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Sodium Ferulate attenuates anoxia/reoxygenation-induced calcium overload in neonatal rat cardiomyocytes by NO/cGMP/PKG pathway

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ABSTRACT

Development of intracellular calcium overload is an important pathophysiological factor in myocardial ischemia/reperfusion or anoxia/reoxygenation injury. Recent studies have shown that Sodium Ferulate (SF) stimulates nitric oxide (NO) production and exerts a cardioprotective effect in the ischemia–reperfused heart. However, it has not been determined whether the cardioprotection of SF is associated with suppression of Ca^{2+} overload via NO/cyclic GMP (cGMP)/cGMP-dependent protein kinase (PKG) pathway. In this work, after cardiomyocytes were incubated with 100, 200, 400, or 800 μM SF for 3 h, anoxia/reoxygenation injury was induced and intracellular Ca^{2+} concentration, NO synthase (NOS) activity, guanylate cyclase activity, NO, and cGMP formation were measured appropriately. The results showed that treatment with SF concentration-dependently inhibited calcium overload induced by anoxia/reoxygenation. We also demonstrated that SF (100–800 μM) concentration dependently enhanced NO and cGMP formation through increasing NOS activity and guanylate cyclase activity in the cardiomyocytes. On the contrary, inhibition of calcium overload by SF was markedly attenuated by addition of an NOS inhibitor, an NO scavenger, an soluble guanylate cyclase inhibitor, and a PKG inhibitor: N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (c-PTIO, 1.0 μM), 1H-[1, 2, 4] oxadiazolo [4, 3-α] quinoxalin-1-one (ODQ, 20 μM) and KT5823 (0.2 μM), respectively. Our findings indicate that SF significantly attenuates anoxia/reoxygenation-induced Ca^{2+} overload and improves cell survival in cultured cardiomyocytes through NO/cGMP/PKG signal pathway.

1. Introduction

Increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) has been implicated in the pathogenesis of myocardial ischemia–reperfusion injury (Piper et al., 2003). Cumulated evidence shows that intracellular Ca^{2+} overload may damage and diminish the function of the myocardium via numerous potentially degenerative states, including hypercontracture (Meissner and Morgan, 1995), arrhythmia (du Toit and Opie, 1994), activation of proteolytic enzymes (Matsumura et al., 1993), mitochondrial Ca^{2+} accumulation (Lochner et al., 1987; Miyamae et al., 1996), reduced ATP levels (Steenbergen et al., 1990), and activation of phospholipases (Moraru et al., 1994). Therefore, limiting Ca^{2+} overload is expected to contribute to cardioprotection in ischemia–reperfusion injury.

Nitric oxide (NO) is a biological active free radical and also an important intracellular and intercellular messenger which is generated in mammalian cells from L-arginine by a family of nitric oxide synthases (NOS), which include endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) (Marletta, 1993). With no doubt the fundamental signaling role of NO in cellular function is the activation of soluble guanylyl cyclase which leads to the formation of cyclic GMP (cGMP), which in turn, leads to cGMP-dependent protein kinase (PKG) activation (Shah and MacCarthy, 2000; Murad, 2006). The signaling cascade plays an important modulatory role in many physiological and pathological conditions (Moncada et al., 1991; Rastaldo et al., 2007). It is noteworthy that the NO/cGMP/PKG signaling pathway is deeply involved in the cardioprotective action in ischemia–reperfusion injury as a survival signal (Burley et al., 2007). Moreover, several studies have demonstrated activation of the NO/cGMP/PKG signaling pathway can provide an inhibitory effect on the elevation of intracellular Ca^{2+} concentrations by phosphorylating target proteins responsible for intracellular Ca^{2+} homeostasis (Ruth et al., 1993; Komalavilas and Lincoln, 1994; Liu et al., 1997). At present, several targets have been proposed for PKG for the control of Ca^{2+} homeostasis in cardiomyocytes. These include the sarcolemmal L-type Ca^{2+} channel, which upon phosphorylation by PKG, inhibits Ca^{2+} entry during phase 2 of the action potential; the Ca^{2+}-activated potassium channel, which upon sarcolemmal hyperpolarisation, decreases influx of Ca^{2+} through the L-type Ca^{2+} channel; phospholamban and the ryanodine receptor, which allows the sarcoplasmic reticulum to

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sequester Ca^{2+}; the inositol-1,4,5-trisphosphate receptor, which reduces Ca^{2+} release from the sarcoplasmic reticulum (Kwan et al., 2000; Lucas et al., 2000; Zucchi et al., 2001; Piper et al., 2004).

Sodium Ferulate (3-methoxy-4-hydroxy-cinnamate sodium, $C_{10}H_9O_4Na$, SF) is sodium salt of ferulic acid, which is an effective component of Chinese medicinal herbs, such as *Angelica sinensis*, *Cimicifuga heracleifolia*, and *Lignsticum chuangxiong*. In vitro and in vivo studies revealed that SF possesses beneficial pharmacological effects, which include anti-platelet aggregation, anti-thrombosis, anti-peroxidation, inhibition of the production of inflammatory mediators, and decrease of serum lipids (Wang et al., 2004; Wang and Ou-Yang, 2005). Recently, Liu et al. found SF can stimulate NO production and protect myocardium against ischemia–reperfusion injury (Liu et al., 2006). However, it has not been determined whether the cardioprotection of SF involves a reduction in myocyte Ca^{2+} accumulation.

