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CARDIOVASCULAR SYSTEM

Cyclooxygenase-2 mediates the delayed cardioprotection induced by hydrogen sulfide preconditioning in isolated rat cardiomyocytes

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Abstract We previously reported that hydrogen sulfide (H_2S) preconditioning (SP) produces cardioprotection in isolated rat cardiomyocytes. The present study was designed to determine the involvement of cyclooxygenase-2 (COX-2) in the SP-induced delayed cardioprotection. Isolated cardiac myocytes were treated with NaHS (100 μM, a H_2S donor) for 30 min and then cultured for 20 h followed by ischemia/reperfusion insults. SP significantly increased cell viability, percentage of rod-shaped cells, and myocyte contractility after 10 min of reperfusion. Given 30 min before and during lethal ischemia, two selective COX-2 inhibitors, NS-398 and celebrex, abrogated SP-induced cardioprotective effects. Moreover, SP upregulated the expression of COX-2 and increased PGE_2 production in the cardiac myocytes. These effects were significantly attenuated by glibenclamide, an ATP-sensitive K^+ channel (K_{ATP}) blocker, and chelerythrine, a selective protein kinase C (PKC) inhibitor, suggesting that activation of both K_{ATP} and PKC is required for the stimulation of COX-2. Additionally, NG-nitro-L-arginine methyl ester, a nitric oxide synthase inhibitor, failed to regulate COX-2 protein expression but inhibited SP-enhanced COX-2 activity and PGE_2 production. In conclusion, we provided the first evidence that SP may produce delayed cardioprotection via K_{ATP}/PKC dependent induction of COX-2 expression and via nitric oxide-induced COX-2 activation.

Keywords Cyclooxygenase-2 · Hydrogen sulfide · Ischemic preconditioning · ATP-sensitive K^+ channel · Protein kinase C · PGE_2

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Introduction

Hydrogen sulfide (H_2S) has recently been recognized as a novel endogenous gasotransmitter [23]. It has been shown that H_2S plays important physiological and pathological roles in different systems including the central nervous system [7, 10, 23, 25], immune system [13], and the cardiovascular system [2, 15]. In heart, H_2S regulates the heart contractility and protects heart from ischemia injury. We recently found that H_2S preconditioning may produce early and delayed cardioprotection against ischemia/reperfusion injury similar to that of ischemic preconditioning (IP) [2, 15, 20]. Moreover, activation of protein kinase C (PKC) and opening of ATP-sensitive potassium (K_{ATP}) channel may serve as triggers to protect the hearts against subsequent ischemic injury. However, so far, the effectors (mediators) of SP-induced delayed cardioprotection remain undefined.

IP is a biphasic phenomenon with an early phase of protection that develops within minutes from the initial ischemic insult and lasts for 2 to 3 h and a late (delayed) phase that becomes apparent 12 to 24 h later and lasts 3 to 4 days. The rapid nature of the early phase suggests that it involves the modification of proteins that are already present. Unlike early IP, late IP requires increased synthesis of new proteins. It is generally accepted that the effectors involved in the late phase of IP may be different depending

on the nature of stimuli, the species of the animals, and so forth [1, 9, 18, 22]. Several proteins have been proposed as possible effectors of protection afforded by IP. These include inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS), and cyclooxygenase-2 (COX-2) [3]. Analysis of COX-2 byproduct levels implies that COX-2 mediates the late phase of cardioprotection via increased production of cytoprotective prostanoids, mainly PGE_2 and PGI_2 [17].

We recently reported that H_2S-induced cardioprotection occurred in two time windows (~1 h and 16–28 h), which is similar to the nature of IP [15]. However, the signal cascades contribute to the H_2S-induced cardioprotection are not well understood, although our previous study showed that PKC and K_{ATP} channel may serve as potential triggers. The present study was therefore undertaken to determine whether COX-2 serves as a mediator and whether it is related to the activation of PKC and K_{ATP} in SP-induced cardioprotection in isolated rat cardiac myocytes by using two structurally unrelated selective COX-2 inhibitors, NS-398 and celebrex. We demonstrated for the first time that H_2S-induced delayed cardioprotection in isolated rat cardiac myocytes is mediated by COX-2 and its downstream product PGE_2. In addition, we found that the induction of COX-2 is downstream of a K_{ATP}/PKC signaling pathway and its activity is dependent on NOS-derived NO release.

Materials and methods

The study protocols were approved by the Institutional Animal Care and Use Committees of National University of Singapore.

