Am J Transl Res 2015;7(10):1883-1895
www.ajtr.org /ISSN:1943-8141/AJTR0014075

*Original Article*

# Syntaxin 1A mediates isoflurane but not hypoxia preconditioning-induced alleviation of hypoxia-reoxygenation injury in rat cardiomyocytes

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Received August 6, 2015; Accepted October 11, 2015; Epub October 15, 2015; Published October 30, 2015

**Abstract:** Preconditioning with ischemia/hypoxia (IPC/HPC) or clinically available volatile anesthetics such as isoflurane (Iso-PC) could activate cardioprotective signaling pathways, thereby reducing myocardial ischemia/reperfusion (IR) injury. However, their molecular targets remain elusive. We herein investigated the roles of syntaxin 1A (Stx-1A) in cardiomyocyte protection induced by HPC and Iso-PC. Both *in vivo* myocardial IR model and *in vitro* cardiomyocyte hypoxia/reoxygenation (HR) model were used to test the effects of IR/HR, IPC/HPC and Iso-PC on Stx-1A protein expression. Stx-1A knockdown and overexpression in cardiomyocytes were achieved by adenovirus infection to define the relationship between Stx-1A levels and IPC/Iso-PC-induced cardioprotection. Cardiac troponin T (cTnT), cell apoptosis rate, and cell viability were introduced as indicators for cardiomyocyte HR injury. Changes of cardioprotective signaling pathways activities including PI3K/AKT/GSK3β, ERK1/2, STAT3 and PKC were also detected using Western blot. Rat cardiomyocyte Stx-1A was upregulated 4 hours after IR or HR. IPC/HPC as well as Iso-PC further increased Stx-1A expression compared with IR/HR. Stx-1A knockdown was accompanied with more cell apoptosis and decreased cell viability while overexpression of Stx-1A seemed cardioprotective. Iso-PC induced decrease in cell apoptosis and increase in cell viability but not HPC-induced cardioprotection was reversed by Stx-1A shRNA transfection. No difference in cell apoptosis or cell viability was found before and after Stx-1A overexpression in each group. Moreover, Stx-1A knockdown were accompanied with increased PI3K/AKT/GSK3β activities irrespective of the treatments. Stx-1A is cardioprotective and a potential target of isoflurane induced cardioprotection. Further studies are needed to test whether stx-1A is regulated by AKT/GSK3β signaling.

**Keywords:** Syntaxin 1A, cardiomyocyte, hypoxia, volatile anesthetics, preconditioning

## Introduction

Perioperative myocardial ischemia/reperfusion (IR) injury which occurs to varying degrees in patients with cardiac surgery and in 1 to 4% of unselected non-cardiac surgical patients, is associated with increased morbidity and mortality [1, 2]. Methods and drugs that reduce perioperative myocardial IR injury (such as ischemia preconditioning (IPC) and adenosine) also improve the prognosis of patients [3-5]. Besides these efforts, the cardioprotective roles of widely used volatile anesthetics such as isoflurane (Iso-PC) and sevoflurane are attracting increased interests [6-8]. However, the molecular mechanisms underlying volatile anesthetics and IPC-induced cardioprotection remain elusive.

Iso-PC and IPC have been found to work probably via similar cardioprotective signaling pathways including PI3K/AKT/GSK3β, ERK1/2, JNK, PKC, $K_{ATP}$ channels to alleviate cardiac IR injury [7, 9]. Moreover, their cardioprotection could be mitigated by similar pathophysiological conditions such as diabetes mellitus [10, 11]. These common traits encourage further search for more cardioprotective candidates that can be

