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North American ginseng inhibits myocardial NOX2-ERK1/2 signaling and tumor necrosis factor-α expression in endotoxemia

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ABSTRACT

Sepsis is a systemic inflammatory response to infection with a high mortality but has no specific treatment despite decades of research. North American (NA) ginseng (*Panax quinquefolius*) is a popular natural health product with anti-oxidant and anti-inflammatory properties. The aim of the present study was to investigate the effects of NA ginseng on pro-inflammatory cytokine expression and cardiac function in endotoxemia, a model of sepsis. Mice were challenged with lipopolysaccharide (LPS) to induce endotoxemia. Myocardial expression of tumor necrosis factor-alpha (TNF-α), a major pro-inflammatory cytokine that causes cardiac dysfunction, was upregulated in mice with endotoxemia, which was accompanied by increases in NOX2 expression, superoxide generation and ERK1/2 phosphorylation. Notably, pretreatment with NA ginseng aqueous extract (50 mg/kg/day, oral gavage) for 5 days significantly inhibited NOX2 expression, superoxide generation, ERK1/2 phosphorylation and TNF-α expression in the heart during endotoxemia. Importantly, cardiac function and survival in endotoxemic mice were significantly improved. Additionally, pretreatment with ginseng extract inhibited superoxide generation, ERK1/2 phosphorylation and TNF-α expression induced by LPS in cultured cardiomyocytes. We conclude that NA ginseng inhibits myocardial NOX2-ERK1/2-TNF-α signaling pathway and improves cardiac function in endotoxemia, suggesting that NA ginseng may have the potential in the prevention of clinical sepsis.

1. Introduction

Sepsis is a systemic inflammatory response syndrome to infection, and the leading cause of death in intensive care units [1,2]. Myocardial dysfunction has been recognized as a major contributor to mortality and morbidity in patients with sepsis [3,4]. Although the precise mechanisms that cause myocardial dysfunction during sepsis are not fully understood, studies have demonstrated that overproduction of pro-inflammatory cytokines in the heart, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, lead to cardiac dysfunction [5,6]. Among these cytokines, TNF-α has been implicated as one of the major factors [4]. Indeed, systemic administration of TNF-α produces the hemodynamic profile seen in sepsis with an impaired cardiac contractile function [7]. In contrast, treatment with anti-TNF-α antibody in patients with septic shock improves cardiac performance [8]. These studies suggest that TNF-α plays a pivotal role in myocardial dysfunction in sepsis.

North American (NA) ginseng (*Panax quinquefolius*) has received increasing attention as an alternative medicine due to its multiple pharmacological effects, including anti-apoptotic, anti-oxidant, and anti-tumor activities [9–11]. Additionally, NA ginseng also modulates inflammatory and immune responses [12–15]. However, the effect of NA ginseng on TNF-α expression was inconsistent depending on different cell types and ginseng extracts. For example, the polysaccharide fraction of NA ginseng was found to stimulate TNF-α release in alveolar macrophages [13], whereas an alcoholic extract of ginseng, which contains ginsenosides but not polysaccharides, suppressed LPS-induced macrophage TNF-α production [16]. To date, the effects of NA ginseng on LPS-induced TNF-α expression in cardiomyocytes are still unknown.

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NADPH oxidase is a major reactive oxygen species (ROS) producing enzyme, which has at least seven isoforms, NOX1-5, Duox1 and Duox2 [4]. NOX2 is the major isoform in the heart. Our previous studies have shown that NOX2 activation is critical to pro-inflammatory response in the heart during endotoxemia via extracellular regulated kinase 1 and 2 (ERK1/2) signaling [17,18]. In the present study, we hypothesized that NA ginseng inhibits myocardial TNF-α expression via downregulation of NOX2-ERK1/2 signaling and improves cardiac function in endotoxemia. To test this hypothesis, an *in vivo* murine model of endotoxemia and cultured primary neonatal mouse cardiomyocytes were employed. Our data showed for the first time that pretreatment with NA ginseng aqueous extract inhibits NOX2-ERK1/2 signaling and TNF-α expression in the heart, and improves cardiac function and survival in mice with endotoxemia.

2. Materials and methods

2.1. Animals

Breeding pairs of C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and produced neonatal and adult mice for this study. Animals were provided food and water *ad libitum* in a 12/12-h light/dark cycle. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health. The study protocols were approved by the Animal Care Committee at the University of Western Ontario, Canada.

2.2. Experimental protocols in endotoxemic mice

Adult male mice were pretreated with aqueous extracts of NA ginseng (50 mg/kg, oral gavage) or vehicle (water) for 5 days. On day 6, mice were challenged with an intraperitoneal (IP) injection of 4 mg/kg LPS (from *Salmonella typhosa*, Sigma) to induce endotoxemia. At various time points (15 min to 4 h) after an LPS challenge, mice were sacrificed. Plasma and heart tissues were collected for measurements of TNF-α levels, O_2^- production, and ERK1/2 phosphorylation. To assess cardiac function, hemodynamic measurements were made at 4 h after LPS treatment under ketamine and xylazine anesthesia. To study animal survival, mice were monitored for 5 days after a high dose of LPS challenge (15 mg/kg, IP).