Materials

AG-490, 2-deoxyglucose (2-DOG), sodium lactate, and sodium hydrogen sulfide were purchased from Sigma-Aldrich (St. Louis, MO, USA). NS-398, COX-2 activity assay kit and polyclonal anti-COX-2 was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Celebrex was obtained from Pfizer. PGE_2 immunoassay kits were purchased from Amersham (Ann Arbor, MI, USA). AG-490 was dissolved in ethanol at a final concentration less than 0.1% (*v*/*v*). Other chemicals were dissolved in distilled water except chelerythrine, glibenclamide, and NS-398, which were dissolved in DMSO at a final concentration less than 0.1% (*v*/*v*).

Isolating rat cardiac myocytes

Sprague–Dawely rats (210–270 g, male) were anesthetized with 0.6 ml/100 g 7% (*w*/*v*) chloral hydrate by intraperitoneal (i.p.) injection followed by i.p. administration of heparin (1,000 IU) to prevent coagulation during removal of the heart. Hearts were rapidly excised, mounted on a Langendorff apparatus, and retrogradely perfused via the aorta with calcium-free Tyrode's solution (in millimolar): 137 NaCl, 5.4 KCl, 1 $MgCl_2$, 10 HEPES, 10 glucose, and pH 7.4 at 37°C. After 5 min, the perfusate was switched to an enzyme solution containing 1 mg/ml collagenase (type I) and 0.28 mg/ml protease (type XIV) and perfused for another 25–30 min. The ventricular tissue was then finely minced in a Petri dish containing pre-warmed Ca^{2+}-Tyrode's solution and shaken gently to ensure adequate dispersion of dissociated cardiac myocytes. After that, the cardiac myocytes were filtered through a 2.5×10^{-4}-m mesh screen, washed three times in Ca^{2+}-Tyrode's solution, and collected by centrifugation (700 rpm for 2 min). Ca^{2+} concentration in the Tyrode's solution was gradually increased to 1.25 mM in 20 min. More than 80% of the cells were rod-shaped and impermeable to Trypan blue dye. The cells were allowed to stabilize for 30 min at room temperature before experimentation.

Experimental protocol

The experimental protocol was indicated in Fig. 1. To mimic ischemia, simulated ischemia solution [i.e., glucose-free Krebs buffer containing 5 mM sodium lactate, 10 mM 2-deoxy-D-glucose (2-DOG), an inhibitor of glycolysis [11], and 10 mM sodium dithionite ($Na_2S_2O_4$), an oxygen scavenger [14], pH 6.6] was used. The cardiac myocytes were subjected to either IP with simulated ischemia buffer or 100 μM NaHS as a donor of H_2S for 30 min. Cells were then washed three times to ensure removal of drugs and thereafter incubated in normal DMEM at humified CO_2 incubator at 37°C for 20 h. Finally, cells were subjected to severe ischemia for 5 min with Kreb's buffer containing 20 mM 2-DOG, 5 mM sodium lactate, and 20 mM $Na_2S_2O_4$, pH 6.6 followed by washout and replacement with DMEM for reperfusion. To examine whether COX-2 is an effector of SP, two COX-2 inhibitors, NS-398 (10 μM) and celebrex (10 μM), were added 30 min before and during severe ischemia. To study the signaling cascades of SP, cells were treated with chelerythrine (1 μM, an inhibitor of PKC) and glibenclamide (10 μM, a K_{ATP} channels blocker) 10 min before and during SP treatment, respectively. NG-nitro-L-arginine methyl ester (L-NAME, 100 μM, a NOS inhibitor) and AG-490 (Janus kinase, JAK inhibitor 10 μM) was added 30 min before SP and remained throughout the experiment.

Cell viability and morphology assay

Trypan blue exclusion was employed as an index of cell viability. At the end of reperfusion, cells were incubated