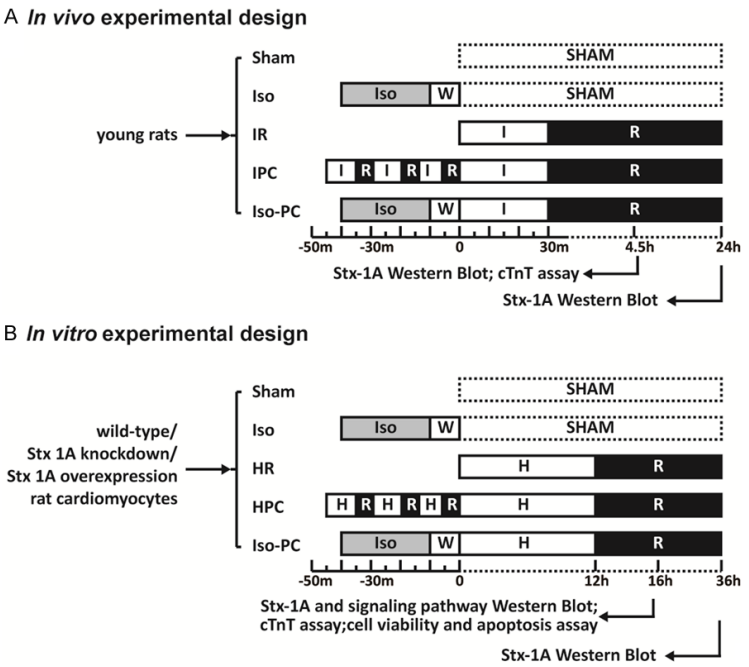



**Figure 1.** Experimental protocol for in vivo IR (A) and in vitro HR (B) model. Sham, Sham group; Iso, Isoflurane treatment without I/R or H/R injury; IR (ischemia/reperfusion) or HR (hypoxia/reoxygenation); IPC (ischemia preconditioning) or HPC (hypoxia preconditioning); Iso-PC (isoflurane preconditioning); W, washout for 10 minutes.

modulated by both volatile anesthetics preconditioning and IPC. Recent studies indicated that syntaxin 1A (Stx-1A), a membrane protein, which forms SNARE (soluble N-ethylmaleimide sensitive factors attachment protein or SNAP receptor) complex together with vesicle-associated membrane protein (VAMP) and SNAP25, might be one choice.

Stx-1A is widely expressed in the brain, endocrine system, the heart as well as other organs [12]. It functions primarily but not exclusively through the SNARE complex-mediated synaptic vesicle fusion [13]. In particular, Stx-1A was found to be able to regulate myocardial IR injury-related signaling pathways such as $K_{ATP}$ channels and calcium channels [12, 14-16]. Moreover, Stx-1A was up-regulated after ischemia [17]. These findings suggested a potential role of Stx-1A in cardiac IR injury. More importantly, volatile anesthetics including halothane and isoflurane were found to interact with Stx-1A to exert hypnosis [18-20].

To test the hypotheses that (1) cardiac Stx-1A levels were regulated by IR, HR, IPC and Iso-PC; (2) cardiomyocyte Stx-1A levels were related with IPC and Iso-PC induced protection against cardiomyocyte HR injury; (3) aforementioned cardioprotective signaling pathways were involved in Stx-1A-mediated cardioprotection, the current study was conducted.

## Materials and methods

### *Animals*

All procedures performed in this study were approved by the Animal Care and Use Committee of the Second Military Medical University (SMMU, Shanghai, China) and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (No. 85-23, revised 1996). Young male (12-16-week-old, 250-300 g) and newborn (P1-3) Sprague-Dawley rats (SIPPR/BK, Shanghai, China) were used in this study. The young rats were kept in a temperature-controlled (22±2°C) environment under a 12-12 hour light-dark cycle (lights on at 8:00 am) with free access to food and water.

### *Experimental design*

The goal of this study was to test the role of Stx-1A in cardioprotection produced by IPC and Iso-PC. As shown in **Figure 1**, both *in vivo* myocardial IR model and *in vitro* cardiomyocyte HR model were used in our study. Rats or neonatal ventricle cells were divided into five groups in each model: Sham (control); Iso (isoflurane exposure only); IR (ischemia/reperfusion) or HR (hypoxia/reoxygenation); IPC (ischemia pre-conditioning) or HPC (hypoxia pre-conditioning) and Iso-PC (isoflurane pre-conditioning).

To test hypothesis 1, Stx-1A protein levels were detected by Western blot *in vivo* and *in vitro*. To test hypothesis 2, Stx-1A knockdown and overexpression in cardiomyocytes were achieved by adenoviral infection. In the experiments designed for hypothesis 3, the activities of PI3K/AKT/GSK3β, ERK1/2, STAT3 and PKC signaling were detected in vitro using Western blot.

