Effect of *n*-butanol Extract from *Potentilla anserina* on Hypoxia-induced Calcium Overload and SERCA2 Expression of Rat Cardiomyocytes

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Abstract: **Objective** To investigate the effect of *n*-butanol extract from *Potentilla anserina* (NP) intervention on hypoxia-induced Ca^{2+} overload and SERCA2 expression of rat cardiomyocytes. **Methods** Primary cultured myocardial cell from SD neonatal rat (1—3 d) was used in the establishment of hypoxia model. After hypoxia for 3 h, the Ca^{2+} concentration of myocardial cells was measured with fura-2/AM fluorescent probe, and the biochemical indicator intracellular Ca^{2+}-ATPase was examined and the mRNA and its protective protein levels of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPases (SERCA2) were assayed with RT-PCR, Western-blotting, and immune-cytochemical staining in each group. **Results** The results showed that NP decreased Ca^{2+} concentration, increased the activity of Ca^{2+}-ATPase, and improved the mRNA and protein expression of SERCA2 in hypoxia-injured myocardial cells as compared with the model group. **Conclusion** These results indicate that NP could attenuate the Ca^{2+} overload. The mechanism might be explained as that NP could elevate the SERCA2 level, increase the activity of myocardium in rats, and further enhance the capacity of SR Ca^{2+} re-uptake.

Key words: cardiomyocyte; hypoxia; intracellular Ca^{2+}; *Potentilla anserina*; sarcoplasmic reticulum

DOI: 10.3969/j.issn.1674-6384.2012.02.008

Introduction

It is well known that Ca^{2+} regulates numerous physiological cellular functions as a second messenger (Bers, 2002), as well as triggers pathological events such as cell injury and death (Gupta and Pushkala, 1999). In the heart, alterations in Ca^{2+} cycling with ischemia are well documented (Tupling *et al*, 2001). It has been shown repeatedly that ischemia leads to a reduction in sarcoplasmic reticulum (SR) Ca^{2+} uptake, when measured *in vitro* (Tupling *et al*, 2001; Chen *et al*, 2006; Periasamy, Bhupathy, and Babu, 2008). Generally, reduction in Ca^{2+} uptake with myocardial ischemia was mainly attributed to parallel reduction in Ca^{2+}-ATPase activity; However, some evidences suggest that increased Ca^{2+} release channel (CRC) activity and excessive Ca^{2+} leakage may also be involved (Premkumar *et al*, 2000; Fill and Copello, 2002).

SR, a membranous structure in skeletal muscle, plays a critical role in the regulation of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). In cardiac muscle, Ca^{2+} is stored primarily in SR, released through CRC to initiate contraction, and reabsorbed into the lumen of SR by the 110 SR Ca^{2+}-ATPase pump to allow relaxation (Periasamy, Bhupathy, and Babu, 2008). Therefore, a normal function of SR is critical for normal cellular function and survival.

The Tibetan herb of *Potentilla anserine* L., belonging to the genus *Potentilla* L., is widely distributed in the western areas of China, especially in Tibet Autonomous Region, Gansu, and Qinghai provinces. For thousands of years, this Tibetan traditional

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Received: November 7, 2011; Revised: February 18, 2012; Accepted: February 29, 2012
Fund: National Natural Science Foundation of China (81073152) and the Great Program of Science Foundation of Tianjin (10JCZDJC21100)

herb has been popularly used for replenishing vital energy (qi) and blood, strengthening spleen, and harmonizing the stomach (Hong, Cai, and Xiao, 2006; Zhang *et al*, 2010). Our previous studies (*in vitro* and *in vivo*) proved that the *n*-butanol extract of *P. anserina* (NP) showed a remarkably protective effect on hypoxia cardiomyocytes (Wang *et al*, 2009). We also observed the protective effects of NP against pituitrin-induced acute myocardial ischemic injury for the first time (Li *et al*, 2009). However, whether or not NP protects the myocardium against hypoxia via intracellular Ca^{2+} has not been verified, and its molecular mechanisms in Ca^{2+} handling remain largely unknown.

In this study, we investigated the effect of NP on $[Ca^{2+}]_i$ in cultured neonatal rat cardiac myocytes under hypoxia conditions. Furthermore, we attempted to investigate the hypothesis that the inhibition of NP on Ca^{2+} overload was related to the improvement of SR Ca^{2+} pump function. To test this hypothesis, the effect of NP on SR Ca^{2+} pump function as well as its gene expression in anoxic cardiomyocytes was measured.