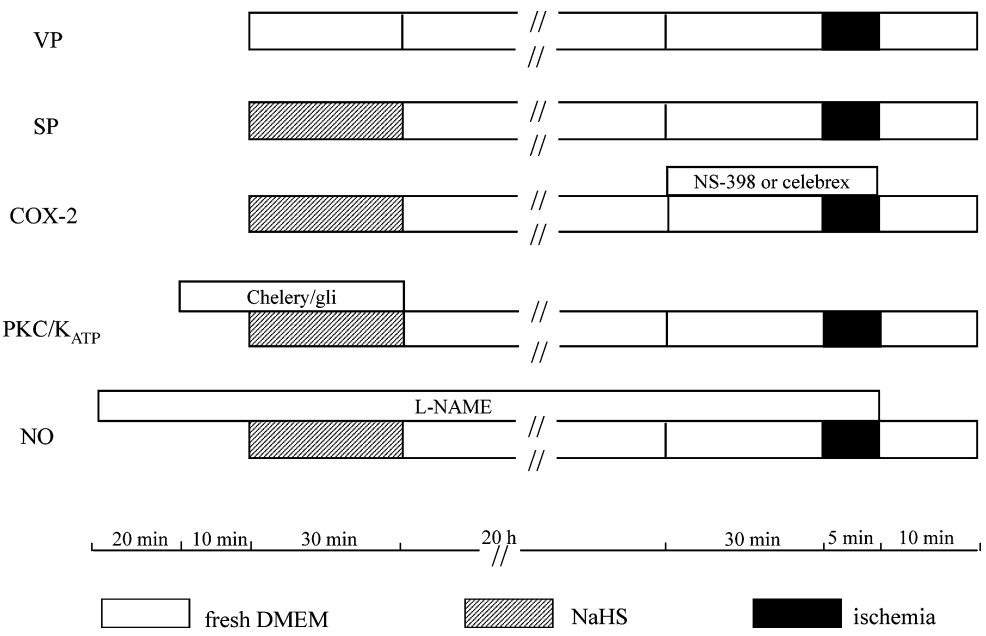

Fig. 1 Experimental protocols. *Open fields*: fresh DMEM solution in isolated cardiac myocytes. *Solid fields*: severe ischemia solution (Kreb's buffer containing 20 mM 2-DOG, 5 mM sodium lactate, and 20 mM $Na_2S_2O_4$, pH 6.6). *Slashed fields*: DMEM containing 100 μM NaHS

with 0.4% (*w*/*v*) Trypan blue dye for 3 min. Only those surviving cells were unstained and called non-blue cells. Cells were counted in a double-blinded manner under an inverted contrast light microscope at 10× magnification. The rod-shaped cells were determined as the length/width ratio more than 3:1. Cell viability was expressed as the percentage of non-blue cells/rod-shaped cells over total cells in each visual field.

Cardiac myocyte contractility measurement

Rod-shaped cardiac myocytes with clear striation were chosen. Contraction amplitude was recorded online through an ×40 objective lens (Nikon) and transmitted to a charge-coupled device (CCD) black and white (B/W) video camera (NL-2332; National Electronic, Canadas). The output from the CCD camera was displayed on a video monitor (National Electronic). Cardiac myocyte edge was measured using a video motion edge detector (VED-105; Crescent Electronics, Canada). Light–dark contrast of the edge of the myocytes provided a marker for measurement of the amplitude of motion. The amplitude of myocyte motion remained unchanged for at least 10 min, indicating the stability of the preparation.

Measurement of PGE_2 levels

Culture medium was collected at the end of severe ischemia insults (Kreb's buffer containing 20 mM 2-DOG, 5 mM sodium lactate, and 20 mM $Na_2S_2O_4$, pH 6.6). Prostaglandin E_2 (PGE_2) levels were measured with a commercial immunoassay kit (Amersham).

COX-2 activity assay

The COX-2 activity was measured according to the manufacturer's instructions. Briefly, cardiac myocytes were collected at the end of severe ischemic insults by centrifugation (2,000×*g* for 10 min at 4°C). Cell pellet was sonicated in cold lysis buffer (0.1 M Tris–HCl, pH 7.8 containing 1 mM EDTA) and centrifuged at 10,000×*g* for 15 min at 4°C. The supernatant was then removed and stored at −80°C for further assay with commercially available COX activity assay kit (Cayman Chemicals).

Western blot analysis

After incubation for 20 h following SP or IP, cells were rinsed with phosphate-buffered saline and lysed with ice cold lysis buffer (150 mM NaCl, 25 mM Tris–HCl pH 7.5, 5 mM EDTA, 1% NP-40, 0.4% deoxycholic acid, and EDTA-free protease inhibitor cocktail tablets; Roche Pharmaceuticals). The samples were rocked for an hour at 4°C followed by centrifuge (13,200×*g*, 4°C) for 10 min. Protein samples were combined with 4× sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis sample buffer [4% (*w*/*v*) SDS, 40% glycerol, 20% (*v*/*v*) β-mercaptoethanol, 0.004% (*w*/*v*) bromphenol blue, 125 mM Tris buffer, pH 6.8] and boiled for 5 min. Proteins (~150 μg) were separated using 12% polyacrylamide/SDS gel and transferred to nitrocellulose membrane. Protein transfer was confirmed by using prestained molecular weight markers (Bio-Rad Laboratories, Hercules, CA, USA). After blocking at room temperature in 10% non-fat dry milk with TBST buffer (10 mM Tris–HCl, 120 mM NaCl, 0.1% Tween-20, pH 7.4) for 1 h, membrane

was incubated with rabbit anti-COX-2 antibody (Cayman) at 4°C overnight and then washed three times with TBST buffer before incubating with 1:10,000 dilutions of HRP-conjugated anti-rabbit IgG at room temperature for 1 h. After washing three times in TBST, membranes were developed using enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA)

Statistical analysis

Experimental data are presented as the mean±SEM. One way analysis of variance was used with a post hoc (Bonferroni) test to determine the difference between groups. The significance level was set at $p<0.05$.

