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Original Article

The PI3K/Akt pathway mediates the protection of SO_2 preconditioning against myocardial ischemia/reperfusion injury in rats

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Aim: To explore the mechanisms underlying the protection by SO_2 preconditioning against rat myocardial ischemia/reperfusion (I/R) injury.
Methods: Male Wistar rats underwent 30-min left coronary artery ligation followed by 120-min reperfusion. An SO_2 donor (1 μmol/kg) was intravenously injected 10 min before the ischemia, while LY294002 (0.3 mg/kg) was intravenously injected 30 min before the ischemia. Plasma activities of LDH and CK were measured with an automatic enzyme analyzer. Myocardial infarct size was detected using Evans-TTC method. The activities of caspase-3 and -9 in myocardium were assayed using a commercial kit, and the levels of p-Akt, Akt, PI3K and p-PI3K were examined with Western blotting.
Results: Pretreatment with SO_2 significantly reduced the myocardial infarct size and plasma LDH and CK activities, as well as myocardial caspase-3 and -9 activities in the rats. Furthermore, the pretreatment significantly increased the expression levels of myocardial p-Akt and p-PI3K p85. Administration of the PI3K inhibitor LY294002 blocked all the effects induced by SO_2 pretreatment.
Conclusion: The results suggest that the PI3K/Akt pathway mediates the protective effects of SO_2 preconditioning against myocardial I/R injury in rats.

Keywords: sulfur dioxide; ischemia/reperfusion; heart infarct; preconditioning; LDH; creatine kinase; caspase-3; caspase-9; PI3K/Akt pathway; LY294002

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Introduction

Ischemia preconditioning was first described by Murry in 1986[1]. The author demonstrated that brief episodes of ischemia reperfusion (I/R) could induce resistance to cell injury following lethal ischemia and reduce infarct size. A similar protective effect of preconditioning was observed in other organs and tissues, which led to the induction of an endogenous protective substance to resist lethal ischemia. However, applying ischemia preconditioning in clinical therapy is not practical. Therefore, pharmacological preconditioning, such as with adenosine, nicorandil and isoflurane, has been found to show a protective effect similar to that of ischemia preconditioning on myocardial I/R[2].

SO_2 was considered to be a pollutant gas and toxic in mammals. SO_2 dissociates to its derivatives bisulfite and sulfite (1:3 *M/M*) in neutral fluid and plasma *in vivo*[3]. Recently, SO_2 was found to be generated endogenously[4]. Sulfite has previously been identified as the physiological form of SO_2[5–7]. SO_2 has physiologic effects on the cardiovascular system, including regulation of negative cardiac function and inhibition of L-type calcium channel activity[8]. In addition, the pathophysiologic effects of SO_2 have recently been found to aggravate myocardial I/R injury[9]. Zhang *et al*[9] observed that SO_2 treatment after I/R could provoke radical generation and aggravate I/R injury. A recent study revealed that low doses (1–10 μmol/kg) of SO2 preconditioning could inhibit myocardial injury[10]. However, the mechanism responsible for protection by SO_2 preconditioning against myocardial I/R injury is not fully understood.

The phosphoinositide 3-kinase (PI3K)/Akt pathway has been identified as a key component of the protective mechanism of ischemia preconditioning[11]. PI3Ks play an important role in the control of cell growth, proliferation, survival and migration[12]. The activation of Akt, which is downstream of

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PI3K, may ameliorate I/R injury[13]. The PI3K/Akt pathway is also involved in the preconditioning of hydrogen sulfide (H_2S), another sulfur-containing gas in the methionine metabolic pathway with effects on myocardial I/R similar to those of SO_2[14]. The aim of the present study was to investigate whether the PI3K/Akt pathway is involved in the cardioprotective effect of SO_2 preconditioning.

Materials and methods

The investigation was approved by the Institutional Authority for Laboratory Animal Care of Peking University and complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 85–23, revised in 1996).