Materials and methods

Drugs and reagents

The roots of *Potentilla anserina* L. were collected from Yushu, Qinghai Province of China in 2003 and transported to Tianjin City after dried in air. The roots were identified by Prof. SUN Qi-shi of Shenyang Pharmaceutical University. A voucher specimen (2003-09-7) is deposited in the Logistics University of Chinese People's Armed Police Forces (Tianjin, China). The air-dried roots of *P. anserine* (10 kg) were powdered and refluxed with 70% ethanol for 3 h (twice) and concentrated under reduced pressure. The crude extract was suspended in hot water (× 10) and partitioned successively with H_2O saturated petroleum ether (× 3), acetic ether (× 3), and *n*-butanol (× 4) in the same volume. The *n*-butanol extracts were filtered, evaporated under vaccum at 45 ℃ and lyophilized, and then NP was got (1 g NP corresponds to 60 g of the roots of *P. anserine*). The content of triterpenes in NP was measured by spectrophotometry and was not lower than 0.9%.

Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F12) from GIBCO was supplemented with Penicillin G (100 U/mL), Streptomycin (100 ng/mL), and 10% fetal bovine serum, and pH value was adjusted to 7.2. Fura-2 acetoxymethyl ester (fura-2/AM) was from Biotium (USA). Anti-SERCA2 antibody was purchased from Santa Cruz Biotechnology Inc. (USA). Trypsin and ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) were purchased from Sigma-Aldrich (USA). Verapamil was purchased from Shanghai Harvest Pharmaceutical Company (China).

Primary culture for ventricular myocytes of neonatal rat

Neonatal rat cardiomyocytes were cultured from 1－3 d neonatal SD rats (Laboratory Animal Center of Academy of Military Medical Sciences) with a modification of Simpson's method (Simpson and Savion, 1982). In brief, the pups were decapitated and their ventricles were removed aseptically and minced in chilled Ca^{2+}- and Mg^{2+}-free Hank's solution. The minced ventricles were digested with 0.25% trypsin with constant agitation (100 cycles/min) at 37 ℃. After digestion each supernatant except the first one was collected every 10 min and centrifuged immediately at 1000 r/min for 10 min. The pellet was dispersed, filtered through a nylon mesh, and suspended in DMEM/F12 containing 10% neonatal bovine serum. After a 30 min period of pre-plating allowing noncardiomyocytes to attach, the nonattached cells were seeded at the concentration of 3×10^5, and cultured in a 95% air humidified incubator with 5% CO_2 at 37 ℃. The medium was added to 0.1 mmol/L 5-bromo-2′-deoxyuridine (Sigma, USA) that presumably prevent noncardiomyocytes proliferation without cardiomyocyte toxicity (Simpson and Savion, 1982). Studies were performed after 4－5 d of culture in cells that had been serum-deprived for 12 h.

Experimental protocols

After 4－5 d of cell culture in normoxic DMEM/F12, the culture medium was changed with anoxic Hank's solution which had been equilibrated in atmosphere of 95% N_2 and 5% CO_2. The myocytes were submitted to anoxic incubation at 37 ℃ in an incubator where normal air was replaced by 95% N_2 and 5% CO_2 to produce hypoxia for 3 h (PO_2 range in the incubator was maintained at < 1%), which mimics the *in vivo* condition of myocardial ischemia (Sun *et al*, 2005; Rui *et al*, 2005). Corresponding control cells were incubated under normoxic conditions at 37 ℃.

For the cardio-protection study, NP (0.250, 0.0625, and 0.0156 mg/mL) was added into the culture media throughout the anoxic experiments. Verapamil (5 μmol/L) was used as a positive control.