Results

Effect of SP on myocyte viability and morphology in the absence and presence of two selective COX-2 inhibitors

As shown in Fig. 2, preconditioning with NaHS (100 μM) for 30 min significantly increased cell viability and percentage of rod-shaped cells over total number of cells after severe ischemia insults. This is consistent with our previous findings that NaHS at 1–100 μM concentration-dependently produced late phase of cardioprotection [15]. To examine the involvement of COX-2 in these effects, two selective COX-2 inhibitors, NS-398 (10 μM) and celebrex (10 μM), were used. Given 30 min before and during lethal ischemia, both NS-398 and celebrex significantly abolished the above cardioprotective effects. These data suggest that COX-2 may serve as a mediator in SP-induced delayed cardioprotection.

Effect of SP on electrically induced myocyte contractility in the cardiac myocytes in the absence and presence of two selective COX-2 inhibitors

To further examine whether COX-2 also mediates SP-induced cardioprotection on cell function, myocyte twitch amplitude was measured in electrically stimulated cardiac myocytes. In VP group, the myocyte twitch amplitude was slightly decreased by ischemia, but abolished by reperfusion (Fig. 3). These observations suggest that reperfusion-induced calcium overload and acidosis may produce severe myocyte injury. SP significantly reversed the impaired myocyte contraction caused by reperfusion, suggesting that SP may protect heart function during ischemia/reperfusion. Blockade of COX-2 with NS-398 and celebrex also abolished the cardioprotective effect of SP on myocyte contraction, confirming that COX-2 may play an important role in the late phase protection of SP.

Effect of SP on COX-2 expression and PGE_2 production in isolated cardiac myocytes in the absence and presence of a PKC inhibitor

To substantiate the involvement of COX-2 in the cardioprotection of SP, we measured COX-2 protein expression 20 h after preconditioning treatment. As shown in Fig. 4a, similar to IP, SP markedly upregulated COX-2 expression as compared to that of VP group. To identify the upstream signaling pathway involved in the induction of COX-2, a selective PKC inhibitor, chelerythrine (1 μM), was applied 10 min before and during SP (Fig. 1, PKC). As shown in Fig. 4a, blockade of PKC with chelerythrine significantly reversed SP-induced COX-2 upregulation.

Similarly, SP significantly increased the PGE_2 level by 1.6-fold at the end of ischemia treatment (Fig. 4b), which was also attenuated by chelerythrine. These data suggest that SP-induced PGE_2 production is via the activation of PKC.

Effect of SP on COX-2 expression and PGE_2 production in isolated cardiac myocytes in the absence and presence of a K_{ATP} blocker

As opening of K_{ATP} channel is involved in SP-induced cardioprotection [15], we examined whether COX-2 acti-

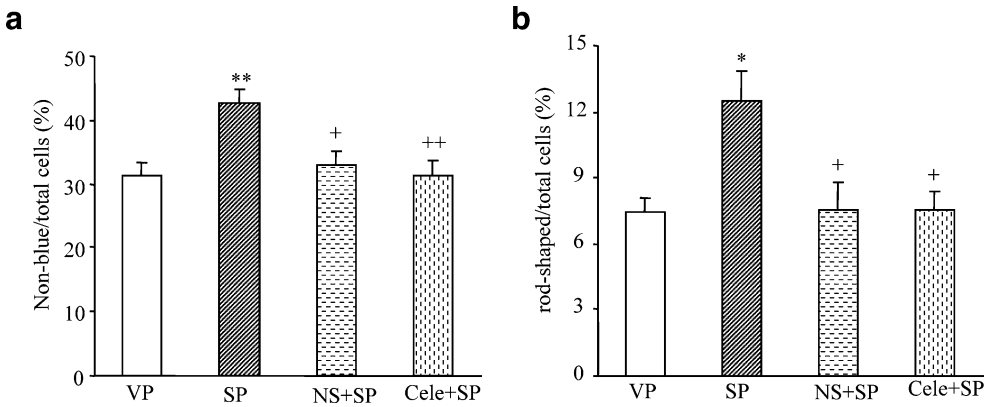

Fig. 2 Blockade of COX-2 with celebrex (10 μM) and NS-398 (10 μM) significantly attenuated the protective effects of SP on cell viability (**a**) and the percentage of rod shaped cells over total cells (**b**) at the end of reperfusion. Mean±SEM; $N=8$. $^{*}P<0.05$, $^{**}P<0.01$ vs VP; $^{+}P<0.05$, $^{++}P<0.01$ vs SP