2.3. In vivo *measurements of cardiac function*

Cardiac function was measured in mice as previously described [18]. Briefly, mice were anesthetized with ketamine (50 mg/kg, i.p.) and xylazine (12.5 mg/kg, i.p.), intubated, and ventilated with a respirator (SAR830, CWE, Ardmore, PA). To maintain the body temperature at 37 °C during anesthesia, animals were kept on a heating pad. A Millar pressure conductance catheter (1.4-Fr, Millar Instruments) was introduced into the right carotid artery to measure mean arterial blood pressure and heart rate, after which the catheter was advanced to the left ventricle (LV) to assess left ventricular end-systolic pressure (LVESP), first derivatives of LV pressure (d*P*/dt), stroke work (SW), cardiac output (CO) and end-systolic pressure-volume relationship (ESPVR). All measurements were recorded using the PowerLab Chart Program (ADInstruments, Colorado Springs, CO). After completion of these measurements, blood and heart were collected. Plasma and LV tissues were stored at −80 °C until further studies.

2.4. Primary neonatal mouse cardiomyocyte culture

Neonatal cardiomyocytes were prepared as we described previously [17]. Briefly, hearts from mice born within 24 h were collected and minced in a normally Ca^{2+} and Mg^{2+}-free Hanks balance solution. Cardiomyocytes were dispersed by incubation with 22.5 μg/ml liberase blendzyme IV (Roche, Mannheim, Germany) at 37 °C for 40 min. The isolated cells were pre-plated for 90 min to remove non-cardiomyocytes, after which cardiomyocytes were cultured in M199 medium containing 10% fetal bovine serum (FBS) in 24-well culture plates pre-coated with 1% gelatin (Sigma). The cells were cultured 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.

2.5. Detection of superoxide

Myocardial superoxide generation was determined using dihydroethidium (DHE, Invitrogen) staining [19]. Briefly, frozen sections (10 μm) of the LV myocardium were treated with DHE (2 μM) with and without diphenyleneiodonium (DPI, 30 μM) at 37 °C for 30 min in a dark, humidified chamber. DHE red fluorescence was imaged at ×400 magnification using a fluorescence microscope (Observer D1, Zeiss, Germany) connected to a digital camera. The fluorescent signal intensities were analyzed and quantified by using AxioVision software.

In cultured cardiomyocytes, superoxide generation was determined using CellROX® deep red reagent (Thermo Scientific, Mississauga, Ontario) as previously described [20,21]. The CellROX deep red reagent is a cell-permeable and fluorogenic probe designed to assess ROS levels in live cells. Briefly, cultured neonatal mouse cardiomyocytes were incubated with CellROX deep red reagent (1 μM) for 15 min with or without pre-treatment with an NADPH oxidase-specific inhibitor DPI (10 μM) for 15 min. The fluorescent signal was visualized under a confocal microscope (ZEISS LSM 800 with Airyscan) and analyzed using Zen software (Zeiss, Germany).

2.6. Measurement of TNF-α protein

Tissues from the LV myocardium were homogenized in phosphate-buffered saline (PBS) followed by centrifugation at 5000*g* for 10 min. The supernatant of the homogenates was used and subjected to Lowry assay to determine protein concentrations. TNF-α protein levels in LV tissue homogenates, plasma and cardiomyocyte culture media were determined using a mouse TNF-α ELISA kit (eBioscience, San Diego, USA) as previously described [22].

2.7. Real-time PCR analysis

Total RNA from the LV myocardium or cultured cardiomyocytes was extracted using TRIzol reagent (Sigma) as previously described [17]. cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen, Burlington, ON). SYBR green PCR master mix was used in accordance with the manufacturer's instructions (Eurogentec, San Diego, CA). 28S rRNA was used as a loading control. The primer sequences were as follows: TNF-α 5′CCG ATG GGT TGT ACC TTG TC3′ (forward) and 5′GGG CTG GGT AGA GAA TGG AT3′ (reverse); Nox2 5′GCT GGA AAC CCT CCT ATG ACT3′ (forward) and 5′TGT ACT GTC CCA CCT CCA TCT3′(reverse); 28S rRNA 5′TTG AAA ATC CGG GGG AGA G3′ (forward) and 5′ACA TTG TTC CAA CAT GCC AG3′ (reverse). Samples were amplified for 34 cycles using an Eppendorf Real-Time PCR machine. The expression of TNF-α in relation to 28S rRNA was determined.

2.8. Western blot analysis

Protein levels of Nox2 and ERK1/2 phosphorylation were assessed by Western blot analysis. Briefly, LV myocardial tissues were homogenized in PBS and cultured cardiomyocytes were

lysed followed by centrifugation to extract proteins. The supernatant of the homogenates was used and subjected to Lowry assay to determine protein concentrations. A total of 50 μg protein was loaded, subjected to 10% SDS-PAGE electrophoresis, and transferred to a nitrocellulose membrane. Immunoblotting was carried out with specific primary antibodies against mouse ERK1/2 (1:1000), phospho-ERK1/2 (1:1000, Cell Signaling), and NOX2 (1:1000, BD Biosciences) and α-actinin (1:1000, Sigma), respectively, followed by incubation with horseradish peroxidase conjugated secondary antibodies (Bio-Rad) [17,20]. Protein bands were visualized on an X-ray film with an enhanced chemiluminescence method or scanned using an Odyssey CLx infrared imaging system (LI-COR Biosciences, Lincoln, NE). Densitometric ratios of phosphorylated to total ERK1/2 and NOX2 to α-actinin were obtained.

2.9. Ginseng extraction procedures

Four-year old North American ginseng (*Panax quinquefolius*) roots were supplied by 5 farms in Ontario, Canada and processed at Naturex (South Hackensack, NJ) using an aqueous extraction procedure, as described in our previous report [9]. The extracts were lyophilized and stored at −20 °C. The aqueous extracts contained 57.3 mg ginsenosides per gram ginseng dry weight as measured by high performance liquid chromatography (HPLC) and were used in the present study.