Creation of myocardial I/R rat model *in vivo*

Male Wistar rats (250–300 g) were from Vital River (Beijing, China). The rats were anaesthetized using urethane (1 g/kg) via intraperitoneal injection. The surgical procedures were performed as previously described[10]. After pericardiotomy, a 5–0 silk suture was placed under the left coronary artery (LCA), which was occluded by tightening the snare using a lightweight hemostatic clamp. After 30 min of myocardial ischemia, the suture was loosened for 120 min for reperfusion[15, 16]. The sham-operated animals underwent the same surgical procedures except that the suture around the LCA was not fastened. Standard lead II electrocardiography was used for monitoring during I/R. Ischemia was confirmed by a transient decrease in blood pressure and cyanosis on the myocardial surface. Reperfusion was indicated by an epicardial hyperemic response and the rapid disappearance of cyanosis.

Experimental protocol for assessing I/R in rats

To determine the effect of SO_2 preconditioning on myocardial I/R injury and expression levels of p-Akt, Akt, PI3K, and p-PI3K in the rat myocardium, healthy, clean-grade male Wistar rats were randomly divided into a sham group, in which rats underwent the LCA surgical procedures except that the suture around the LCA was not fastened (n=6); an I/R group, in which LCA ligation was performed as described above (n=7); an I/R+SO_2 group (n=7); and a 0.3 mg/kg LY294002 plus 1 μmol/kg SO_2 preconditioning group (I/R+LY294002+SO_2 group, n=7). For rats in the I/R+SO_2 group and I/R+LY294002+SO_2 group, 10 min before ischemia, an SO_2 donor ($NaHSO_3$ and Na_2SO_3, 1:3 MM ratio) was intravenously injected at 1 μmol/kg for 5 min. In the sham group, the rats were intravenously injected with the same volume of normal saline. A total of 0.3 mg/kg LY294002 was given via right external jugular intravenous administration 30 min before ischemia in rats in the I/R+LY294002+SO_2 group.

To determine the effect of SO_2 treatment on expression levels of p-Akt, Akt, PI3K, and p-PI3K in the rat myocardium, rats that did not undergo surgical operation were divided into a control group, in which rats were intravenously injected with the same volume of normal saline for 5 min (n=6); a 1 μmol/kg SO_2 treatment group, in which an SO_2 donor at 1 μmol/kg was intravenously injected for 5 min (SO_2 group, n=7); and a 0.3 mg/kg LY294002 plus 1 μmol/kg SO_2 group (LY294002+SO_2 group, n=7), in which an SO_2 donor at 1 μmol/kg was intravenously injected 20 min after 0.3 mg/kg LY294002 had been given via right external jugular intravenous administration. At the end of the experiment, plasma myocardial enzymes [creatine kinase (CK) and lactate dehydrogenase (LDH)], myocardial infarct size and myocardial caspase-3 and -9 activities were detected. The expression levels of p-Akt, Akt, PI3K, and p-PI3K in the rat myocardium were detected by Western blotting.

Measurement of LDH and CK activities in rats with I/R

After treatment, plasma samples were obtained from carotid aorta using a heparinized syringe and immediately centrifuged. The activities of LDH and CK were assayed using an automatic enzyme analyzer (Hitachi 7080, Hitachi, Japan).

Determination of myocardial infarct size in rats with I/R

At the end of the experiment, the LCA was reoccluded, and the area at risk (AAR) in the heart was delineated by injecting Evans blue dye (1 mL of a 3% solution) via the external jugular vein. The heart was rapidly excised, washed with 0.9% saline and cut into 5 transverse slices of equal thickness (2.0 mm) from the apex to the base. The slices were incubated for 10 min in phosphate-buffered 1% 2,3,5-triphenyltetrazolium chloride (TTC) at 37 °C and then fixed with 10% formalin solution. The AAR was the area that was not stained with Evans blue dye. The AARs not stained by TTC were defined as the area of infarction (AI). The AAR, AI and ventricle size (VS) were assessed by a blinded observer using computer-assisted planimetry (NIH Image 1.57 software). The procedures for determining the AAR and AI were as previously described[17].