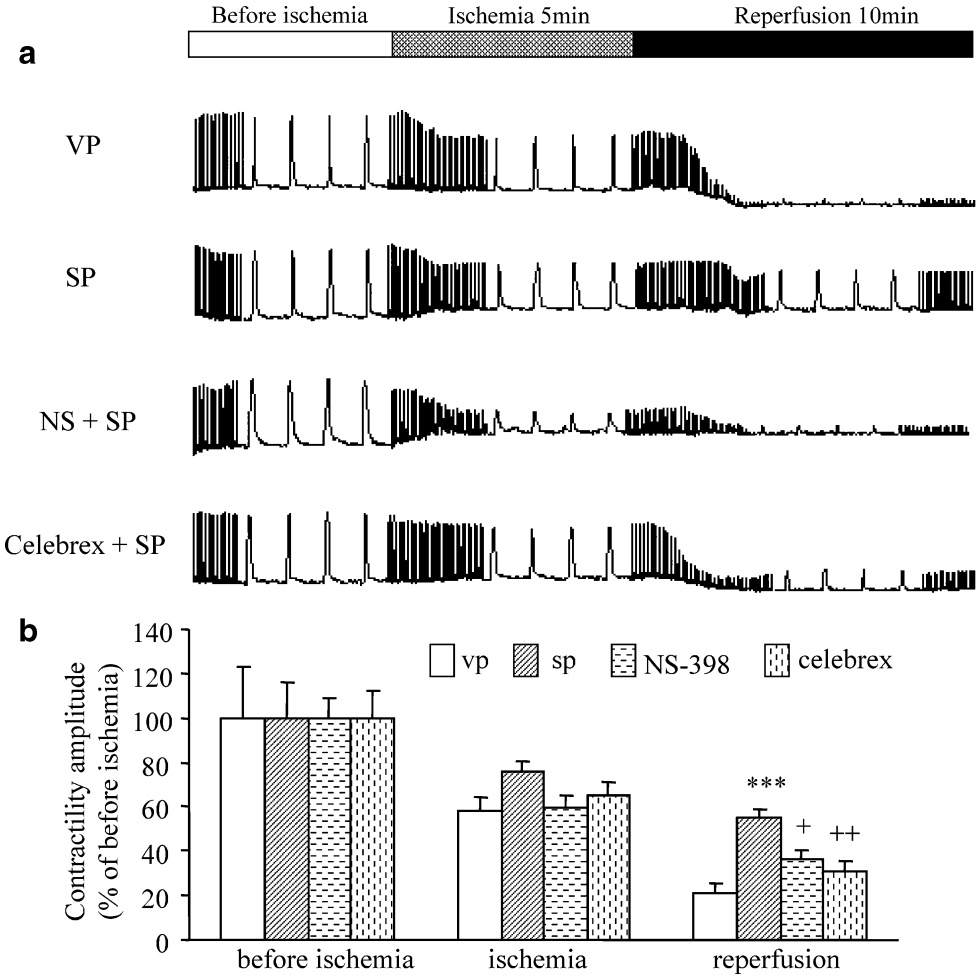

Fig. 3 COX-2 mediates the cardioprotective effect of SP on myocyte contractile function. **a** Representative tracings of electrically induced myocyte contraction in the VP, SP, NS-398 (NS) +SP and celebrex + SP groups. **b** Group results showing that both NS-398 and celebrex reversed the cardioprotective effects of SP on myocyte contraction during reperfusion. Mean ±SEM; N=6. ***P<0.001 vs VP; ^{+}P<0.05, ^{++}P<0.01 vs SP

vation is secondary to K_{ATP} channel opening. As shown in Fig. 5a, treatment with glibenclamide (10 μM), a K_{ATP} channel blocker, 10 min before and during SP abrogated SP-induced COX-2 upregulation. The similar results were also observed when PGE_2 production was measured in the cell culture medium (Fig. 5b). These findings suggest that opening of K_{ATP} channel is necessary for the induction of COX-2 afforded by SP.