*Rat in vivo myocardial IR model*

Rats were anesthetized with intraperitoneal pentobarbital sodium (40 mg/kg) and ventilated via endotracheal intubation on a rodent respirator. A thoracotomy was performed at the fourth intercostal space, and a reversible coronary artery snare was placed around the left anterior descending coronary artery. Myocardial IR was performed by tightening the snare for 30 minutes and then loosening it for 4 hours or 24 hours. IPC was induced by 3 episodes of 10-minute ischemia followed by 5-minute reperfusion before the 30-minute ischemia [21], while isoflurane preconditioning was fulfilled by an exposure to 1.5% isoflurane (approximately 1 MAC for rats) [22] for 30 minutes before ischemia. The concentration of isoflurane was monitored by Cardiocap 5 (Datex-Ohmeda, Madison, WI, USA) and isoflurane was driven by 100% oxygen.

*Cardiomyocyte in vitro HR model*

Cardiomyocyte HR was performed as previously described [23] (**Figure 1**). In brief, HPC was induced by 3 episodes of 10-minute hypoxia followed by 5-minute reoxygenation before the 12-hour hypoxia, while isoflurane preconditioning was performed by an exposure to 1.5% isoflurane for 30 minutes before hypoxia. Cells assigned to Iso group only received isoflurane exposure and suffered from no HR.

*Determination of myocardial injury by identifying cardiac troponin T (cTnT) release*

The cTnT levels were detected using Enzyme-linked immunosorbent assay (ELISA) for *in vivo* blood samples and *in vitro* cell culture medium (**Figure 1**). Briefly, approximately 1 mL of blood was drawn from rat left ventricle, and transferred to a 1.5 mL tube. After clotting, the blood was centrifuged at 3000 RPM for 5 minutes. For primary cardiomyocytes, the culture medium was collected at the same time point. All sera and culture samples were frozen and stored at -80°C until determination. The quantitation was performed by the department of clinical laboratory, Changhai hospital affiliated to SMMU.

*Cell viability assay*

Cardiomyocyte viability was detected using MTT assay. In short, cells were plated in 96-well plates at a density of $1\times10^4$ cells per well. After reperfusion, 50 μL of MTT (5 mg/mL in PBS; Sigma, St Louis, MO, USA) was added to each well and incubated for 4 hours. Then the medium was removed carefully, and 200 μL of DMSO was added to each well. The plates were shaken for 10 minutes, and delivered to a micro plate reader for absorbance recording at 570 nm. An increase in absorbance at a wavelength of 570 nm represents a better cell viability.

*Detection of cell apoptosis*

Cell morphological changes were observed by flow cytometry. Cells were stained with Annexin-V and propidium iodide (PI) to quantify apoptosis using a commercial Annexin-V apoptosis detection kit APC (eBioscience, San Diego, CA, USA). Prepared cells were washed twice with ice-cold PBS and re-suspended in 200 μL of binding buffer. A total of 5 μL of APC and 5 μL of PI were then added to the cell suspension, which were then analyzed with a FACSCalibur flow cytometer.

*Gene silence and overexpression by adenovirus infection*

The recombinant adenovirus vector for silencing of Stx-1A expression (Stx-1A-siRNA), adenovirus vector for Stx-1A overexpression and negative adenovirus vector were purchased from Shanghai GeneChem Company (Shanghai, China). The target sequence of siRNA against rat Stx-1A was 5'-UCUUCUUAAUGUCCGACAUGAGCUC-3', as described by Chen *et al* [24]. The recombinant adenovirus was added into the culture medium at $10^6$ PFU/ml and co-incubated with cardiomyocytes 36 hours ahead of hypoxia protocol. The efficiency of gene knockdown and overexpression were assessed by Western blot.

*Western blot*

The ventricles of adult rats and neonatal cardiomyocytes were lysed in ice-cold RIPA lysis buffer with 1% protease and phosphatase cocktail. Lysates were collected and protein concentration was determined by the bicinchoninic acid (BCA) protein assay kit (Beyotime, Jiangsu, China). Equal amounts of protein were separated in 10% SDS-polyacrylamide gel and subsequently transferred to a nitrocellulose membrane. After blocking, membranes were incubated overnight with anti-Stx1A (1:1000;