2.10. Statistical analysis

Data were expressed as mean ± SEM. Statistical analysis was performed by using the GraphPad Prism 5 (GraphPad Software). One-way ANOVA followed by Newman-Keuls test was used to determine statistical differences between multiple groups. Survival curves were created by the method of Kaplan and Meier, and compared by Gehan-Breslow-Wilcoxon test. Two-tailed $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of ginseng on LPS-induced TNF-α expression in hearts and cardiomyocytes

Low levels of TNF-α mRNA in the myocardium were detected in control mice. Treatment with ginseng aqueous extract (50 mg/kg/day, oral gavage) for 5 days did not alter their TNF-α mRNA levels. Notably, myocardial TNF-α mRNA levels were significantly increased 2 h after LPS (4 mg/kg, IP) stimulation ($P < 0.01$, Fig. 1A). Ginseng pretreatment for 5 days significantly attenuated LPS-induced TNF-α mRNA expression in the heart ($P < 0.05$). Likewise, the inhibitory effect of ginseng on TNF-α expression was also observed at the protein level. After 4 h of LPS treatment, the increased TNF-α protein levels in the LV myocardium were diminished by ginseng pretreatment ($P < 0.05$, Fig. 1B). Additionally, increases in plasma TNF-α protein levels during LPS stimulation were inhibited by pretreatment with ginseng ($P < 0.05$, Fig. 1C).

In primary neonatal mouse cardiomyocyte cultures, pretreatment with ginseng for 48 h dose-dependently (0.1, 1 and 10 μg/ml) inhibited LPS-induced TNF-α protein production ($P < 0.05$, Fig. 2A). Additionally, increases in cardiomyocyte TNF-α mRNA expression were prevented by ginseng pretreatment (10 μg/ml) during LPS stimulation ($P < 0.05$, Fig. 2B).

3.2. Effects of ginseng on myocardial function in endotoxemia

In vivo cardiac function was determined in mice four hours after LPS (4 mg/kg, IP) injection with and without ginseng pretreatment. As shown in Table 1, LV end-systolic pressure (LVESP), LV +d*P*/dt, stroke work, and end-systolic pressure-volume relationship (ESPVR) were decreased in the LPS group compared to the control group ($P < 0.05$), indicating myocardial dysfunction. These hemodynamic variables were improved in the LPS + Ginseng group compared to the LPS group ($P < 0.05$). Mean arterial pressure was lower in LPS and LPS + Ginseng groups ($P < 0.05$) without any significant changes in heart rate.

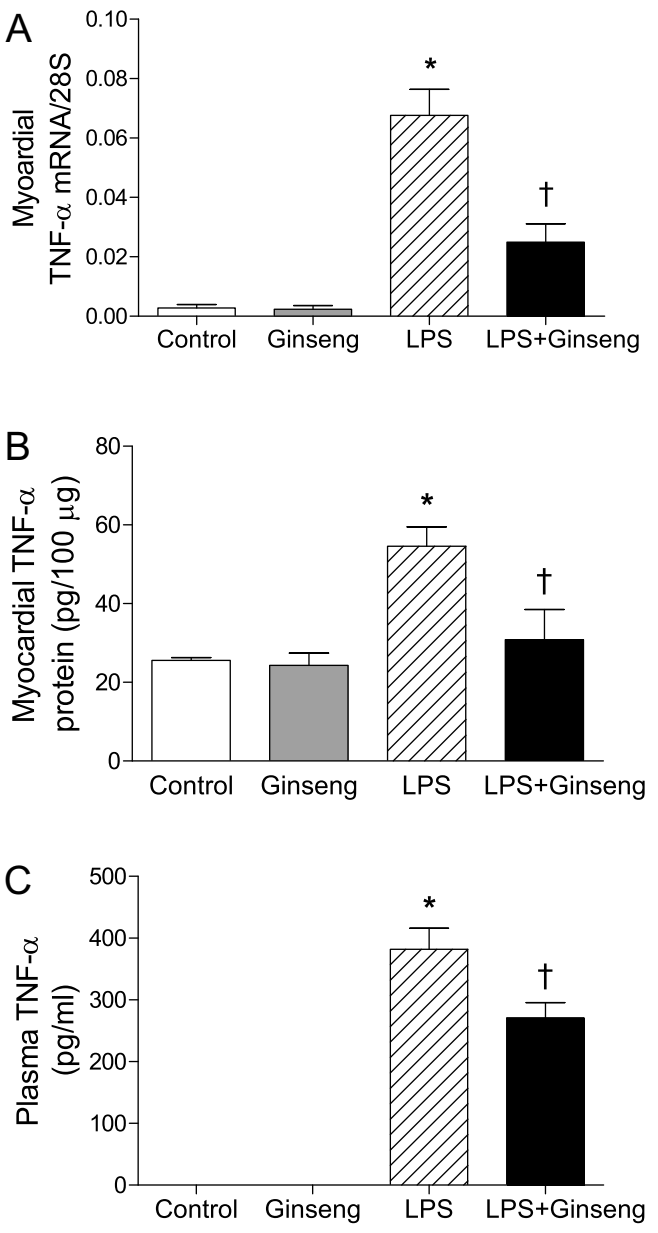


Fig. 1. Ginseng inhibits myocardial TNF-α production in mice with endotoxemia. (A) Real-time PCR was performed to detect myocardial TNF-α mRNA levels at 2 h after LPS treatment (4 mg/kg, IP). n = 7–8 per group. (B) At 4 h after LPS treatment, myocardial TNF-α protein levels were measured using ELISA. n = 3–6 per group. (C) At 2 h after LPS treatment, plasma TNF-α protein levels were determined using ELISA. n = 4 per group. Data were analyzed by one-way ANOVA followed by Newman-Keuls test. *$P < 0.01$ vs. control; †$P < 0.05$ vs. LPS.