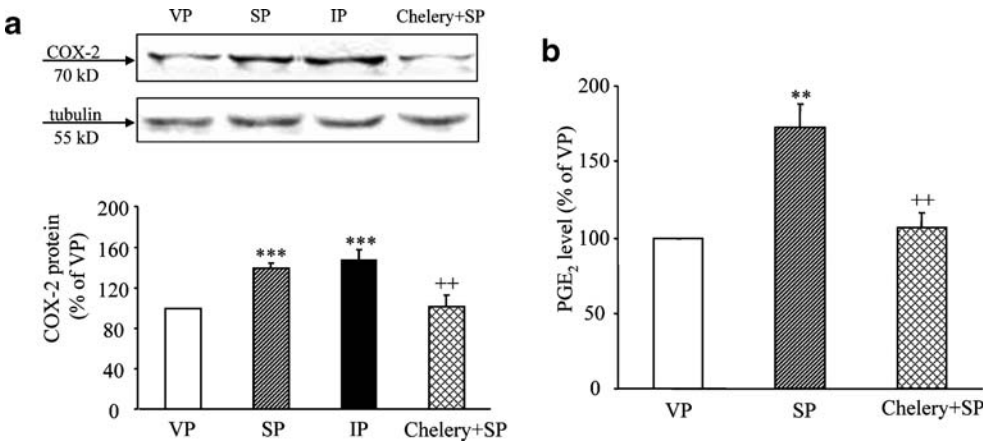

Fig. 4 PKC mediates the effect of SP on COX-2 expression and PGE_2 production in rat ventricular myocytes. Representative Western blots and densitometric analysis of COX-2 protein (**a**) and the level of PGE_2 in the cardiomyocytes (**b**) showing that chelerythrine attenuated SP-induced upregulation of COX-2 protein and PGE_2 formation. In all samples, the densitometric measurements of COX-2 expression and PGE_2 level were expressed as a percentage of the corresponding value measured in the VP group. Mean±SEM; N=4-5. **P<0.01, ***P< 0.001 vs VP; $^{++}$p<0.01 vs SP

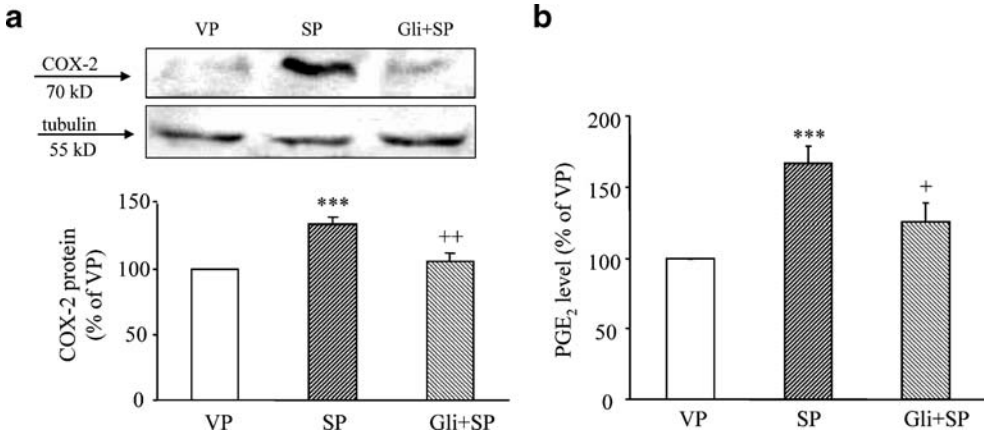

Fig. 5 Effect of SP on COX-2 expression and PGE_2 production in rat ventricular myocytes involves opening of K_{ATP} channels. Representative Western blots and densitometric analysis of COX-2 protein (**a**) and the level of PGE_2 in the cardiomyocytes (**b**) showing that glibenclamide attenuated SP-induced upregulation of COX-2 protein and PGE_2 formation. Mean±SEM; N=5-9. ***P< 0.001 vs VP; ^{+}P<0.05, $^{++}$p< 0.01 vs SP

Effect of SP on COX-2 expression and PGE_2 production in isolated cardiac myocytes in the absence and presence of a NOS inhibitor

NO is another important trigger in the cardioprotection of SP [15]. To study whether NOS contributes to the upregulation of COX-2 and PGE_2, an isoform-nonselective NOS inhibitor L-NAME (100 μM), was administrated 30 min before and throughout the experiment (Fig. 1, NO). As shown in Fig. 6a, L-NAME failed to antagonize the increase of COX-2 expression induced by SP. Interestingly, the elevated PGE_2 production induced by SP was significantly attenuated in the presence of L-NAME (Fig. 6b). We then further observed whether the lowered PGE_2 level induced by L-NAME was caused by the inhibition of COX-2 activity. As shown in Fig. 6c, L-NAME significantly blocked the SP-enhanced COX-2 activity, suggesting that the effect of NO on PGE_2 is dependent on COX-2 activity.