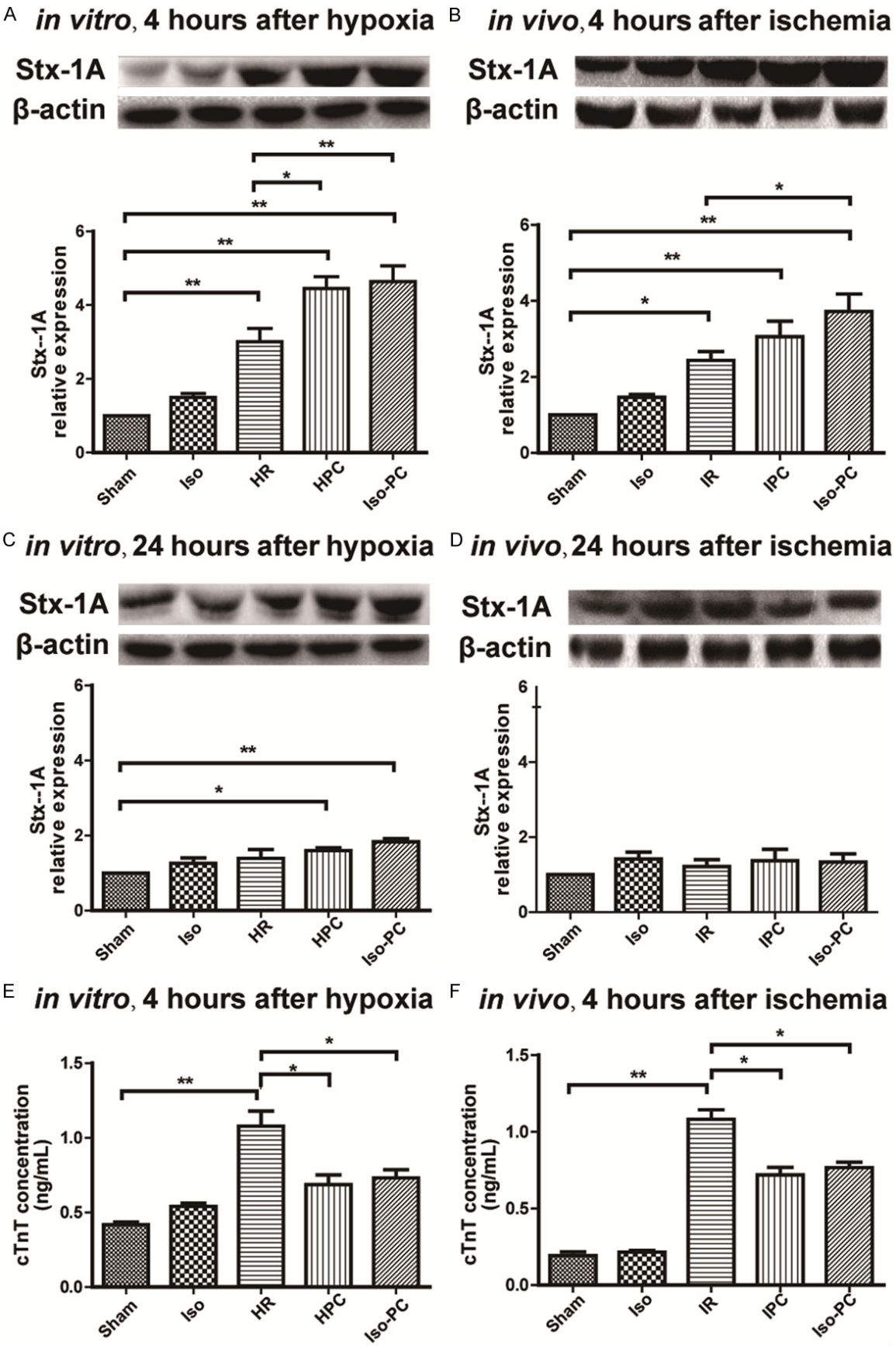

A in vitro, 4 hours after hypoxia
Stx-1A
β-actin
Stx--1A relative expression
Sham
Iso
HR
HPC
Iso-PC
B in vivo, 4 hours after ischemia
Stx-1A
β-actin
Stx--1A relative expression
Sham
Iso
IR
IPC
Iso-PC
C in vitro, 24 hours after hypoxia
Stx-1A
β-actin
Stx--1A relative expression
Sham
Iso
HR
HPC
Iso-PC
D in vivo, 24 hours after ischemia
Stx-1A
β-actin
Stx--1A relative expression
Sham
Iso
IR
IPC
Iso-PC
E in vitro, 4 hours after hypoxia
cTnT concentration (ng/mL)
Sham
Iso
HR
HPC
Iso-PC
F in vivo, 4 hours after ischemia
cTnT concentration (ng/mL)
Sham
Iso
IR
IPC
Iso-PC