Therefore, in this study, we investigated whether SF could rescue Ca^{2+} overload in cultured neonatal rat cardiomyocytes under anoxia/reoxygenation conditions as an in vitro ischemia/reperfusion model (Chlopcikova et al., 2001; Rui et al., 2005). Furthermore, we investigated whether the NO/cGMP/PKG signaling pathway implicated in the inhibitory effect of SF.

2. Materials and methods

2.1. Animals

All animals used in this study were cared for in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health (NIH Publication No. 85-23, revised 1996), and all procedures were approved by the Nanchang University Medical College Animal Care Review Committee.

2.2. Chemicals and reagents

Sodium Ferulate was purchased from Laimei Pharmaceutic Co. Ltd. (Chongqing, China) and was dissolved in distilled water; Cell culture products were obtained from Life Technologies (Paisley, Scotland); L-NAME, c-PTIO, ODQ, KT5823, Fluo-2/AM, Pluronic F-127, and other chemical agents were all purchased from Sigma Chemical Company (St Louis, MO, USA); L-NAME was dissolved in distilled water; c-PTIO was dissolved in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); ODQ and KT5823 were dissolved in dimethyl sulfoxide (DMSO); Anti-iNOS, Anti-nNOS, and anti-eNOS primary antibodies, as well as the HRP-linked secondary antibody, were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); cGMP enzyme immunoassay Kit were purchased from Assay Designs Inc.(Ann Arbor, MI, USA).

2.3. Isolation of neonatal rat cardiomyocytes

Primary cultures of neonatal rat cardiomyocytes were prepared from 1-to 3-day-old Sprague–Dawley rats by a modification of a previously published protocol (Spector et al., 1998). Briefly, the hearts were rapidly excised, minced, and dissociated with 0.1% trypsin. The dissociated cells were preplated for 1 h to enrich the culture with myocytes. The nonadherent myocytes were then plated at a density of 2000 cells/mm^2 in a nutritive medium consisting of Eagle's minimum essential medium supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 U/ml) and maintained at 37 °C in the presence of 5% CO_2 in a humidified incubator. Bromodeoxyuridine (0.1 mM) was included in the medium for the first 3 days after plating to inhibit fibroblast growth. Using this method, we routinely obtained enriched cultures containing more than 93% cardiomyocytes, as verified by the proportion of cells showing spontaneous contraction or displaying muscle specific markers on immunohistologic examination. Four-day cardiomyocyte cultures were used in subsequent experiments.

2.4. Anoxia/reoxygenation injury model

The in vitro model of anoxia/reoxygenation used in the present study was similar to that described by Koyama et al. (1991). Briefly, cardiomyocytes were initially equilibrated in normal Tyrode solution at 37 °C, pH 7.4 (control). Subsequently, the confluent beating cardiomyocytes in 24-well plates were exposed to anoxia with simulated ischaemia buffer (in mM: NaCl 98.5, KCl 10, $MgSO_4$ 1.2, $CaCl_2$ 1.0, HEPES 20, sodium lactate 40, pH 6.8, 37 °C) for 3 h and then re-oxygenated for 2 h with simulated reoxygenation buffer (in mM: NaH_2PO_4 0.9, $NaHCO_3$ 20.0, $CaCl_2$ 1.0, $MgSO_4$ 1.2, HEPES 20.0, NaCl 129.5, KCl 5.0, glucose 5.5, pH 7.4, 37 °C). Anoxic conditions were obtained by equilibrating a small humidified plexiglass chamber containing cardiomyocytes with 95% N_2 and 5% CO_2 via a gas transfusive apparatus (Changjing Biotech Co., China) and confirmed by measuring chamber pO_2 (chamber pO_2 fell to 0 mm Hg within 5 min after initiation of perfusion with the anoxic gas). Reoxygenation was achieved by exposing cells to room air (CO_2 incubator).

2.5. Experimental protocols for drug treatments

At the start of each experiment, the cells were rinsed in phosphate buffered saline and incubated for 3 h in fresh medium with or without various concentrations of SF (100, 200, 400, or 800 μM) prior to anoxia/reoxygenation. In a different series of experiments, 100 μM L-NAME (N^G-nitro-L-arginine methyl ester, an NOS inhibitor), 1.0 μM c-PTIO (2-(4-carboxyphenyl)-4, 4, 5, 5-tetramethylimidazole-1-oxyl-3-oxide, an NO scavenger), 20 μM ODQ (1H-[1, 2, 4] oxadiazolo [4, 3-α] quinoxalin-1-one, an soluble guanylate cyclase inhibitor), or 0.2 μM KT5823 (a PKG inhibitor) were added to the cell cultures 30 min before and during 800 μM SF treatment. Additionally, same final concentrations of solvent were also included in all respective control experiments. Cell viability, intracellular Ca^{2+} concentration, and lactate dehydrogenase activity were measured at the end of the reoxygenation times. Other parameters measurements were performed after 3 h of incubation with SF.