Fluorescence measurements of $[Ca^{2+}]_i$

The cell suspensions were incubated with 1 μmol/L Fura-2/AM for 30 min at 37 ℃, washed twice by centrifugation, resuspended in buffer, and maintained at room temperature. Fluorescence was recorded from 2 mL aliquots of magnetically stirred cellular suspension (1×10^6 cells/mL) by using a Shimadzu spectrofluorophotometer (RF-5301). The excitation wavelengths were 340 and 380 nm, and emission wavelength was fixed at 510 nm. The $[Ca^{2+}]_i$ was calculated according to the equation described by Grynkiewicz, Poenie, and Tsien (1985):

$$[Ca^{2+}]_i = K_d \times \frac{R - R_{min}}{R_{max} - R} \times (F_D/F_S)$$

Where F is the fluorescence intensity, $R=F_{340}/F_{380}$, $R_{min}=F_{340min}/F_{380min}$ (F_{340min} and F_{380min} are the fluorescence intensity at 340 and 380 nm, respectively when fura-2 does not combine with Ca^{2+}), $R_{max} = F_{340max}/F_{380max}$ (F_{340max} and F_{380max} are the fluorescence intensity at 340 and 380 nm, respectively when fura-2 combines completely with Ca^{2+}). K_d = 224 nmol, is the dissociation constant of compound [Fura-2·Ca^{2+}]. F_D and F_S are the maximal and minimal fluorescence intensity values respectively after excitation with 380 nm.

Ca^{2+}-ATPase activities

After 4—5 d cell culture, cells were digested with 0.25% trypsin and centrifugated (1000 r/min, 10 min). Supernatant was discarded, and physiological saline (300 μL) was added into the cell suspension. Cells were crushed with ultrasound wave (5 s × 5, the time interval was 10 s). Ca^{2+}-ATPase activity was determined by enzyme-linked assay according to the manufacturer's protocol.

RT-PCR

Total RNA was extracted from cardiomyocytes by Trizol reagent according to the manufacturer's protocol [TaKaRa RNA PCR Kit (AMV) Ver. 3.0]. The total RNA concentration was determined by absorption at 260 nm. The same amount of total RNA (2 μg) was used from each sample. The upstream SERCA2 primer was 5'-CTCACACAAAGACCGTGGAGG-3' and the downstream primer was 5'-GTCAGCCGGAACTT TGTCACC-3'(476 bp). β-Actin was used as an internal RNA loading control for each sample, with an upstream primer of 5'-AAAGACCTCTATGCCAACA-3' and a downstream primer of 5'-TTGTCAAAGAAAGGGT GTAA-3' (301 bp). The cDNA was amplified under the following cycle conditions: one cycle at 95 ℃ for 3 min, 30 cycles at 95 ℃ for 30 s, 60 ℃ for 1 min, and 72 ℃ for 1 min, and the final extension for 8 min at 72 ℃. The PCR products were analyzed by 1.5% agarose gel stained with ethidium bromide and UV irradiation. This semiquantitative determination for SERCA2 mRNA was expressed as a ratio to β-actin mRNA.

Immunocytochemical staining

The samples for immunocytochemical analysis were fixed in acetone. After endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide (H_2O_2), the sections were blocked for 30 min at room temperature with blood serum fluid and then incubated with primary antibodies (diluted by 1:200) overnight at 4 ℃. The slides were rinsed with PBS and incubated with rabbit anti-rat IgG horseradish peroxidase-labeled antibody (Sigma-Aldrich, Tokyo, Japan) for 30 min at room temperature. After color reaction, the tissues were counterstained with hematoxylin. The sections were scanned at ×200 magnification. The images were then digitalized and the integrated absorbance (A) of SERCA2 and β-actin were calculated by Image-Pro plus5.1 software.

Western-blotting analysis

Neonatal rat ventricular myocytes were collected and lysed in a buffer (pH 7.4) containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 40 mmol/L NaF, 5 mmol/L EDTA, 5 mmol/L EGTA, 2.175 mmol/L sodium orthovanadate, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 0.1% aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride. The lysate was centrifuged at 14 000 r/min for 10 min (4 ℃) and the supernatant was collected. The protein concentration was measured by Lowry's method. Protein extracts (50 μg) were separated by SDS-PAGE and transferred to PVDF membranes. After transference, the membranes were blocked with 5% fat-free milk for 1 h and then incubated with primary antibodies in 1:1000 dilution at 4 ℃ overnight. The membranes were then washed with TBS/Tween 20 (TBST) solution and incubated with horseradish peroxidase

conjugated secondary antibodies, and immunodetection was carried out by DAB. The densities of bands were scanned and quantified by image analysis system. Normalized densities were determined with a ratio of band density of SERCA2 to band density of β-actin.