3.3. Effects of ginseng on animal survival in endotoxemia

To assess animal survival in endotoxemia, mice were pretreated with aqueous ginseng extract orally for 5 days followed by a high dose of LPS (15 mg/kg, IP) to induce lethal endotoxemic shock.

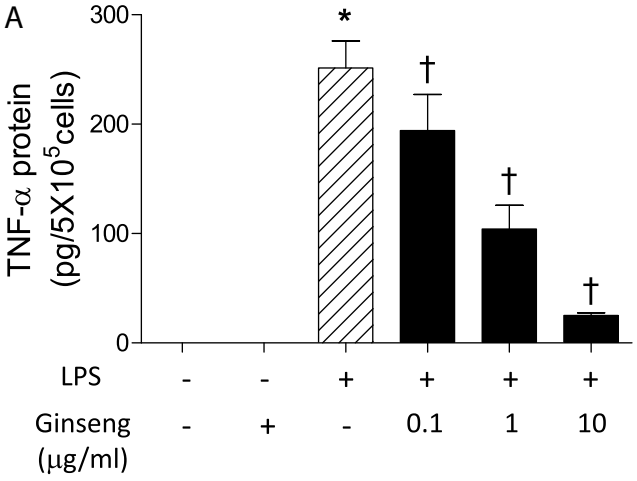
Table 1
Effects of ginseng on cardiac function in mice with endotoxemia.

Parameters	Control	Ginseng	LPS	LPS + Ginseng
n	6	9	7	7
MAP, mmHg	95.8 ± 3.6	88.4 ± 5.5	62.1 ± 4.9*	67.6 ± 4.3*
HR, beats/min	417.6 ± 31.4	424.8 ± 16.1	498.6 ± 16.0	472.7 ± 18.6
LVEDP	9.0 ± 2.5	9.3 ± 3.4	4.3 ± 0.4	8.8 ± 3.0
LVESP	105.1 ± 6.5	100.4 ± 5.3	72.6 ± 3.5*	87.1 ± 4.7†
LV +d*P*/dt, mmHg/s	8113 ± 349	7965 ±434	3733 ± 259*	4902 ± 437†
LV −d*P*/dt, mmHg/s	7632 ± 396	7144 ± 357	4185 ± 365*	5022 ± 499
Stroke work, mmHg.μl	1092 ± 199	1529 ± 101	385 ± 94*	829 ± 171†
Cardiac output, μl/min	5907 ± 784	8653 ± 772	4691 ± 500	7380 ± 534†
ESPVR	2.9 ± 0.4	2.9 ± 0.7	0.6 ± 0.8*	3.5 ± 0.7†

MAP, Mean arterial pressure; HR, heart rate; LVEDP, LV end-diastolic pressure; LVESP, LV end-systolic pressure; LV d*P*/dt, first derivatives of LV pressure; ESPVR, end-systolic pressure-volume relationship. All data are mean ± SEM and analyzed by one-way ANOVA followed by Newman-Keuls test.

* $P < 0.05$ vs. control.

† $P < 0.05$ vs. LPS.



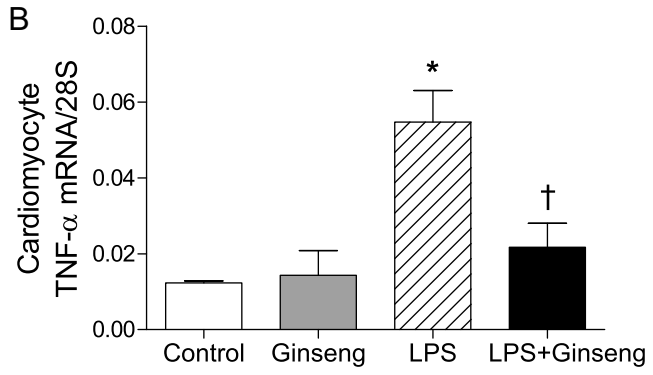


Fig. 2. Ginseng inhibits LPS-induced TNF-α production in cardiomyocytes. (A) Cultured neonatal cardiomyocytes were pretreated with ginseng aqueous extract (0.1, 1 and 10 μg/ml) for 48 h and then challenged with LPS (1 μg/ml) for 5 h. TNF-α protein levels in media were examined using ELISA. (B) Cardiomyocytes were pretreated with ginseng aqueous extract (10 μg/ml) for 48 h followed by LPS (1 μg/ml) treatment for 3 h. TNF-α mRNA levels in cardiomyocytes were determined using real-time PCR. n = 4 per group. Data were analyzed by one-way ANOVA followed by Newman-Keuls test. *$P < 0.01$ vs. control; †$P < 0.05$ vs. LPS alone.

Mouse survival was monitored for 5 days after LPS injection. The results showed that only 33% of the control mice survived within 5 days after an LPS challenge, while oral administration of ginseng improved the survival of endotoxemic mice to 56% during the same period ($P < 0.05$, Fig. 3).

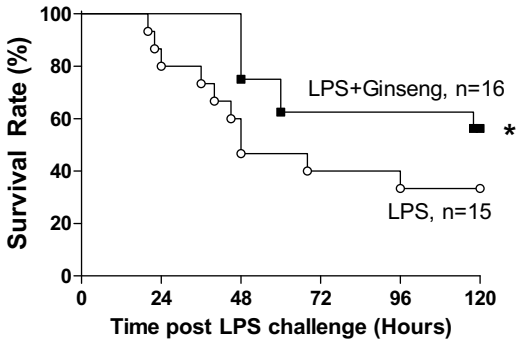


Fig. 3. Ginseng improves animal survival in endotoxemia. Adult male mice were pretreated with ginseng aqueous extract (50 mg/kg/day) or vehicle (water) by oral gavage for 5 days followed by a high dose of LPS (15 mg/kg, IP) injection. Animal survival was monitored for 5 days after LPS treatment. n = 15–16 mice per group. Kaplan and Meier survival curves were compared using Gehan-Breslow-Wilcoxon test. *$P < 0.05$ vs. LPS.