Effect of SP on myocyte viability in the absence and presence of JAK inhibitor

As it has been reported that IP-induced upregulation of COX-2 protein expression is mediated by JAK/STAT signaling cascade [4], we therefore examined the role of JAK/STAT in SP-induced cardioprotection in isolated rat cardiac myocytes. As shown in Fig. 7, treatment with AG-490 (10 μM), a JAK inhibitor, 30 min before SP and throughout the experiment did not attenuate SP-induced protection in cell viability. These data suggest that JAK/STAT signaling cascade may not play an important role in the cardioprotection of SP in the rat cardiac myocytes.

Discussion

COX is the rate-limiting prostaglandin synthase and regulates the synthesis of prostaglandins by catalyzing the

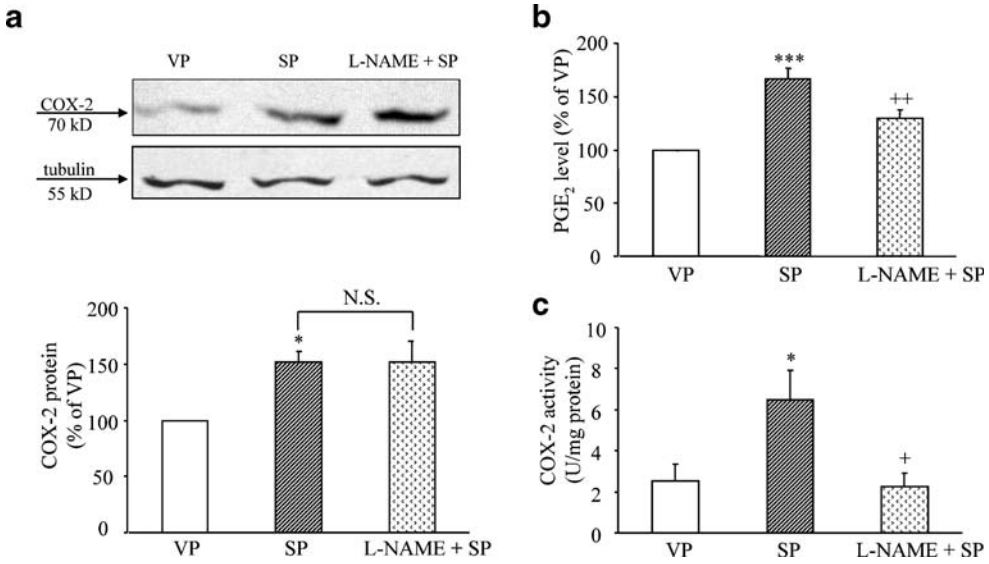

Fig. 6 Effect of SP on COX-2 expression, activity and PGE_2 production in rat ventricular myocytes in the presence and absence of a NOS inhibitor. **a** The densitometric analysis of COX-2 protein shows that L-NAME failed to inhibit SP-induced upregulation of COX-2 expression; **b** L-NAME partially reversed SP-induced increase of PGE_2 production in myocytes. **c** L-NAME abolished SP-elevated COX-2 activity in myocytes. Mean±SEM; N=4–9. *P<0.05, ***P<0.001 vs VP; $^{+}$p<0.05, $^{+}$ $^{+}$p<0.01 vs SP; *NS* indicates non-significance between SP and L-NAME + SP group

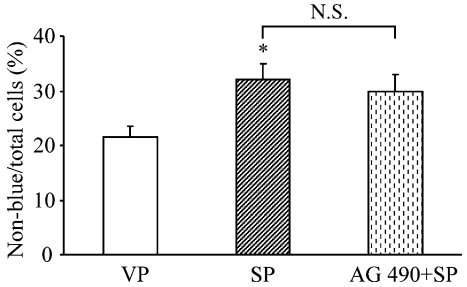

Fig. 7 Blockade of JAK with AG-490 (10 μM) did not block the protective effects of SP on cell viability. Mean±SEM; N=8. *P<0.05 vs VP; *NS* indicates non-significance between SP and AG-490 + SP group

conversion of arachidonic acid to PGH_2, the common precursor of bioactive prostaglandins. Two distinct COX isoforms have been characterized. COX-1 is responsible for constitutive prostaglandin formation, whereas COX-2 is usually induced in response to stress. There are accumulating evidence that COX-2 plays an essential role in mediating the late phase, but not the early phase, of IP-induced cardioprotection [24, 27]. A series of in vivo studies have also well established that COX-2 activity is necessary for the protective effects of various triggers (such as δ-opioid receptor and NO donor) induced delayed preconditioning [17]. In clinical trials, it was also found that arthritis patients with selective COX-2 inhibitor therapy are at higher risks for developing cardiovascular complications. All these studies position COX-2 as an important cardioprotective mediator.