2.6. Assay of cell viability

Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolim bromide (MTT) assay. The cardiomyocytes were spreaded in 96-well plates at 1×10^5 cells/well. After experiment treatment, the cardiomyocytes were washed with warm phosphate buffered saline and incubated with 0.5 mg/ml MTT in phosphate buffered saline for 4 h at 37 °C. The reaction was stopped by the addition of 150 μl diphenylamine solution, and the absorbance of the blue formazan derivative was read at 570 nm using a microplate reader (Bio-Rad Laboratories, CA, USA).

2.7. Assay of lactate dehydrogenase activity

To measure lactate dehydrogenase (LDH) activity in the culture medium of cardiomyocytes, after experiment treatment, 0.1 ml of culture medium was taken and analyzed with an automatic biochemical analyzer (Hitachi 7060, Japan).

2.8. Assay of NO production

NO production in cells was measured by Griess method (Schmidt and Kelm, 1996) and according to the indication on the NO assay kit (Beyotime Biotech Inc., Jiangsu, China). Briefly, 50 μl of culture medium, after SF treatment, was collected and was reacted with an equal volume of Griess reagents (1% sulfanilamide, 0.1% N-[1-naphthyl] ethylenediamine dihydrochloride and 2.5% phosphoric acid) in a

multiwell microtiter plate. The absorbance was measured at a wavelength of 548 nm on a microplate reader, and nitrite concentration was determined using a curve calibrated from sodium nitrite standards. After washing, cells were dissolved in 0.2 ml of 1% sodium dodecyl sulphate and used for protein assay (BCA protein assay kit; Sigma) with bovine serum as a standard. Nitrite levels were corrected by protein measurements, and data are shown as nmol/mg protein.

2.9. Determination of NOS activity and eNOS, nNOS, iNOS expression

For an assay of NOS activity we used a fluorimetric detection system FCANOS1 (Sigma), based on the principle of fluorescence of triazolofluoresceine, which is formed after interaction of NO with 4,5-diaminofluoresceine, which is formed from 4,5-diaminofluoresceine diacetate under the action of intracellular esterases. The wavelength of excitation/emission was 492/515 nm. Detailed methods referred to the manufacture's instructions. Enzyme activity was evaluated in units of fluorescence (UF) per min per 10^6 cells.

NOS protein in cardiomyocytes was assessed by Western blotting. Briefly, cells were lysed with lysis buffer containing 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 1% (v/v) TritonX-100, 1 mM NaF, 1 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Equivalent amounts of protein were resolved on 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking with 5% non-fat milk, the membranes were blotted with a polyclonal anti-eNOS, anti-eNOS, or anti-iNOS antibody (1:1000 dilution), and detected with a horseradish peroxidase-linked secondary antibody (1:2000 dilution). The specific bands were detected using chemiluminescence reagents on a chemiluminescence film. Densitometric analysis of the Western blot was performed with GDS-8000 UVP photo scanner and LAB WOEK45 Image software (Bio-rad). Normalization of results was ensured by running parallel western blots with β-actin antibody.

2.10. Determination of guanylate cyclase activity

After SF treatment, the cardiomyocytes were lysed by sonication for 45 s and kept on ice in 25 mM HEPES buffer (pH 7.5) containing dithiothreitol (1 mM), phenylmethylsulphonyl fluoride (10 μg/ml), trypsin inhibitor (10 μg/ml), leupeptin (10 μg/ml), antipain (10 μg/ml), chymostatin (10 μg/ml) and pepstatin (10 μg/ml). The lysate solution was then centrifuged at 78 000 g for 20 min at 4 °C to obtain supernatant used for guanylate cyclase activity assay, 100 μl of supernatant (1 μg protein) was added into the prewarmed (37 °C) buffer (pH 7.2) containing 25 mM Tris–HCl, 3 mM GTP, 5 mM $MgCl_2$, 1 mM 3-isobutyl-1-methylxanthine for 20 min of incubation. Then, the reaction was terminated by boiling for 3 min, and the amount of cGMP in the mixture was measured by enzyme immunoassay kit. The guanylate cyclase activity was expressed as pmol/min per mg of protein.

2.11. Determination of intracellular cGMP

After SF treatment, the cardiomyocytes were lysed in 0.1 M hydrochloric acid. 50 μl aliquots of the cell lysates were transferred into 96-well plates and subjected to enzyme immunoassay for intracellular cGMP quantification using a colorimetric cGMP enzyme immunoassay Kit, according to the manufacturer's instructions. Optical absorbance was measured on a microplate reader at a wavelength of 415 nm. cGMP levels were corrected by protein measurement, and data are shown as pmol/mg protein.