3.4. Effects of ginseng on myocardial ERK1/2 phosphorylation during endotoxemia

To further study the potential mechanism by which ginseng inhibits LPS-induced TNF-α expression, we evaluated the effects of ginseng on ERK1/2 phosphorylation after LPS treatment in the LV myocardium *in vivo* and cultured cardiomyocytes *in vitro*. As shown in Fig. 4A and B, pretreatment with ginseng significantly diminished ERK1/2 phosphorylation both in the myocardium and cardiomyocytes at 1 h and 30 min after LPS treatment, respectively ($P < 0.05$). Decreased ERK1/2 signaling may contribute to decreased TNF-α expression in the heart during endotoxemia by ginseng pretreatment.

3.5. Effects of ginseng on NOX2-mediated O_2^- production in the heart during endotoxemia

To examine the effects of ginseng on NOX2 signaling, NOX2-mediated O_2^- generation in the cultured cardiomyocytes and the myocardium was determined using CellROX deep red reagent and *in situ* DHE staining, respectively. Our results showed that LPS significantly induced O_2^- generation in cultured cardiomyocytes, which was significantly inhibited by pretreatment with ginseng ($P < 0.05$, Fig. 5). Additionally, myocardial O_2^- generation was significantly increased in mice with endotoxemia ($P < 0.01$, Fig. 6). Pretreatment with ginseng (50 mg/kg/day, p.o.) for 5 days significantly inhibited myocardial O_2^- levels in mice with endotoxemia ($P < 0.05$, Fig. 5). Furthermore, LPS-induced O_2^- production was inhibited by an NADPH oxidase inhibitor DPI in cultured cardiomyocytes and in the myocardium ($P < 0.05$, Figs. 5 and 6).

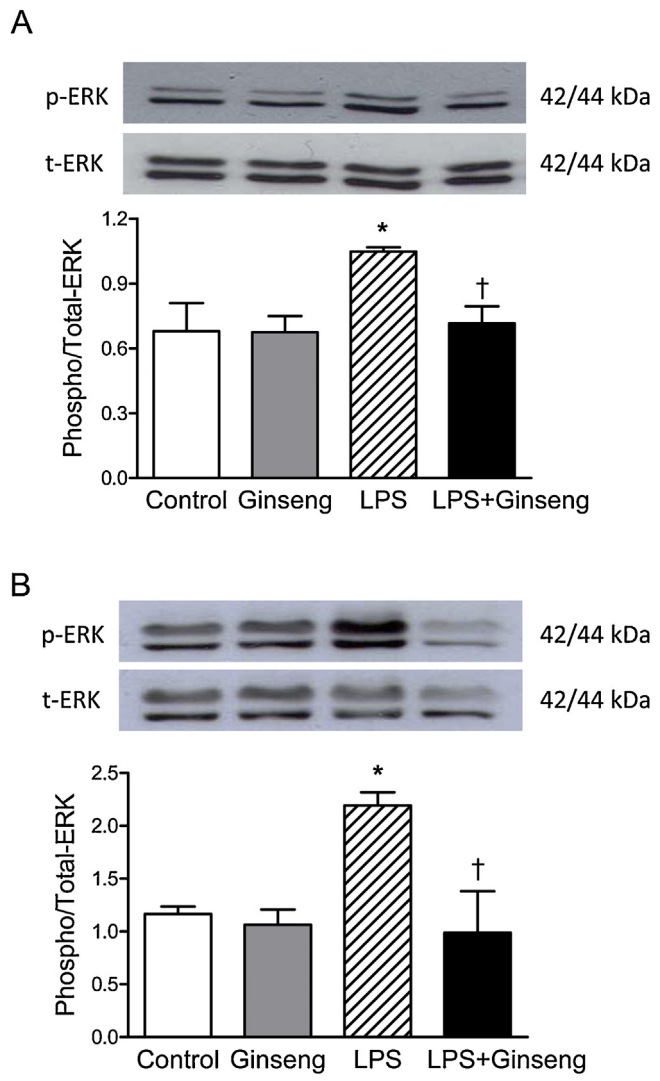


Fig. 4. Ginseng inhibits LPS-induced ERK1/2 phosphorylation in the myocardium and cultured cardiomyocytes. (A) Adult mice were pretreated with ginseng (50 mg/kg/day, oral gavage) for 5 days followed by LPS (4 mg/kg, IP) treatment for 1 h. The LV tissues were collected and subjected to Western blot analysis to examine phosphorylated ERK1/2 (p-ERK) and total ERK1/2 (t-ERK) protein levels. (B) Cultured neonatal cardiomyocytes were pretreated with ginseng (10 μg/ml) for 48 h, and followed by LPS (1 μg/ml) stimulation. p-ERK and t-ERK protein levels were examined at 30 min after LPS treatment using Western blot analysis. Ratio of p-ERK to t-ERK protein levels is presented. n = 3–4 per group. Data were analyzed by one-way ANOVA followed by Newman-Keuls test. *$P < 0.05$ vs. Control; †$P < 0.05$ vs. LPS.

To further study the effects of ginseng on NOX2 expression, myocardial NOX2 mRNA and protein levels were determined in mice with endotoxemia. In response to LPS stimulation, myocardial NOX2 mRNA and protein levels were increased ($P < 0.01$, Fig. 7), which were significantly inhibited by pretreatment with ginseng in mice ($P < 0.05$, Fig. 7). The decreased NOX2 expression may contribute to the inhibitory effect of ginseng on NOX2-mediated O_2^- production in the heart during endotoxemia.