**Figure 2.** Stx-1A expression and cTnT release after HR and IR. A. Stx-1A expression in primary cardiomyocytes 4 hours after hypoxia. B. Stx-1A expression in hearts 4 hours after ischemia. C. Stx-1A expression in primary cardiomyocytes 24 hours after hypoxia. D. Stx-1A expression in hearts 24 hours after ischemia. E. cTnT release 4 hours after hypoxia. F. cTnT release 4 hours after ischemia. *, $P<0.05$ and **, $P<0.01$.

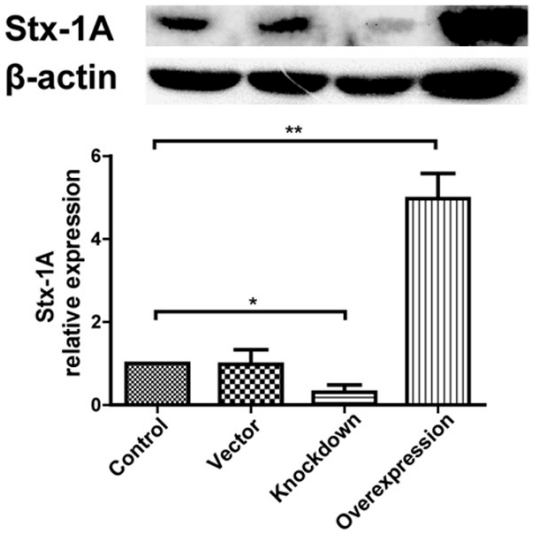



**Figure 3.** Stx-1A expression after adenovirus transfection of plasmid vector, siRNA or rat Stx-1A. *, P<0.05 and **, P<0.01.

SYSY, Geottingen, Germany), anti-ERK 1/2 (1:1000), anti-pERK1/2$^{Thr202/Tyr204}$ (1:1000), anti-AKT (1:1000), anti-pAKT$^{Ser473}$ (1:1000), anti-GSK3β (1:1000), anti-pGSK3β$^{Ser9}$ (1:1000), anti-STAT3 (1:1000; Epitomics, Burlingame, CA, US), anti-pSTAT3$^{Tyr705}$ (1:1000; Epitomics, Burlingame, CA, US) or anti-PKC (1:1000). All antibodies were from Cell Signaling Technology (Danvers, MA, USA) unless specified. Then the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody. The blots were visualized by an enhanced chemiluminescence reaction (ECL) system and photographed by ChemiDoc™ XRS+ System (Bio-Rad, Hercules, CA, USA). β-actin (1:5000; Bioworlde, Jiangsu, China) was used as an internal control. Band densitometry analysis was performed using QuantityOne software (Bio-Rad).

*Statistical analysis*

Statistics were calculated using Graphpad Prism 6.0 (San Diego, CA, USA). Data were expressed as mean±SD (standard deviation) from at least three experiments. Statistical values were analyzed using one-way ANOVA and variations of different groups were compared with the Tukey's *post hoc* test. A *P*-value of smaller than 0.05 (two-tailed) was considered statistically significant.