Measurement of myocardial caspase-3 and -9 activities in rats with I/R

We used the AAR of the ischemic heart as a sample to determine the activity of caspase-3 and -9. The activities of caspase-3 and -9 were assayed using the caspase activity quantitative detection kit (Genmed Scientifics Inc, Shanghai, China) according to the manufacturer's instructions.

Western blot analysis

We used the AAR of the ischemic heart as a sample for Western blot analysis of Akt and PI3K expression. After homogenization with lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% Nonidet P-40, and 0.5% sodium deoxycholate), the supernatants were boiled and separated using sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The antibodies against Akt and PI3K were purchased from Cell Signaling Technology (Boston, MA, USA). The antibody against GAPDH (0411) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rabbit and anti-mouse HRP-labeled antibodies and the ECL detection reagents were purchased from Santa Cruz Biotechnology. The X-ray

film used for Western blotting was obtained from Kodak (Rochester, NY, USA). The developed signal was visualized using an enhanced chemiluminescence (ECL) detection kit and quantified using AlphaImager (San Leandro, CA, USA)[18].

Statistical analysis

All data were expressed as the mean±SD. Significant differences were evaluated by a one-way ANOVA followed by a *post hoc* test (least significant difference, LSD test). Statistical significance was set at $P<0.05$. All analyses were performed using SPSS 16.0 (Chicago, IL, USA).

Results

SO_2 preconditioning reduced the myocardial infarct size of rats with I/R

We determined the infarct size using the Evans-TTC method to evaluate the direct effect of SO_2 preconditioning on myocardial I/R injury[17]. The area at risk was expressed as a ratio of AAR to VS (AAR/VS), and the area of the infarct size was expressed as a ratio of AI to AAR (AI/AAR). There were no differences in AAR/VS between the I/R, I/R+SO_2, and I/R+LY294002+SO_2 groups. In rats treated with I/R, the percentage of AI/AAR was approximately 43.37%. Compared with the I/R group, the I/R+SO_2 group showed significant reductions in myocardial infarct size by 32.91%. By contrast, pretreatment with LY294002 successfully abolished the effect of 1 μmol/kg SO_2 pretreatment, and the myocardial infarct size was increased by 58.70% ($P<0.01$) (Figure 1A).

SO_2 preconditioning reduced plasma LDH and CK activities in rats with I/R

To evaluate the extent of myocardial injury in rats with I/R, we detected plasma LDH and CK activities. At the end of the experiment, compared with activities in the sham group, the plasma CK and LDH activities in the I/R group were significantly increased — by 105.38% and 549.24%, respectively ($P<0.01$). Pretreatment with SO_2 significantly reduced plasma LDH and CK activities by 32.91% and 27.13%, respectively ($P<0.01$), compared with the I/R group ($P<0.05$). However, the plasma LDH and CK activities in the I/R+LY294002+SO_2 group were higher than those in the I/R+SO_2 group ($P<0.05$) (Figure 1B).

SO_2 preconditioning reduced myocardial caspase-3 and -9 activities

Compared with levels in the sham group, the myocardial caspase-3 and -9 activities in the I/R group were significantly increased—by 103% and 82.69%, respectively ($P<0.01$). Pretreatment with an SO_2 donor significantly reduced the increased myocardial caspase-3 and -9 activities by 44.05% and 38.3%, respectively ($P<0.01$), as compared with the I/R group. However, the myocardial caspase-3 activities in the I/R+LY294002+SO_2 group were higher than those in the I/R+SO_2 group ($P<0.05$) (Figure 2).