4. Discussion

Cardiac dysfunction has been recognized as a major factor contributing to multiple organ failure and high mortality in sepsis [23–25]. TNF-α plays a critical role in the pathogenesis of myocardial depression in the septic heart. This pro-inflammatory cytokine is predominantly produced from macrophages at early stages of sepsis. However, it is important to point out that myocardial TNF-α production is an important source of TNF-α affecting cardiac function, especially at later stages of sepsis [3,4]. Thus, inhibition of TNF-α expression in the heart may improve cardiac function in sepsis.

Ginseng has been used as a nutritional supplement in East Asia for thousands of years and is becoming popular in the West in the recent two decades [26]. Asian ginseng (*Panax ginseng*) and NA ginseng are the most popular ginseng species in the world. Active constituents found in most ginseng species include ginsenosides, polysaccharides, peptides, polyacetylenic alcohols, and fatty acids. Although studies have shown that ginseng possesses a wide range of pharmacological effects and much progress has been made in understanding the mechanisms of its action, most of these results were obtained from experiments with Asian ginseng [27,28], which is very different in its composition and pharmacology from NA ginseng. To date, the efficacy of NA ginseng and its mechanisms of action is still not fully understood, which significantly limits the development and clinical application of this herb. In the cardiovascular system, NA ginseng has been shown to protect against myocardial ischemia-reperfusion injury [9], decrease cardiomyocyte hypertrophy [29], and prevent diabetic cardiomyopathy [30].

The present study was designed to examine the effects of ginseng on myocardial TNF-α expression and cardiac function in sepsis. To this end, we employed an *in vivo* mouse model of sepsis, endotoxemia, and an *in vitro* cell culture model of neonatal mouse cardiomyocytes. Our results showed that ginseng potently inhibits TNF-α expression in the myocardium and in cultured cardiomyocytes. Subsequently, we evaluated whether the cardiac function could be improved by ginseng pretreatment by *in vivo* hemodynamic analysis. Consistent with our hypothesis, NA ginseng significantly improved cardiac function and animal survival in endotoxemia. However, when ginseng was concomitantly administered to mice or cultured cardiomyocytes with an LPS challenge, no significant effects on cardiac function, myocardial or cardiomyocyte TNF-α expression were observed (data not shown). It appears that pretreatment of ginseng is essential to achieve its beneficial effects. It should be noted that the beneficial effects of ginseng observed in this study were derived from an aqueous extract of North American ginseng rather than any individual ginsenosides, which are major active components of ginseng. HPLC analysis revealed that Re and Rb1 are predominant ginsenosides in the ginseng we used in the present study [9]. A recent study by Lim et al. demonstrated that ginsenoside Re inhibits TNF-α levels, and ameliorates ischemia and reperfusion injury in the heart [31], suggesting that the beneficial effects of NA ginseng reported in the study may be contributable to ginsenoside Re. In addition, ginsenoside Re and Rb1 protect cardiomyocytes against oxidative injury [32,33]. Furthermore, recent studies from our consortium show that polysaccharide fractions of ginseng extracts modulate immune response in cultured alveolar macrophages [34]. However, whether Re, Rb1 and/or polysaccharides mediated the cardioprotective effects of NA ginseng described in the current study remains to be determined in future studies. Additionally, ginseng is metabolized extensively by intestinal bacteria and the absorption rate of ginseng saponins is low after oral administration [35]. In humans, the elimination half-life ($T_{1/2}$) of the tested saponins is generally less than 24 h. The pharmacokinetics of ginseng during endotoxemia requires further investigation.

NOX2-ERK1/2 signaling has been shown to be pivotal in promoting pro-inflammatory response in sepsis [4,17,18]. To this end, we have previously demonstrated that inhibition of NOX2-ERK1/2 signaling results in decreased myocardial TNF-α expression and improvement in cardiac function in endotoxemia [4]. However, the effect of NA ginseng on NOX2-ERK1/2 signaling is not known. In the present study, pretreatment with NA ginseng inhibited