2.12. Determination of intracellular Ca^{2+} concentration

A spectrofluorometric method with Fura-2/AM as the Ca^{2+} indicator was used for measurement of $[Ca^{2+}]_i$ (Xu et al., 1997). Briefly, after experiment treatment, cardiomyocytes were incubated for 40 min at 37 °C in the dark with 5 μM Fura-2/AM dissolved in HEPES-buffer (in mM: NaCl, 110; KCl, 2.6; $MgSO_4$, 1.0; $CaCl_2$, 1.0; glucose, 11 and HEPES, 25) supplemented with 0.02% pluronic acid F-127 and then washed twice to remove any extracellular dye. Ca^{2+} fluorescence recorded at excitation wavelengths of 340 and 380 nm using a Fluoroskan Ascent fluorimeter. Quantitative intracellular Ca^{2+} values were obtained from the observed fluorescence ratio 340:380 (R) and fluorescence calibration following the procedure described by Tsien (Tsien et al., 1982). In brief, to obtain the minimum fluorescence ratio (R_{min}), 400 mM EGTA (pH 8.7) and 16 μM ionomycin were added sequentially, followed by 10 mM $CaCl_2$ to obtain the maximum fluorescence ratio (R_{max}) and 5 mM $MnCl_2$ to measure the autofluorescence. After correction of R, R_{min} and R_{max} for autofluorescence, the $[Ca^{2+}]_i$ levels were calculated by computer according to the formula: $[Ca^{2+}]_i = 224 \times [(R - R_{min})/(R_{max} - R)] \times sf/sb \times K_d$ where sf/sb is the ratio of fluorescence values measured before and after $CaCl_2$ addition at a wavelength of 380 nm, and K_d is the dissociation constant of Fura-2/AM for Ca^{2+}.

2.13. Statistical analyses

Values are expressed as means ± S.E.M. One-way analysis of variance followed by the Student–Newman–Keuls test was applied to calculate the statistical significance between various groups. A value of $P<0.05$ was considered to be statistically significant.

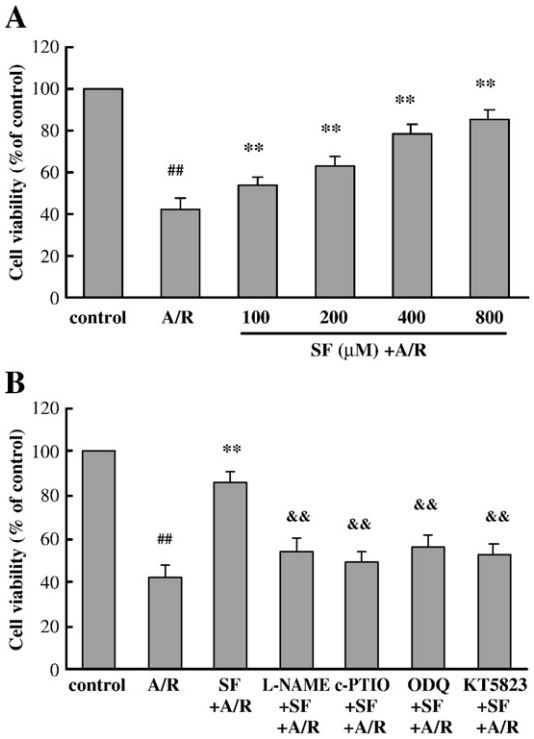

Fig. 1. Effects of SF and combination with L-NAME, c-PTIO, ODQ or KT5823 on viability of cardiomyocytes subjected to anoxia/reoxygenation (A/R). (A) Cardiomyocytes were incubated for 3 h with or without various concentrations of SF (100, 200, 400, or 800 μM), and then subjected to A/R. (B) Cardiomyocytes were incubated 3 h with SF (800 μM) in the presence or absence of NOS inhibitor L-NAME (100 μM), NO scavenger c-PTIO (1.0 μM), soluble guanylate cyclase inhibitor ODQ (20 μM) or PKG inhibitor KT5823 (0.2 μM), followed by A/R. Cell viability was measured by MTT assay as described in the Materials and methods section. All data were presented as mean ± S.E.M ($n=6$). $^{\#\#}P<0.01$ vs control; $^{**}P<0.01$ vs A/R; $^{\&\&}P<0.01$ vs SF + A/R.

3. Results

3.1. Effect of treatment with SF on anoxia/reoxygenation damage in cardiomyocytes

The releases of LDH and cell viability were usually used as indexes of cardiomyocyte injury. As shown in Fig. 1A, the viability of cardiomyocytes subjected to anoxia/reoxygenation was only 42.63% of untreated cells (control). However, pretreatment with SF (100, 200, 400, or 800 μM) significantly increased the viability of cardiomyocytes to 54.07, 63.34, 78.49, and 86.05%, respectively, indicating dose-dependent cardioprotection against anoxia/reoxygenation. When 100 μM L-NAME (an NOS inhibitor), 1.0 μM c-PTIO (an NO scavenger), 20 μM ODQ (an soluble guanylate cyclase inhibitor) or 0.2 μM KT5823 (a PKG inhibitor) was administered 30 min before and during 800 μM SF pretreatments, the protective effect of SF was abolished (Fig. 1B), whereas L-NAME, c-PTIO, ODQ or KT5823 itself did not affect viability of cardiomyocytes (data not shown). It indicates that the protective effect of SF was associated with NO/cGMP/PKG signaling pathway. Similarly, SF significantly lowered the LDH activity compared with the anoxia/reoxygenation group (Fig. 2A), and the effects were abolished in the presence of 100 μM L-NAME, 1.0 μM c-PTIO, 20 μM ODQ or 0.2 μM KT5823 (Fig. 2B).