Data analysis and statistics

Data were presented as $\bar{x} \pm s$. For all measurements, a one-way ANOVA was used to test differences between means. Comparisons between groups were made by using least significant difference (LSD). Statistical analysis was performed by using SPSS11.5 software. Differences were considered significant when $P < 0.05$.

Results

Effect of NP on $[Ca^{2+}]_i$

In cells subjected to anoxic insults, a significant increase in dissociative $[Ca^{2+}]_i$ was observed [hypoxia (474.614 ± 22.161) nmol/L *vs* control (112.740 ± 13.694) nmol/L, $P < 0.01$]. In cells treated with NP (0.25, 0.0625, and 0.0156 mg/mL), the increase in dissociative $[Ca^{2+}]_i$ induced by hypoxia was attenuated in a dose-dependent manner (Fig. 1). The Verapamil-treated group attenuated the dissociative $[Ca^{2+}]_i$ more significantly (Fig. 1).

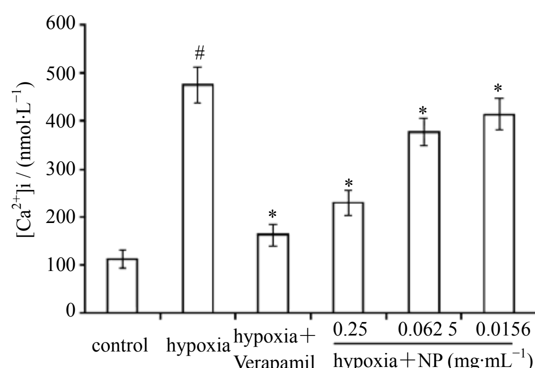

Fig. 1 Effect of NP on $[Ca^{2+}]_i$

$^{\#}P < 0.01$ *vs* control group; $^{*}P < 0.01$ *vs* hypoxia group, same as below

Effect of NP on Ca^{2+}-ATPase activity

To propose a mechanism for the effects of NP on $[Ca^{2+}]_i$, the intracellular Ca^{2+}-ATPase activity in the ventricular myocardium was measured (Fig. 2). A significant depression (55%) of Ca^{2+}-ATPase activity was observed in the hypoxia group, and this reduction was attenuated by NP and Verapamil treatment.

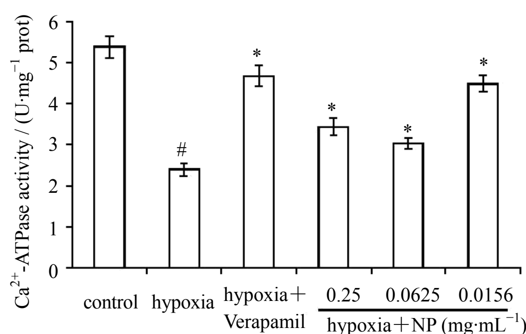

Fig. 2 Effect of NP on intracellular Ca^{2+}-ATPase activity

Effect of NP on SERCA2 mRNA level

To determine the relationship between the inhibition of NP on Ca^{2+} overload and changes in mRNA levels of SERCA2 in cardiomyocytes, the relative level of SERCA2 mRNA was examined by RT-PCR (Fig. 3). The results showed that the level of SERCA2 mRNA reduced in the model group. Both NP and Verapamil improved the SERCA2 mRNA level in hypoxic cardiomyocytes.