## Results

*Stx-1A was up-regulated by IR, HR and isoflurane*

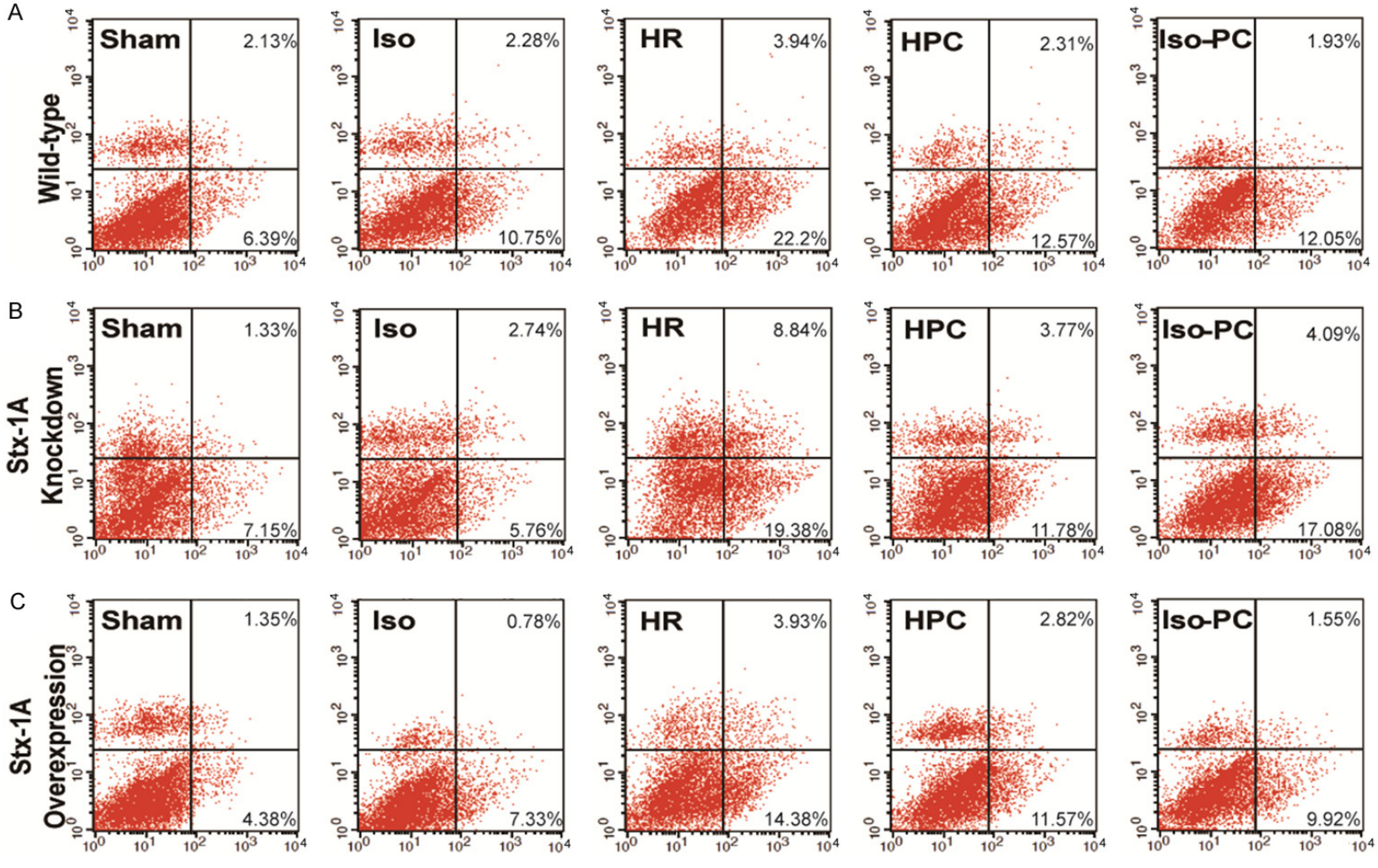
To explore whether there was a change of Stx-1A levels during IPC/HPC and Iso-PC induced cardioprotection, we firstly induced HR injury on neonatal ventricular myocytes. After 4 hours of reoxygenation following hypoxia, a significant increase of Stx-1A protein was observed. Meanwhile, Stx-1A levels were further increased by HPC and Iso-PC in comparison with HR. Isoflurane alone could also increase Stx-1A expression (**Figure 2A**). The enhanced Stx-1A expression by IR, IPC, Iso and Iso-PC were similarly detected *in vivo* (**Figure 2B**). After 24 hours of reperfusion or reoxygenation, Stx-1A level returned to normal (**Figure 2C** and **2D**).

In the cardiomyocytes, HPC and Iso-PC significantly reduced the cTnT levels compared with HR. Similar results of cTnT concentrations were also observed *in vivo*. Taken together, these results suggested a potential role of Stx-1A in cardiomyocyte protection (**Figure 2E** and **2F**).

*Knockdown of Stx-1A exaggerates HR injury and overexpression of Stx-1A was cardioprotective*

Stx-1A knockdown and overexpression was performed in rat neonatal ventricular myocytes 36 hours ahead of hypoxia. As shown in **Figure 3**, our adenovirus transfection resulted in successful inhibition (about 70%) or enhancement (about 5 fold) of Stx-1A respectively.