3.2. Effects of SF on $[Ca^{2+}]i$ of cardiomyocytes subjected to anoxia/reoxygenation

To investigate whether the cardioprotective effect of SF is related to a suppression of intracellular calcium accumulation, we examined the effects of SF on intracellular Ca^{2+} concentration. As shown in Fig. 3A,

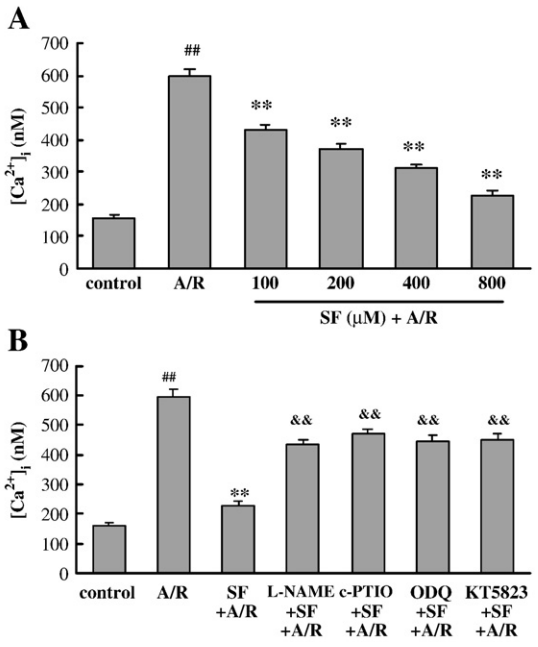

Fig. 3. Effect of SF and combination with L-NAME, c-PTIO, ODQ or KT5823 on intracellular calcium concentration ($[Ca^{2+}]_i$) in cardiomyocytes undergoing anoxia/reoxygenation (A/R). (A) Cardiomyocytes were incubated for 3 h with or without various concentrations of SF (100, 200, 400, or 800 μM), and then subjected to A/R. (B) Cardiomyocytes were incubated with SF (800 μM) in the presence or absence of NOS inhibitor L-NAME (100 μM), NO scavenger c-PTIO (1.0 μM), soluble guanylate cyclase inhibitor ODQ (20 μM) or PKG inhibitor KT5823 (0.2 μM), followed by A/R. Intracellular Ca^{2+} concentration was measured as described in the Materials and methods section. All data were presented as mean±S.E.M (n=6). $^{\#\#}P<0.01$ vs control; $^{**}P<0.01$ vs A/R; $^{\&\&}P<0.01$ vs SF+A/R.

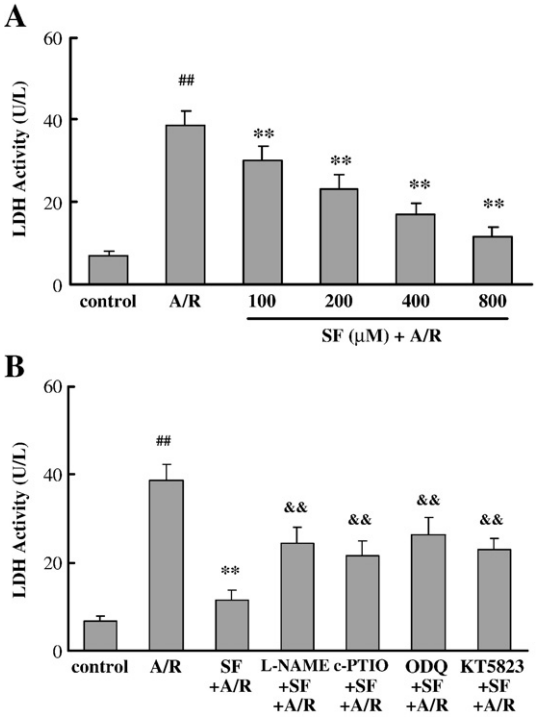

Fig. 2. Effects of SF and combination with L-NAME, c-PTIO, ODQ or KT5823 on LDH activity in cardiomyocytes subjected to anoxia/reoxygenation (A/R). (A) Cardiomyocytes were incubated for 3 h with or without various concentrations of SF (100, 200, 400, or 800 μM), and then subjected to A/R. (B) Cardiomyocytes were incubated 3 h with SF (800 μM) in the presence or absence of NOS inhibitor L-NAME (100 μM), NO scavenger c-PTIO (1.0 μM), soluble guanylate cyclase inhibitor ODQ (20 μM) or PKG inhibitor KT5823 (0.2 μM), followed by A/R. All data were presented as mean±S.E.M (n=6). $^{\#\#}P<0.01$ vs control; $^{**}P<0.01$ vs A/R; $^{\&\&}P<0.01$ vs SF+A/R.

