ($P < 0.05$, Fig. 4A and B), suggesting an important role of eNOS in ginseng-induced cardioprotection.

To study the role of PI3-kinase/Akt pathway in the cardioprotective effects of ginseng, mice were treated with ginseng extracts (50 mg/kg/day, oral gavage) or water for 7 days followed by myocardial I/R. LY294002 (7.5 mg/kg, i.p.) treatment was given at 15 min before reperfusion. Treatment with LY294002 abrogated the beneficial effects of ginseng during I/R ($P < 0.05$, Fig. 4A and B). Taken together, our results showed that ginseng pretreatment limits I/R-induced myocardial injury via PI3-kinase/Akt/eNOS pathway.

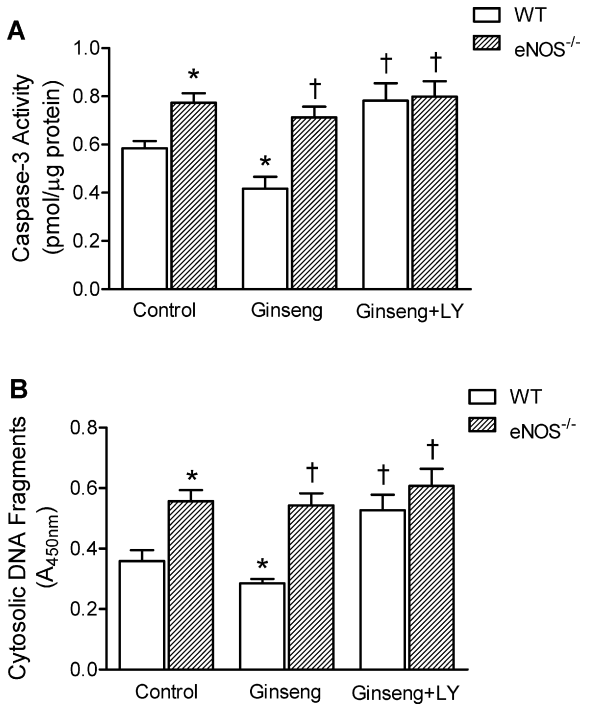

Fig. 5. Effects of ginseng on I/R-induced apoptosis in the peri-infarct myocardium. WT and eNOS$^{-/-}$ mice were treated with ginseng (50 mg/kg/day) or water (control) by oral gavage for 7 days followed by I/R. Some animals were treated with LY294002 (LY, 7.5 mg/kg, i.p.) at 15 min before reperfusion. Myocardial caspase-3 activity (A) and cytosolic DNA fragments (B) were determined. Data are mean ± SEM, $n = 6$ per group. *$P < 0.05$ vs. WT control; †$P < 0.05$ vs. WT + ginseng.

3.6. Effects of ginseng on I/R-induced myocardial apoptosis in WT and eNOS$^{-/-}$ mice

Myocardial apoptosis is a major contributor to infarct size after myocardial infarction [28]. To elucidate whether the cardioprotective effects of ginseng are attributed to inhibition of myocardial apoptosis, we measured two important hallmarks of apoptosis, caspase-3 activity and cytosolic DNA fragments in the peri-infarct myocardium after I/R. Pretreatment with ginseng for 7 days significantly decreased I/R-induced myocardial apoptosis as determined by caspase-3 activity and cytosolic DNA fragments in WT mice ($P < 0.05$, Fig. 5A and B). However, the anti-apoptotic effects of ginseng were abrogated in eNOS$^{-/-}$ mice and in WT mice treated with LY294002 ($P < 0.05$, Fig. 5A and B). These results imply that ginseng protects the heart against I/R-induced myocardial apoptosis via PI3-kinase/Akt/eNOS pathway.

4. Discussion

The present study employed an *in vivo* model of myocardial I/R and cultured cardiomyocytes to investigate the effect of ginseng on myocardial I/R injury and its underlying mechanisms. We demonstrated that ginseng upregulated eNOS expression via PI3-kinase/Akt signaling. We further demonstrated that ginseng decreased infarct size and myocardial apoptosis during I/R, and these cardioprotective effects of ginseng were abrogated in eNOS$^{-/-}$ mice. Our study showed for the first time a pivotal role of eNOS in the cardioprotective effects of North American ginseng during myocardial I/R.