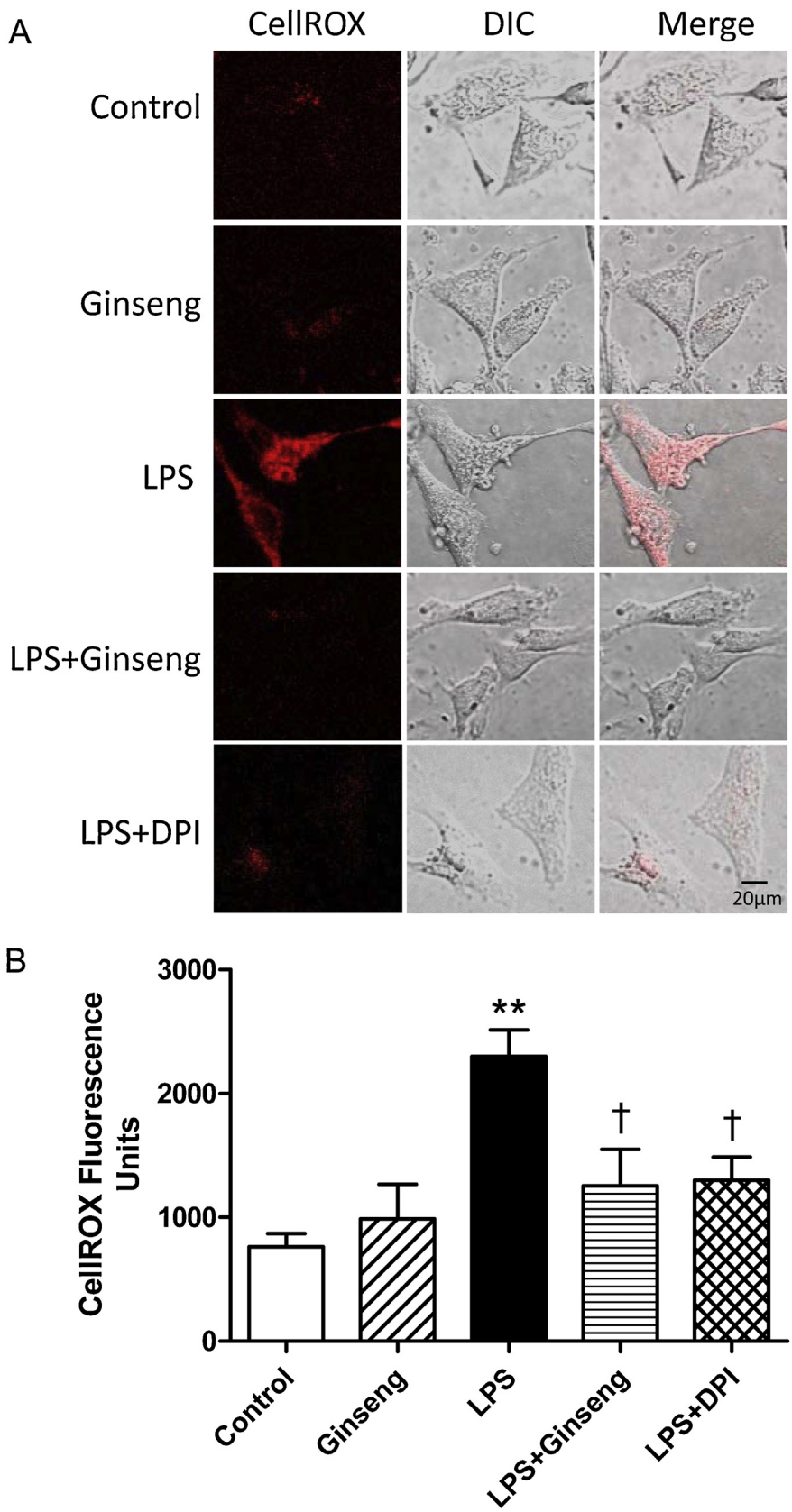


Fig. 5. Ginseng inhibits O_2^- production in cardiomyocytes. Cultured neonatal mouse cardiomyocytes were pretreated with ginseng (10 μg/ml) for 48 h, and followed by LPS (1 μg/ml) stimulation for 30 min. O_2^- production was assessed using CellROX deep red reagent. (A) Representative fluorescent images from each corresponding group. Differential interference contrast (DIC) images were taken to show cardiomyocytes. (B) Quantification of CellROX red fluorescence intensity. n = 4 independent cell cultures per group. Data were analyzed by one-way ANOVA followed by Newman-Keuls test. $^{**}P < 0.01$ vs. Control; †$P < 0.05$ vs. LPS. DPI, diphenyleneiodonium.