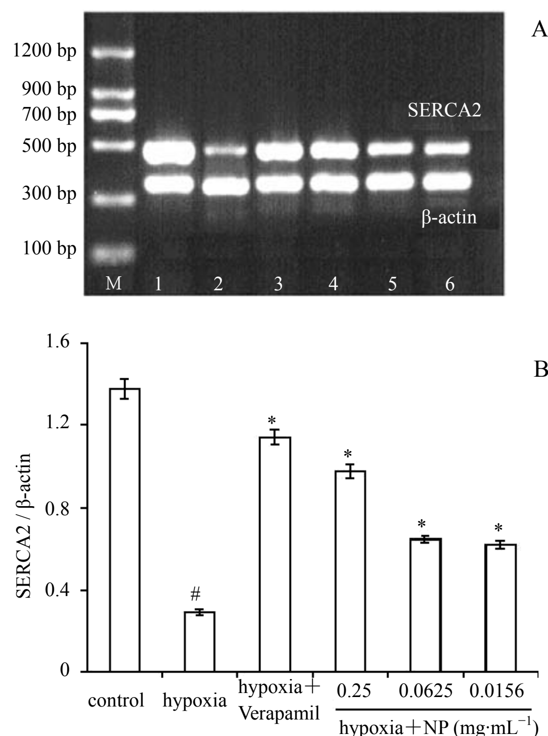

Fig. 3 Effect of NP on SERCA2 mRNA level

A: RT-PCR result for SERCA2 and β-actin in samples obtained from the control (lane 1), hypoxia (lane 2), hypoxia + Verapamil (lane 3), hypoxia + NP: 0.25, 0.0625, 0.0156 mg/mL (lanes 4—6), and DNA Marker (M)

B: summarized data showing SERCA2 gene expression normalized to β-actin gene expression

Effect of NP on SERCA2 protein level

To decipher whether changes in SERCA2 mRNA levels paralleled those with protein levels, the relative content of SERCA2 protein was examined by immunocytochemical staining (Fig. 4) and Western-blotting (Fig. 5). Normalized to β-actin, the SERCA2 protein level reduced in cardiomyocytes exposed to hypoxia. Treatment of NP and Verapamil significantly prevented this decrease in protein content.

Discussion

The main findings of the present study were as follows: (1) NP inhibited Ca^{2+} overload in hypoxia-induced myocardial injury, and (2) the treatment with NP prevented the reduction in Ca^{2+}-ATPase activity as well as SERCA2 mRNA and protein levels. It was the first time to establish that NP was helpful in maintaining the intracellular Ca^{2+} homeostasis of cardiomyocytes via SR mechanism against hypoxia injury at the cellular level.