Ginseng has been used as a herbal medicine and nutritional supplement in East Asia for thousands of years and is gaining popularity in the West in the recent two decades [1,2]. Although ginseng has been used as an alternative medicine to treat various diseases for a

long time, its pharmacological activities are still not well characterized due to the lack of carefully designed clinical and experimental studies. This is especially true with regard to its mechanisms of action in pathological conditions such as ischemic heart disease and I/R injury. In the present study, we showed that oral administration of ginseng aqueous extract significantly decreased infarct size and myocardial apoptosis following myocardial I/R. To investigate the mechanisms by which ginseng protects the heart from I/R injury, myocardial eNOS expression was determined. Previous work from our lab and others has shown that eNOS mediates cardioprotection during myocardial I/R, and eNOS expression is upregulated via PI3-kinase/Akt signaling [16,29,30]. In the present study, oral ginseng treatment increased eNOS protein levels in the myocardium. In addition, ginseng treatment also upregulated eNOS expression in cultured cardiomyocytes. Furthermore, ginseng-induced eNOS expression in cardiomyocytes and in the myocardium was inhibited by LY294002, a PI3-kinase inhibitor. Our results suggest that ginseng upregulates myocardial eNOS expression via PI3-kinase/Akt signaling.

To definitively study the role of eNOS in the cardioprotective effects of ginseng, $eNOS^{-/-}$ mice were employed. Consistent with our previous studies [16], myocardial apoptosis and infarct size were increased in $eNOS^{-/-}$ mice following myocardial I/R. Importantly, the protective effects of ginseng were abrogated. In the heart, eNOS is expressed in several cell types including endothelial cells and cardiomyocytes [14,15]. To investigate the role of eNOS in cardiomyocytes in ginseng's cardioprotection, cultured neonatal cardiomyocytes from $eNOS^{-/-}$ and WT mice were subjected to A/R to simulate I/R *in vivo*. Our data showed that the anti-apoptotic effects of ginseng were abolished in $eNOS^{-/-}$ cardiomyocytes. Taken together, these results demonstrate a pivotal role of eNOS within cardiomyocytes in mediating the cardioprotection of ginseng during myocardial I/R.

PI3-kinase/Akt plays a key role in the reperfusion injury salvage kinase (RISK) pathway and its activation leads to cardiac protection during myocardial I/R [31–33]. In the present study, ginseng treatment increased Akt phosphorylation in the myocardium and in cultured cardiomyocytes. To further study the role of Akt activation in the cardioprotective effects of ginseng, animals and cardiomyocytes were treated with LY294002 to inhibit PI3-kinase/Akt activity. Our data showed that the protective effects of ginseng during I/R *in vivo* and A/R *in vitro* were negated. The results support an important role of PI3-kinase/Akt signaling in the cardioprotection of ginseng as demonstrated in recent studies [13].

In the present study, the aqueous extract of North American ginseng was employed. HPLC analysis showed that the primary ginsenosides in the extract were Rb1 and Re representing 37% and 42% of total ginsenosides, respectively, whereas the content of minor ginsenosides (Rg1, Rb2, Rd and Rc) was between 2% and 9% [26]. Interestingly, ginsenoside Rb1 has been demonstrated to protect the heart from I/R injury [13]. In the present study, we also showed that ginsenoside Rb1 inhibits A/R-induced cardiomyocyte apoptosis. While it is important to show the effects of individual ginsenosides, the use of the whole extract which contains about 40 ginsenosides [3] is in fact more clinically relevant as it represents the consumption of ginseng in humans. Additionally, the dose of ginseng in the present study is also within the dose range of clinical use in human population [34]. A limitation of the present study is that only one dose of ginseng was studied for cardiomyocyte apoptosis *in vitro* (2 μg/ml) and myocardial I/R injury *in vivo* (50 mg/kg/day, oral gavage). It is possible that higher doses of ginseng may have a more pronounced cardioprotective effects. In fact, higher doses of ginseng (100 mg/kg/day) have been shown to improve cardiac function after myocardial infarction in rats [8]. Further studies are required to investigate a full dose response of ginseng on cardiac protection.

In summary, our results demonstrated that the aqueous extract from North American ginseng protects the heart from I/R injury via PI3-kinase/Akt-dependent eNOS expression. The present study suggests that ginseng may be utilized as a cardioprotective agent to limit infarct size in patients with acute myocardial infarction [35] and in the management of ischemic heart disease where I/R injury is expected e.g., during coronary artery angioplasty and bypass surgery.

Acknowledgments

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