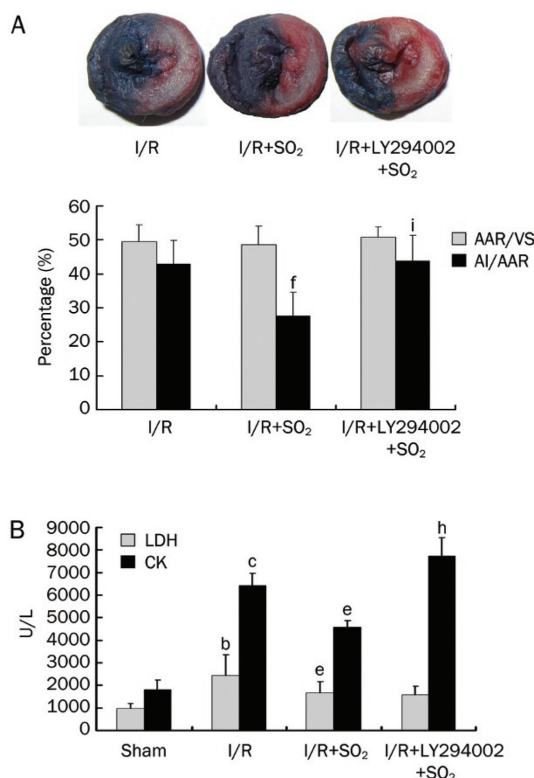

Figure 1. Effect of SO_2 preconditioning on myocardial injury. Effect of SO_2 preconditioning on myocardial infarct size (A), n=21. Effect of SO_2 preconditioning on plasma lactate dehydrogenase (LDH) and creatine kinase (CK) activities (B), n=27. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* the sham group. $^eP<0.05$, $^fP<0.01$ *vs* the I/R group. $^hP<0.05$, $^iP<0.01$ *vs* the I/R+SO_2 group.

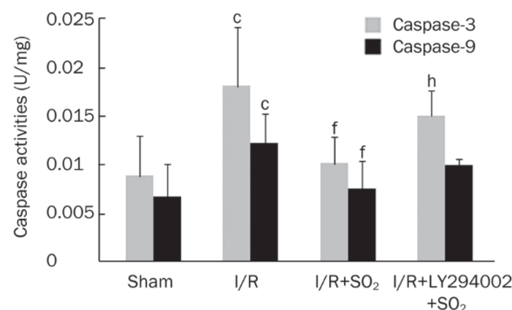

Figure 2. Effect of SO_2 preconditioning on myocardial caspase-3 and -9 activities. Mean±SD. n=27. $^cP<0.01$ *vs* the sham group. $^fP<0.01$ *vs* the I/R group. $^hP<0.05$, $^iP<0.01$ *vs* the I/R+SO_2 group.

SO_2 preconditioning induced myocardial PI3K expression prior to ischemia in rats

To investigate whether PI3K/Akt is involved in cardioprotection via SO_2 preconditioning, we detected PI3K phosphorylation levels using Western blot analysis. Rat hearts were extracted 10 min after SO_2 injection and prior to myocardial ischemia. LY294002, which was originally identified as an

inhibitor of PI3K, was used in the experiment. Compared with the control group, pretreatment with 1 μmol/kg of SO_2 increased myocardial p-PI3K p85 expression by 45.21% (P<0.01) but did not affect the expression levels of p-PI3K p55. However, pretreatment with LY294002 significantly decreased the expression levels of p-PI3K p85 by 18.89% (P<0.05) but did not affect the expression levels of p-PI3K p55 (Figure 3).