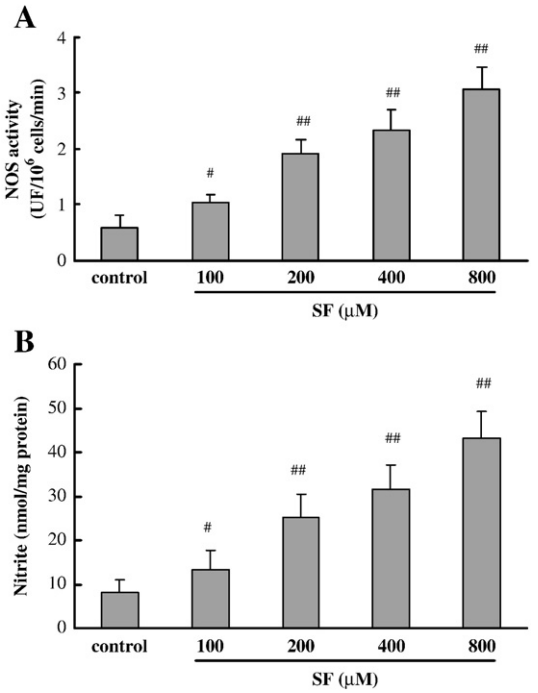

Fig. 4. Effects of SF on NOS activity (A) and NO release (B) in cardiomyocytes. Cardiomyocytes were incubated for 3 h with or without various concentrations of SF (100, 200, 400, or 800 μM). NOS activity and NO release were measured as described in the Materials and methods section. All data were presented as mean±S.E.M (n=6). $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs control.

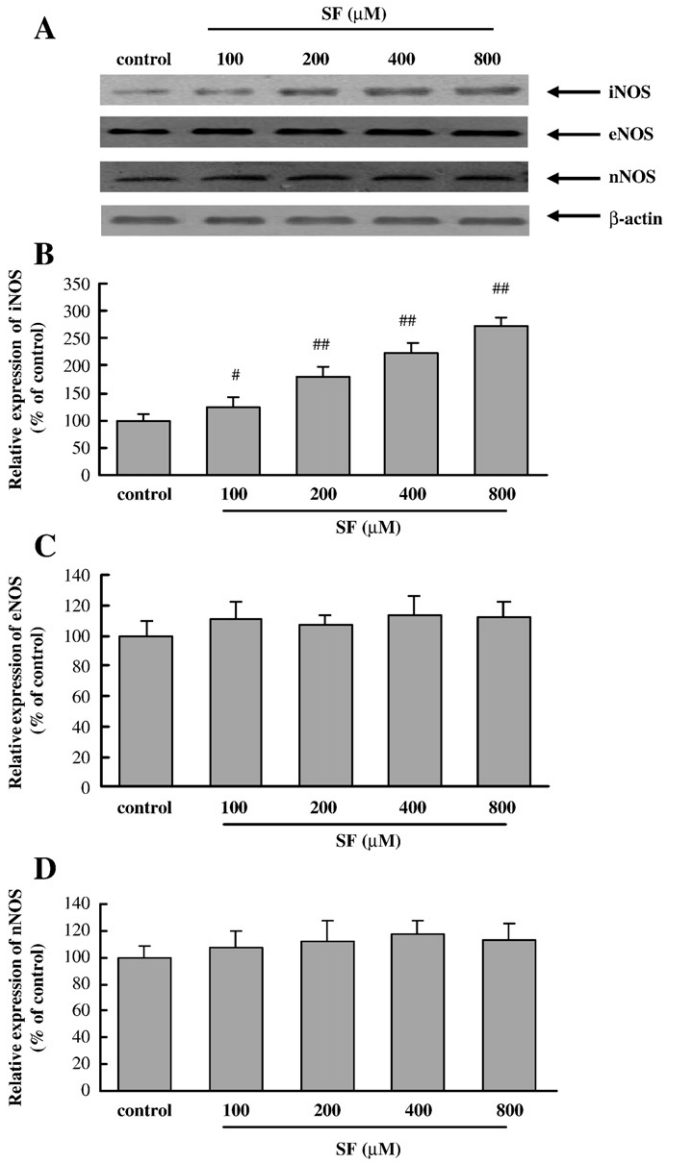

Fig. 5. The effect of SF on the expression of iNOS, eNOS, and nNOS in cardiomyocytes. Cardiomyocytes were incubated for 3 h with or without various concentrations of SF (100, 200, 400, or 800 μM). The expression of iNOS, eNOS, and nNOS were assessed by Western blotting as described in the Materials and methods section. (A) shows a representative Western blot for iNOS, eNOS, and nNOS; (B) Relative levels of iNOS protein as assessed by densitometry; (C) Relative levels of eNOS protein as assessed by densitometry; (D) Relative levels of nNOS protein as assessed by densitometry. Quantitations were normalized to value obtained for β-actin protein expression. Values are expressed as mean±S.E.M. of four independent experiments (n=4). $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared with control.