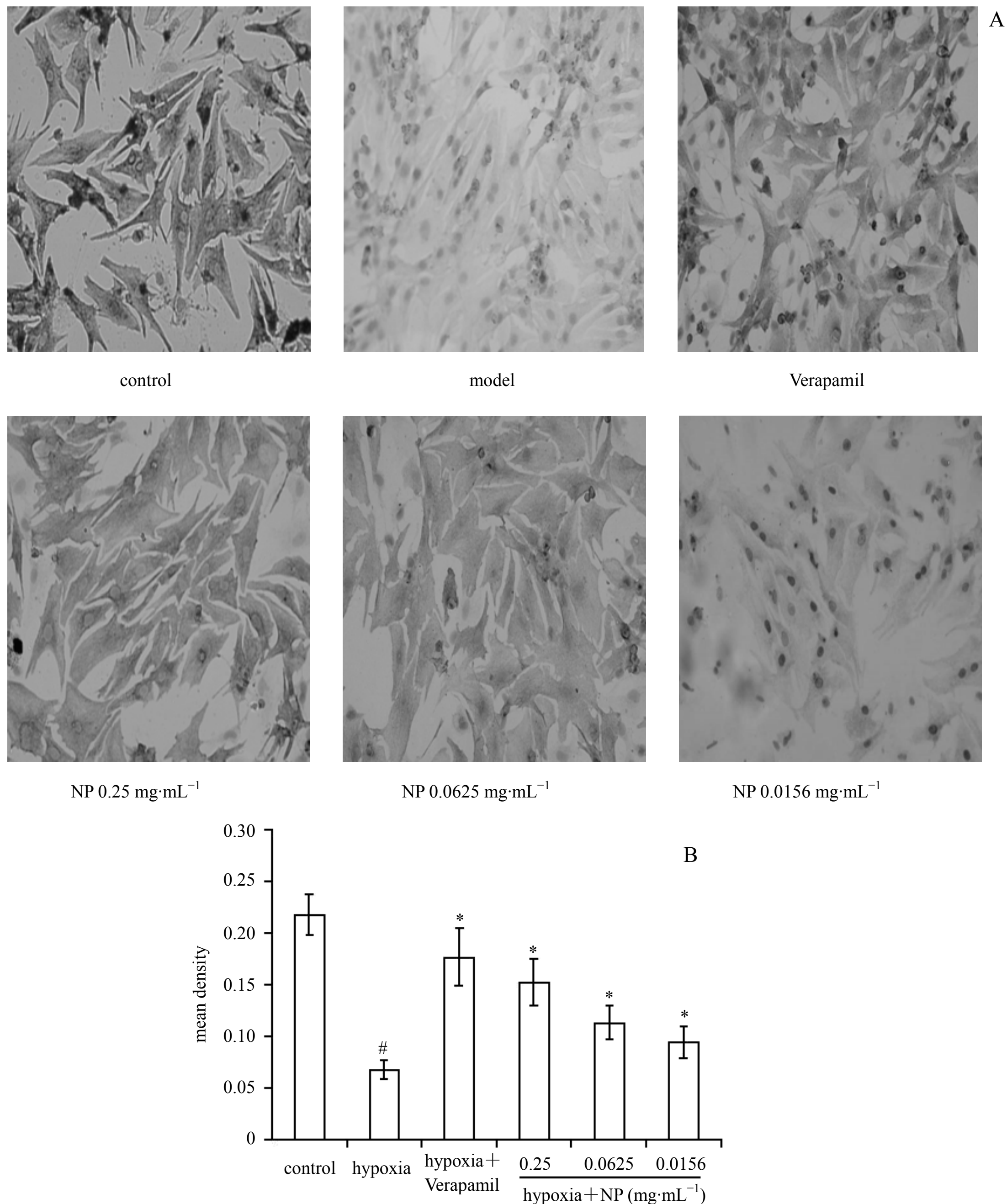

Fig. 4 Immunocytochemical staining analysis of SERCA2 in cardiomyocytes

A: representative photographs indicating immunocytochemical staining SERCA2 in samples

B: summarized data showing SERCA2 protein expression normalized to β-actin protein expression

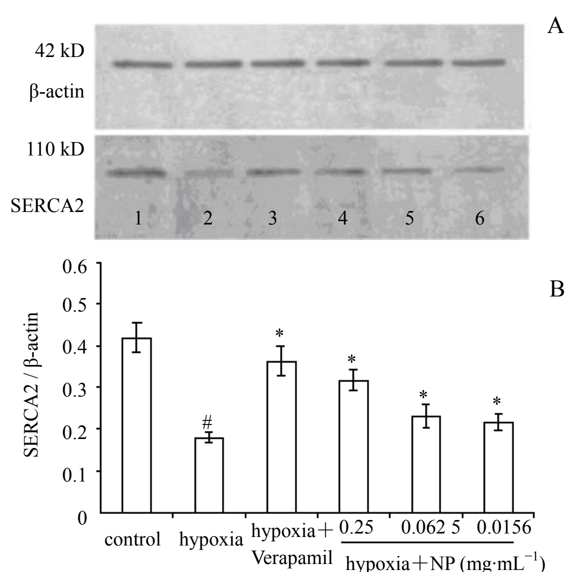

Fig. 5 Western-blotting analysis of SERCA2

A: Western-blotting for SERCA2 and β-actin in samples obtained from the control (lane 1), hypoxia (lane 2), hypoxia + Verapamil (lane 3), and hypoxia + NP: 0.25, 0.0625, 0.0156 mg·mL^{-1} (lanes 4—6)

B: summarized data showing SERCA2 protein expression normalized to β-actin protein expression

Cytosolic Ca^{2+} accumulation has been proposed as a mediator of the pathogenic changes that occur during myocardial hypoxia (Zucchi, Ronca, and Ronca-Testoni, 2001). Therefore, we tested the ability of NP to prevent cytosolic Ca^{2+} overload after hypoxia. As shown in this study, NP delayed the $[Ca^{2+}]_i$ increase in cardiomyocytes caused by hypoxia (Fig. 1).

Then we attempted to investigate the molecular mechanisms underlying the inhibition on the increase in diastolic $[Ca^{2+}]_i$ by NP during myocardial injury. In cardiac muscle, SR plays a critical role in the contraction and relaxation cycle by regulating the intracellular Ca^{2+} levels (Vangheluwe *et al*, 2003; Aoyagi *et al*, 1999). During diastole, muscle relaxation occurs as Ca^{2+} is again removed from cytosol, predominantly by accumulation into SR via the action of SERCA2 (92%) (Bassani, Bassani, and Bers, 1994). Therefore, we focused on examining whether the Ca^{2+}-ATPase activity and SERCA2 expression altered with NP treatment in the anoxic myocardium.