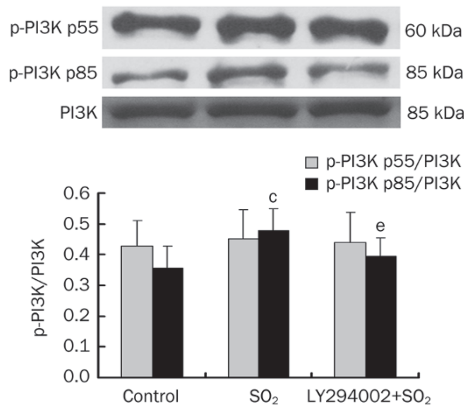

Figure 3. Effect of SO_2 on PI3K expression prior to ischemia. Mean±SD. n=20. ^cP<0.01 *vs* the control group. ^eP<0.05 *vs* the SO_2 group.

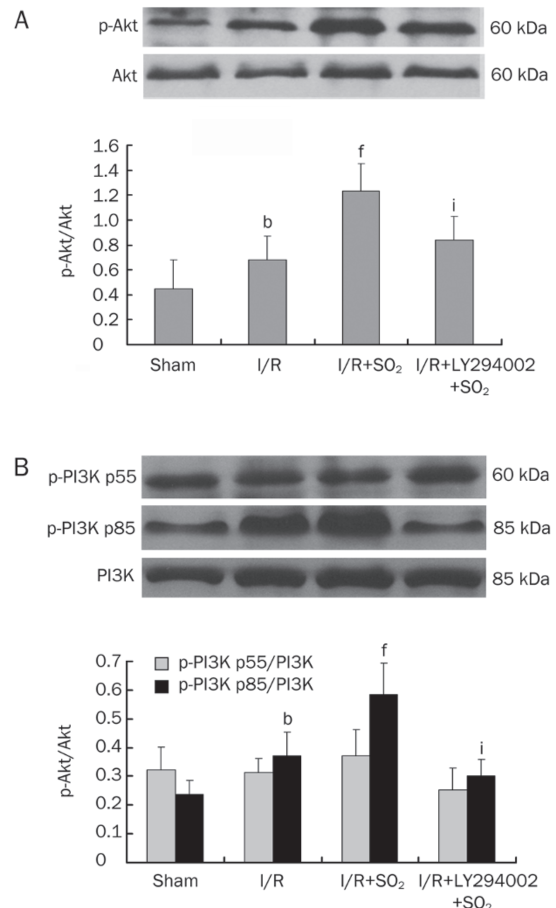

Figure 4. Effect of preconditioning with SO_2 on the expression of Akt (A) and PI3K (B) after ischemia reperfusion. Mean±SD. n=27. ^bP<0.05 *vs* the sham group. ^fP<0.01 *vs* the SO_2 group. ^iP<0.01 *vs* the I/R+SO_2 group.

SO_2 preconditioning further increased myocardial Akt and PI3K expression levels in rats with I/R

Furthermore, we assessed the expression of Akt and PI3K in rats with myocardial I/R. LY294002, a PI3K inhibitor, was also used in this experiment. Compared with the sham group, rats with I/R alone showed an increase in Akt and PI3K p85 phosphorylation levels by 97.64% and 65.36%, respectively (P<0.05 or P<0.01), whereas the expression levels of p-PI3K p55 did not change. In addition, pretreatment with 1 μmol/kg of SO_2 further increased Akt and PI3K p85 phosphorylation levels by 89.29% and 58.07%, respectively (P<0.05 or P<0.01), but the expression levels of p-PI3K p55 did not change. Pretreatment with LY294002 reversed the above effect, and myocardial Akt and PI3K p85 phosphorylation levels were decreased by 33.60% and 52.11% (both P<0.01), respectively, but the expression levels of p-PI3K p55 did not change (Figure 4).

Discussion

The present study revealed that the PI3K/Akt pathway is involved in the cardioprotective effect of SO_2 preconditioning against myocardial I/R injury in rats.