cardiomyocytes subjected to anoxia/reoxygenation insults had an increase in intracellular Ca^{2+} concentration (596.72±23.29 nM) as compared with untreated cells (158.67±11.01 nM). Treatment with SF (100, 200, 400 or 800 μM) produced a significant reduction in intracellular Ca^{2+} concentration in cardiomyocytes undergoing anoxia/reoxygenation (430.59±15.36, 373.34±13.71, 310.87±13.98, and 226.51±14.77 nM, respectively), whereas SF applied alone, under normoxic conditions, without anoxia/reoxygenation, did not affect intracellular Ca^{2+} concentration (data not shown). These results suggest that SF can significantly attenuate Ca^{2+} overload induced by anoxia/reoxygenation in a dose-dependent manner. In addition, data presented in Fig. 3B indicate that the inhibitory potency of SF on Ca^{2+} overload induced by anoxia/reoxygenation was significantly attenuated by addition of L-NAME (100 μM), PTIO (1.0 μM), ODQ (20 μM), or KT5823 (0.2 μM) compared with those cardiomyocytes treated with 800 μM SF alone. However, L-NAME, c-PTIO, ODQ or KT5823 itself did not affect Ca^{2+} overload induced by anoxia/reoxygenation (data not shown). These data suggest that NO/cGMP/PKG signaling pathway might be involved in the inhibition effect of SF on Ca^{2+} overload induced by anoxia/reoxygenation in cardiomyocytes.

3.3. Effects of SF on NOS activity and NO production in cardiomyocytes

To further ensure the potential involvement of NO/cGMP/PKG signaling pathway in the inhibitory effect of SF on Ca^{2+} overload induced by anoxia/reoxygenation, the effects of SF on NOS activity and NO production were measured directly. Data presented in Fig. 4A showed that SF elicited a concentration-dependent increase in NOS activity that was significant (P<0.05) as NOS activity increased by 75.86, 225.86, 304.44, and 429.31% for, respectively, 100, 200, 400, and 800 μM SF compared to control (untreated) cardiomyocytes. Consistent with the NOS activation pattern, SF stimulated NO production in a concentration dependent manner, reaching a maximal level at 800 μM SF (Fig. 4B).

3.4. Effects of SF on the expression of iNOS, nNOS, and eNOS in cardiomyocytes

Next we examined whether the activation of NOS is mediated by upregulation of protein expression. All three known NOS isoforms, nNOS, eNOS, and iNOS are shown to be expressed in cardiomyocytes (Massion et al., 2005). Thus, we examined effects of SF on these NOS isoforms expression. Results from Western blot analysis showed that the expression of eNOS and nNOS were not significantly altered by SF treatment. However, a concentration-dependent increase in iNOS expression was observed in SF-treated cardiomyocytes compared with untreated cardiomyocytes (Fig. 5).

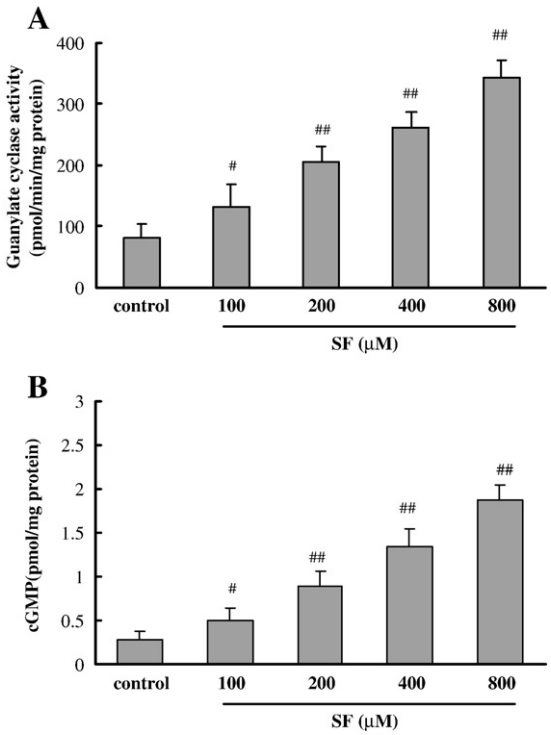

Fig. 6. Effects of SF on guanylate cyclase activity (A) and cGMP formation (B) in cardiomyocytes. Cardiomyocytes were incubated for 3 h with or without various concentrations of SF (100, 200, 400, or 800 μM). Guanylate cyclase activity and cGMP formation were measured as described in the Materials and methods section. All data were presented as mean±S.E.M (n=6). $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs control.

3.5. Effects of SF on soluble guanylate cyclase activity and cGMP formation in cardiomyocytes

In addition, we went further to investigate effects of SF on soluble guanylate cyclase activity and cGMP levels in cardiomyocytes, As shown in Fig. 6, SF concentration dependently further increased soluble guanylate cyclase activity (Fig. 6A) and the formation of cGMP (Fig. 6B).

4. Discussion

The major findings of the present study are that SF has a concentration dependent inhibitory effect on anoxia/reoxygenation-induced Ca^{2+} overload in a neonatal rat cardiomyocyte model and that the inhibitory effect of SF appears to be mediated by a mechanism that involves the NO/cGMP/PKG signaling pathway. To our knowledge, this is the first study to establish that SF is helpful in maintaining the intracellular calcium homeostasis of cardiomyocytes via NO/cGMP/PKG signaling mechanism against anoxia/reoxygenation injury at the cellular level.