We previously reported that SP protects the isolated heart and cardiac myocytes against ischemia insults in two windows. The first window occurs at about 1 h, while the second window occurs after 16 h [15]. In the present study, we concentrated on observing the role of COX-2 in the late phase of cardioprotection induced by SP. We found that blockade of COX-2 with selective COX-2 inhibitors abolished the cardioprotective effects of SP on cell viability, morphology, and myocyte contractility. More importantly, COX-2 was markedly upregulated after SP treatment. This is similar to the previous findings that the delayed IP could also induce upregulation of COX-2 [24]. Our findings provided the evidence that COX-2 is an essential effector mediating the delayed cardioprotection induced by SP in the rat isolated cardiac myocytes.

PGE_2 is one of the two main cardioprotective products of COX-2 in the heart. We also determined the PGE_2 production in the cardiac myocytes. It was shown that SP significantly increased PGE_2 formation at the end of lethal ischemia, indicating that PGE_2 is likely one of the mediators of COX-2-dependent protection produced by SP. Previous studies suggested that the anti-ischemic action of prostaglandins is mediated by receptors specific for E-type prostaglandins (EP receptors) [12]. Different subtypes of EP receptors, namely EP_1, EP_2, EP_3 and EP_4, were identified. Among these receptors, EP_3 has been reported to play an important role in the cardioprotection of IP [12]. However, no commercial specific EP_3 receptor antagonist is available to date. More experiments are therefore warranted to test the involvement of the subtype(s) of PGE_2 receptor.

The mechanism for the cardioprotection of PGE_2 has been reported to be associated with the inhibition of adenylyl cyclase, decrease of $[cAMP]_i$, inhibition of Ca^{2+} overloading [12], activation of K_{ATP} channels [6], reduction of oxygen consumption, and attenuation of neutrophil infiltration [21]. PGE_2 therefore reduces myocardial infarct size and exerts significant cardioprotection during ischemia/reperfusion injury. In in vivo studies, it has been previously reported that exogenous administration of NaHS protects hearts from injury [5, 20, 28]. The findings in the present study provide direct evidence and reveal the mechanism for the cardioprotective effects of H_2S observed in the in vivo studies.

K_{ATP} channels and PKC play obligatory roles in the cardioprotection induced by SP against ischemia/reperfusion insults [2, 15]. Based on the present findings that both glibenclamide and chelerythrine reversed SP-enhanced COX-2 expression and PGE_2 production, it is likely that both opening of K_{ATP} channels and activation of PKCε contribute to the upregulation of COX-2 and the late phase of cardioprotection [3]. This is supported by the recent findings that PKCε activation triggers COX-2 induction in IP-conferred neuroprotection [8]. JAK/STAT is another important pathway to induce COX-2 activity in late phase of IP in mouse [26]. In the present study, we therefore examined the role of JAK/STAT in SP-induced delayed preconditioning. We found that AG-490, a JAK inhibitor, failed to abolish the SP-induced protective effects in cardiac myocytes. Thus, JAK/STAT signaling pathway may not be responsible for SP-induced cardioprotection and COX-2 activity.

In the present study, we also observed whether NO mediates the upregulation of COX-2 protein expression and activity. Of great interest, we found that L-NAME reversed SP-induced augmentation of COX-2 activity and PGE_2 production, but did not inhibit the upregulation of COX-2 expression. These results imply that NOS is essential for SP-induced enzymatic activity, but not for the synthesis of COX-2. This was supported by previous findings that COX-2 is activated by NO derived from NOS in the late phase of preconditioning in conscious rabbits [16, 19, 24].

In conclusion, the present observations expand our understanding of the signaling pathways involved in SP-induced protection against ischemia/reperfusion insults. Our findings identify a crucial role of K_{ATP}/PKC/COX-2 pathway in the induction of cardioprotection via H_2S preconditioning and demonstrate that NOS-derived NO is required for the COX-2 activity.

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