It has been reported that SO_2 can induce negative inotropic effects and relax isolated aortic rings and vascular smooth muscles[19, 20]. Previously, we found that SO_2 treatment after reperfusion aggravated myocardial I/R injury. Therefore, we further explored the cardioprotective effect of SO_2 preconditioning on I/R injury. We recently found that low doses (1–10 μmol/kg) of SO_2 preconditioning had a protective effect on rat myocardial I/R injury[10]. The heart infarct size and increased plasma myocardial enzymes are characteristics of I/R injury in the heart[21]. In the present study, treatment with I/R significantly increased plasma CK and LDH activities in rats with myocardial I/R. In addition, the infarct size was also observed using Evans blue-TTC staining. Pretreatment with SO_2 significantly decreased the infarct size and plasma CK and LDH activities, which agrees with previous reports[10]. Additionally, in the present study, we observed that SO_2 pretreatment significantly decreased caspase-3 and -9 activities in the myocardium of rats with I/R. In a previous investigation, we also showed that preconditioning with SO_2 improved cardiac functionality in rats with myocardial I/R injury[10]. However, the mechanism underlying SO_2 preconditioning in I/R injury is not fully understood.

PI3K/Akt was thought to be involved in I/R and plays an important role in ischemia preconditioning[11, 22, 23]. PI3Ks play a key role in the control of cell growth, proliferation, survival and migration[12]. It was reported that the adenoviral gene transfer of activated phosphatidylinositol 3′-kinase and Akt inhibited the apoptosis of hypoxic cardiomyocytes *in vitro*[24]. Additionally, Akt activation ameliorated cardiac function and prevented injury after transient cardiac ischemia *in vivo*[25].

However, whether the PI3K/Akt pathway was involved in the cardioprotective effect of SO_2 preconditioning was not clear.

We previously found that, after injecting 1 μmol/kg SO_2 donor ($NaHSO_3$ and Na_2SO_3, 1:3 MM ratio), the circulatory SO_2 level increased from 7.07±0.42 μmol/L to 16.3±3.44 μmol/L[10]. In the present study, we observed that treatment with an SO_2 donor significantly increased PI3K p85 phosphorylation expression levels but did not affect the expression levels of p-PI3K p55. An inhibitor of PI3K, LY294002, reversed the above effect of SO_2 and reduced the PI3K p85 phosphorylation levels.

Next, the expression levels of Akt and PI3K were detected after I/R. We found that SO_2 preconditioning markedly increased the levels of Akt and PI3K p85 phosphorylation and protected the rats from I/R myocardial injury. However, pretreatment with LY294002 successfully reversed the above cardioprotective effect of SO_2 along with inhibition of the PI3K/Akt pathway. These data indicated that the PI3K/Akt pathway mediated the cardioprotective effect of SO_2 preconditioning. However, the mechanism underlying the SO_2-mediated activation of the PI3K-Akt signaling pathway is not clear. Previous studies indicated that SO_2 could increase oxidative stress in myocardial tissues[26], and the over-production of oxidants upregulated the PI3K-Akt signaling pathway[27, 28]. Therefore, SO_2 may have activated the PI3K-Akt signaling pathway by stimulating the oxidative response. However, further work is needed to determine the exact mechanisms through which SO_2 upregulates the PI3K/Akt pathway in rats.

In conclusion, SO_2 preconditioning had a cardioprotective effect on rats with myocardial I/R injury. The PI3K/Akt pathway was likely involved in the process of SO_2 preconditioning. Further studies are needed to determine the exact mechanisms through which SO_2 preconditioning protects against I/R myocardial injury in rats.

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Author contribution

Jun-bao DU, Hong-fang JIN, and Xin-bao WANG designed the research; Man-man ZHAO, Jin-yan YANG, and Xin-bao WANG performed the research and contributed new analytical reagents and tools; Man-man ZHAO, Hong-fang JIN, Jin-yan YANG, and Xin-bao WANG collected and analyzed data; Xin-bao WANG and Chao-shu TANG interpreted the data; Man-man ZHAO, Jin-yan YANG, and Xin-bao WANG wrote the paper; Chao-shu TANG and Hong-fang JIN revised the paper.

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