Numerous studies of ischemic myocardial injury implicate the myocardial Ca^{2+} overload induced by ischemia–reperfusion is an important pathophysiological factor that contributes to mechanical dysfunction, myocardial cell death and arrhythmia (Kusuoka et al., 1987; Silverman and Stern, 1994; Piper et al., 2003). In the present study, we also found that cardiomyocytes subjected to anoxia/reoxygenation have a rapid and significant increase in intracellular Ca^{2+} level and concomitantly increase of LDH activity and reduction of cell viability, further suggesting that dysregulation of intracellular calcium homeostasis plays a significant role in the myocardial cell injury induced by ischemia/reperfusion or anoxia/reoxygenation. Therefore, prevention of Ca^{2+} overload has been considered to be a reasonable therapeutic strategy to alleviate ischemia/reperfusion or anoxia/reoxygenation injury.

SF is a biologically active ingredient extracted from the Chinese medicinal plant, such as *A. sinensis*, *C. heracleifolia*, and *L. chuangxiong*, and has gained considerable attention due to its wide range of biological and pharmacological properties. Recent studies showed SF could stimulate NO production and protect against myocardial ischaemia/reperfusion injury in rat hearts (Liu et al., 2006). In the present study, we also found that SF could suppressed the increase of LDH activity and the decrease of viability resulted from anoxia/reoxygenation in a dose-dependent manner, when SF (100, 200, 400 or 800 μM) was applied 3 h prior to anoxia/reoxygenation. The observations further indicate that SF can exert direct cardioprotection from anoxia/reoxygenation injury in a neonatal rat cardiomyocyte model.

Considering that intracellular calcium accumulation has been proposed as a mediator of the pathogenic changes that occur during myocardial ischemia/reperfusion, we further tested the ability of SF to prevent intracellular Ca^{2+} overload after anoxia/reoxygenation. As shown in this study, treatment with SF significantly attenuated Ca^{2+} overload induced by anoxia/reoxygenation in a dose-dependent manner up to 800 μM. It implied that the cardioprotective effect of SF is related to a suppression of intracellular calcium accumulation.

We next attempted to investigate the signaling mechanisms underlying the inhibition of the increase in $[Ca^{2+}]_i$ by SF during myocardial injury. It is well known that regulation of intracellular Ca^{2+} levels are complicated and associated with several intracellular signaling cascades. One such signaling cascade is NO/cGMP/PKG signaling pathway, which has been shown to provide an inhibitory effect on the elevation of intracellular Ca^{2+} concentrations by inhibiting phosphoinositide hydrolysis, intracellular calcium mobilization, and voltage-dependent activation of L-type Ca^{2+} channels (Ruth et al., 1993; Komalavilas and Lincoln, 1994; Liu et al., 1997). Moreover, numerous evidence have suggested that the NO/cGMP/PKG signaling pathway is deeply involved in the cardioprotective action in ischemia–reperfusion (I/R) injury (MacCarthy and Shah, 2000; Hanafy et al., 2001). Therefore, we further examined whether the NO/cGMP/PKG signaling pathway was involved in the inhibitory effect of SF on Ca^{2+} overload induced by anoxia/reoxygenation.

In this study, there was clear evidence that the inhibitory effect of SF on anoxia/reoxygenation-induced Ca^{2+} overload was associated with an enhanced NO/cGMP/PKG-mediated process. First, the inhibitory effect of SF on anoxia/reoxygenation-induced Ca^{2+} overload was significantly attenuated when NO formation was blocked by an NOS inhibitor, L-NAME, or an NO scavenger, c-PTIO. Second, the inhibitory effect of SF on anoxia/reoxygenation-induced Ca^{2+} overload was also reduced if the biosynthesis of cGMP was inhibited by an soluble guanylate cyclase inhibitor, ODQ. Third, the inhibitory effect of SF on anoxia/reoxygenation-induced Ca^{2+} overload was also significantly attenuated when the activity of PKG was inhibited by KT5823. Fourth, the strongest evidence was that SF could directly enhance NO and cGMP production in cardiomyocytes through increasing NOS and soluble guanylate cyclase activity. These findings strongly indicated that the mechanisms by which SF inhibited anoxia/reoxygenation-induced Ca^{2+} overload might be mediated, in part, by an NO/cGMP/PKG-dependent process.

Additionally, another interesting finding in the present study is that SF increased iNOS expression in a concentration-dependent manner, accompanied by increases of NOS activity and NO production in cardiomyocytes. However, the expression of eNOS and nNOS was not significantly altered by SF treatment. These findings suggest SF-induced iNOS expression may play a central protective role against Ca^{2+} overload damage. However, signaling mechanism of up-regulation of iNOS induced by SF remains to be determined. This question is important and interesting, and deserves further exploration.

In summary, the present study provides unequivocal evidence for the first time that SF significantly attenuates anoxia/reoxygenation-induced Ca^{2+} overload and improves cell survival in cultured cardiomyocytes, and that the effect is accomplished through NO/cGMP/PKG signaling pathway. Our studies provide a new mechanism to explain cardioprotective effects of SF. Moreover, our results expose the role of iNOS, rather than eNOS and nNOS, as the major contributing NO synthase during SF treatment. Future studies should focus on the natural mechanism of iNOS protein up-regulation induced by SF. These approaches will be essential to lighten the cellular effect and pharmacological profiles of the ever-increasingly used SF.

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