Stx-1A knockdown or overexpression caused no difference in cell apoptosis (**Figure 4**) or viability (**Figure 5**). When these cells underwent H/R, a trend towards, although not significantly, higher cell apoptosis rate and lower cell viability were seen in cells subject to Stx-1A knockdown while lower apoptosis rates and higher cell viability were detected in cells with Stx-1A overexpression (**Figures 4** and **5**).


A
Wild-type
Sham
2.13%
6.39%
Iso
2.28%
10.75%
HR
3.94%
22.2%
HPC
2.31%
12.57%
Iso-PC
1.93%
12.05%
B
Stx-1A Knockdown
Sham
1.33%
7.15%
Iso
2.74%
5.76%
HR
8.84%
19.38%
HPC
3.77%
11.78%
Iso-PC
4.09%
17.08%
C
Stx-1A Overexpression
Sham
1.35%
4.38%
Iso
0.78%
7.33%
HR
3.93%
14.38%
HPC
2.82%
11.57%
Iso-PC
1.55%
9.92%

D

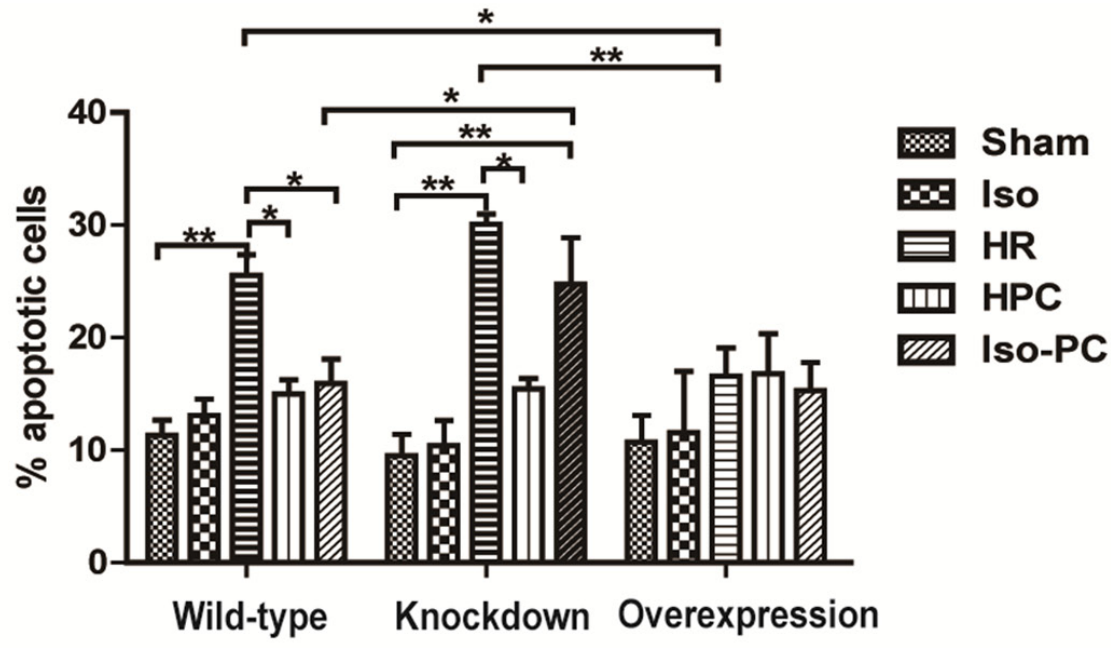



**Figure 4.** Cell apoptosis in wild-type cardiomyocytes and cardiomyocytes subjected to Stx-1A knockdown and overexpression. A-C. Representative results of flow cytometry for wild-type cardiomyocytes and cardiomyocytes subjected to Stx-1A knockdown and overexpression. D. Histograms showing apoptosis rates in three kinds of cells. *, $P<0.05$ and **, $P<0.01$.