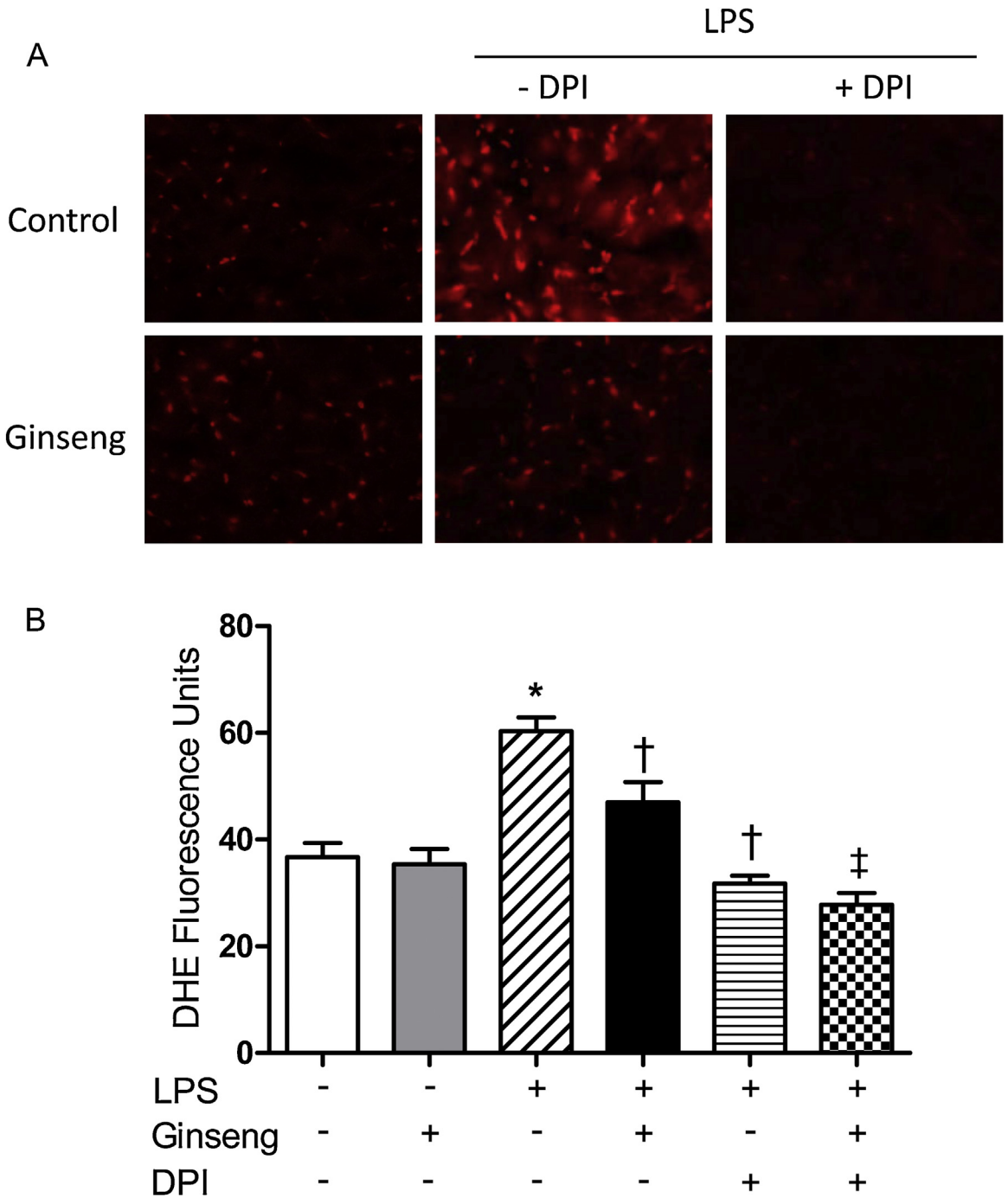


Fig. 6. Ginseng inhibits myocardial O_2^- production in endotoxemia. Adult mice were pretreated with ginseng (50 mg/kg/day, oral gavage) for 5 days, followed by LPS (4 mg/kg, IP) treatment for 15 min. Myocardial O_2^- production was assessed by dihydroethidium (DHE) staining. (A) Representative fluorescent images from each corresponding group. (B) Quantification of DHE fluorescence intensity. n = 7–8 per group. Data were analyzed by one-way ANOVA followed by Newman-Keuls test. *$P < 0.01$ vs. Control; †$P < 0.05$ vs. LPS;. ‡$P < 0.05$ vs. LPS + ginseng. DPI, diphenyleneiodonium.

myocardial NOX2 expression and superoxide production. Furthermore, LPS-induced ERK1/2 phosphorylation was diminished in the myocardium and in cultured cardiomyocytes by NA ginseng pretreatment. These results show that pretreatment with NA ginseng downregulates NOX2-ERK1/2 signaling in the heart, leading to decreased myocardial TNF-α expression during endotoxemia.

In summary, the present study is the first to reveal the beneficial effects of NA ginseng on myocardial TNF-α expression, cardiac function and animal survival in endotoxemia. Our data show that these beneficial effects of NA ginseng are associated with a downregulation of NOX2-ERK1/2 signaling. Thus, inhibition of NOX2-ERK1/2-TNF-α pathway may represent a novel mechanism by which ginseng improves cardiac function and survival in endotoxemia. Since pretreatment is essential to achieve its beneficial effects, our study suggests that NA ginseng may have the therapeutic potential in the prevention of sepsis in patients who are at a high risk of developing this disease. Whether regular consumption of NA ginseng decreases the incidence and severity of sepsis, which often occurs in the aged and immunocompromised population, is an interesting question and remains to be investigated in clinical settings.

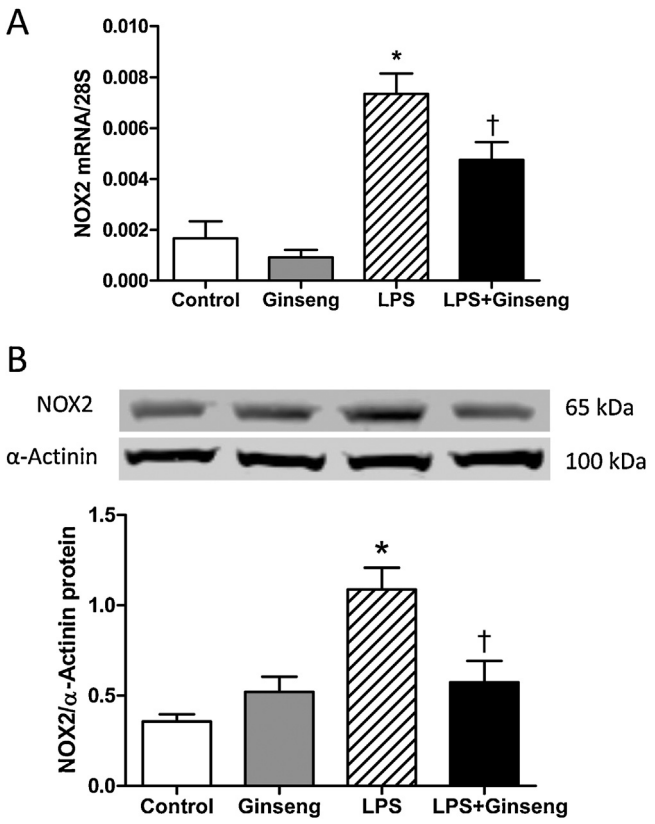


Fig. 7. Ginseng inhibits myocardial NOX2 expression in endotoxemia. Mice were pretreated with ginseng (50 mg/kg/day) for 5 days, followed by LPS (4 mg/kg, IP) for 4 h. (A) Myocardial NOX2 mRNA levels were analyzed by qPCR. (B) Myocardial NOX2 protein levels were assessed by Western blot analysis. NOX2 to α-actinin protein band density ratios are presented. n = 4–5 per group. Data were analyzed by one-way ANOVA followed by Newman-Keuls test. *$P < 0.01$ vs. Control. †$P < 0.05$ vs. LPS.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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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.phrs.2016.06.010.

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