Three different SERCA genes, SERCA1, SERCA2, and SERCA3 were identified by molecular cloning analyses. SERCA2, the main form in the heart, facilitated the storage and distribution of Ca^{2+} in the SR (Ji *et al*, 1999; Wang and Wang, 2007). The SERCA2 utilizes hydrolysis of ATP as a source of energy for Ca^{2+} transport from the cytosol to the lumen of SR (Wang and Goldhaber, 2004; Asahi *et al*, 2003). In anoxic myocardium, hypoxia occurs with acidosis and lactate accumulation, which may disturb energy production. The SR Ca^{2+}-ATPase, in fact, seems to be the most sensitive ATPase in response to reduction in free energy released from ATP hydrolysis, and therefore SERCA2 activity could be affected by the changes in the energetics and ATP supply (Maack and O'Rourke, 2007). Moreover, studies suggested the decrease in ATP hydrolysis may directly result in defects in excitation contraction coupling and contractile function (Pepe, 2000).

Our study showed that NP prevented the decrease in cardiac intracellular Ca^{2+}-ATPase activity caused by hypoxia damage. In addition, we found that the depression in the levels of SERCA2 mRNA and protein was partially restored upon subjecting the hypoxia injured cardiomyocytes to NP treatment. Thus, our results support the concept that treatment of cardiomyocytes with NP prevented the changes in mRNA level and protein content of SERCA2 caused by hypoxia. NP preserved the activity of Ca^{2+}-ATPase and thus might increase the reuptake and refilling of Ca^{2+} in SR, thereby leading to a decrease in the diastolic $[Ca^{2+}]_i$ in cardiomyocytes.

In addition, the reduction in Ca^{2+} uptake with hypoxia is probably related to free radical-induced damage to the Ca^{2+}-ATPase. The Ca^{2+}-ATPase contains 24 cysteine residues and the SR Ca^{2+}-ATPase may be a principal target for modulation of muscle function by reactive oxygen species (Zima *et al*, 2006), as it has been demonstrated in numerous *in vitro* studies (Kang *et al*, 2006). Since it has been reported that NP could scavenge the oxygen free radicals generated from hypoxia-insulted myocardium (Li *et al*, 2009), we could not rule out the contribution of the anti-oxidative effects of NP in improving SR function and cardiomyocyte Ca^{2+} handling abnormalities in anoxic hearts.

In this study, Verapamil as a positive control significantly improved the SR Ca^{2+} transport in the injured myocytes, which is consistent with other reports (Takeo *et al*, 2000). Since NP had a similar effect as Verapamil, an *L*-type Ca^{2+} channel blocker, further study to better understand the beneficial mechanisms of

Ca^{2+} handling, should test whether NP has effects on *L*-type Ca^{2+} channel and other Ca^{2+} regulatory proteins, such as phospholamban, ryanodine receptor, and Na^{+}-Ca^{2+} exchanger.

Fourteen compounds (seven triterpenoids, four isoflavones, two sterols, and one nucleoside) were isolated from NP and structurally identified. These compounds were ursolic acid (**1**), euscaphic acid (**2**), tormentic acid (**3**), rosamultin (**4**), kaji-ichigoside F1 (**5**), 2α,3β,19α-trihydroxyolean-12-en-28oic acid-(28→1)-β-*D*-glucopyranosyl ester (**6**), 2-oxo-3β,19α-dihydroxyurs-12-en-28-oic acid-(28→1)-β-*D*-glucopyranosyl ester (**7**), β-sitosterol (**8**), β-daucosterol (**9**), adenosine (**10**), daidzin (**11**), puerarin (**12**), 3′-methoxy puerarin (**13**), and daidzein 8-*C*-apiosyl (1→6) glucoside (**14**), respectively. We conclude that adenosine (**10**) (Sommerschild *et al*, 1999) and four isoflavone compounds (**11** — **14**) might partly be responsible for its antihypoxia ability (Miao, Zhao, and Qin, 2005; Sun and Liu, 1999).

In conclusion, our findings demonstrated that NP could resist on hypoxia injury by inhibiting Ca^{2+} overload in rat cardiomyocytes, which may be associated with the prevention of changes in cardiac SR protein and gene expression. Stabilizing SERCA2 levels could prevent alterations in SR and cytosolic Ca^{2+} content, so as to attenuate the myocardial cell damage.

Acknowledgements

Professor CUI Xue-jun provided language help during the research.

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