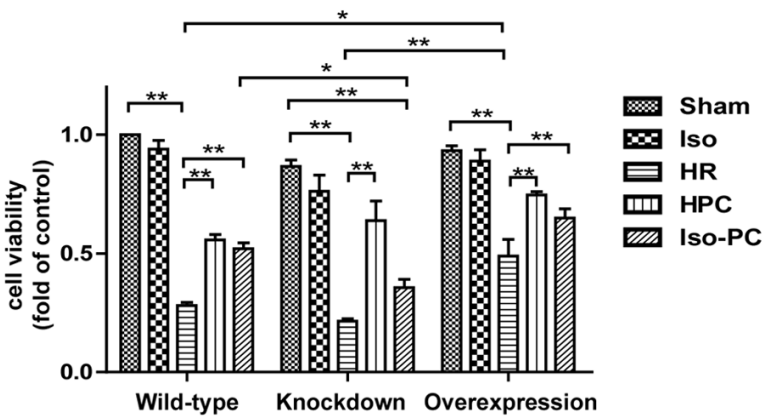



**Figure 5.** Cell viability assay results in wild-type cardiomyocytes and cardiomyocytes subjected to Stx-1A knockdown and overexpression. *, P<0.05 and **, *P*<0.01.

*Stx-1A mediates Iso-PC induced but not HPC induced cardioprotection*

We further tested the role of Stx-1A in cardioprotection produced by HPC and Iso-PC. As shown in **Figures 4** and **5**, Stx-1A knockdown or overexpression did not affect HPC-induced cell survival and higher cell viability. However, the protective effect of Iso-PC on cell survival after HR was nearly completely blocked by Stx-1A knockdown (**Figure 4**). Meanwhile, there was a significant decrease in cell viability when Stx-1A knockdown was conducted before Iso-PC (**Figure 5**). No significant changes in cell apoptosis were detected in cells subjected to Iso-PC and HR when Stx-1A levels were artificially increased (**Figure 4**). These findings suggested that Stx-1A was essential for Iso-PC but not HPC-induced cardiomyocyte protection.

*AKT/GSK3β activity was up-regulated by Stx-1A knockdown*

We further detected the activity changes of pre-mentioned cardioprotection signaling pathways using Western blot. As shown in **Figure 6**, HPC up-regulated ERK1/2, STAT3, PKC, AKT and GSK3β activities in all types of cardiomyocytes, but only AKT and GSK3β activities showed Stx-1A level-dependent changes. Stx-1A knockdown was accompanied with significant increases in pAKT (**Figure 6A** and **6B**) and pGSK3β relative levels (**Figure 6A** and **6C**) in all three groups. No significant difference were found between wild-type and Stx-1A-overexpressed cells in pAKT or pGSK3β levels.

We further tested the influence of Stx-1A levels on Iso-PC induced cardioprotection signaling pathways. Similar to HPC, Iso and Iso-PC caused significant increases in ERK1/2, STAT3, PKC, AKT and GSK3β activities in all types of cardiomyocytes and only AKT and GSK3β activities was regulated by Stx-1A knockdown. Cells with Stx-1A overexpression caused no significant changes in pAKT or pGSK3β levels compared with wild-type cardiomyocytes (**Figure 6D-F**). Together with results shown in **Figures 4** and **5**, a role of AKT/GSK3β signaling in Iso-PC induced cardioprotection mediated by Stx-1A might be found.

## Discussion

In the present study, we found that 1) Rat cardiomyocyte Stx-1A level was up-regulated by ischemia *in vivo* and hypoxia *in vitro*, and HPC/IPC as well as Iso-PC could further increase Stx-1A expression. 2) Artificial regulation of Stx-1A levels had an influence on HR-induced cardiomyocyte apoptosis and cell inviability as well as the cardioprotective ability of Iso-PC but not HPC. 3) Stx-1A knockdown reversed Iso-PC induced cardioprotection and increased AKT/GSK3β activities. Taken together, these findings suggest that Stx-1A was cardioprotective and isoflurane preconditioning might activate AKT/GSK3β signaling to increase Stx-1A expression and exert cardioprotection.

During stress or injury such as ischemia, Stx-1A expression was rapidly increased [17, 25]. Increased Stx-1A might participate in cell salvage or repair through mediating neurotransmitter release and plasma membrane recycling thereby exerting protection [26, 27]. Previous studies have shown that Stx-1A is present in rat cardiomyocytes and regulates $K_{ATP}$ channels during stress [16, 28]. Our current study found that cardiomyocytes Stx-1A level was increased shortly after cardiac IR and HR and returned to baseline thereafter (**Figure 2**). Furthermore, Stx-1A overexpression partially reversed cell injury caused by hypoxia (**Figures 4** and **5**). These results identified a role of Stx-1A in car-

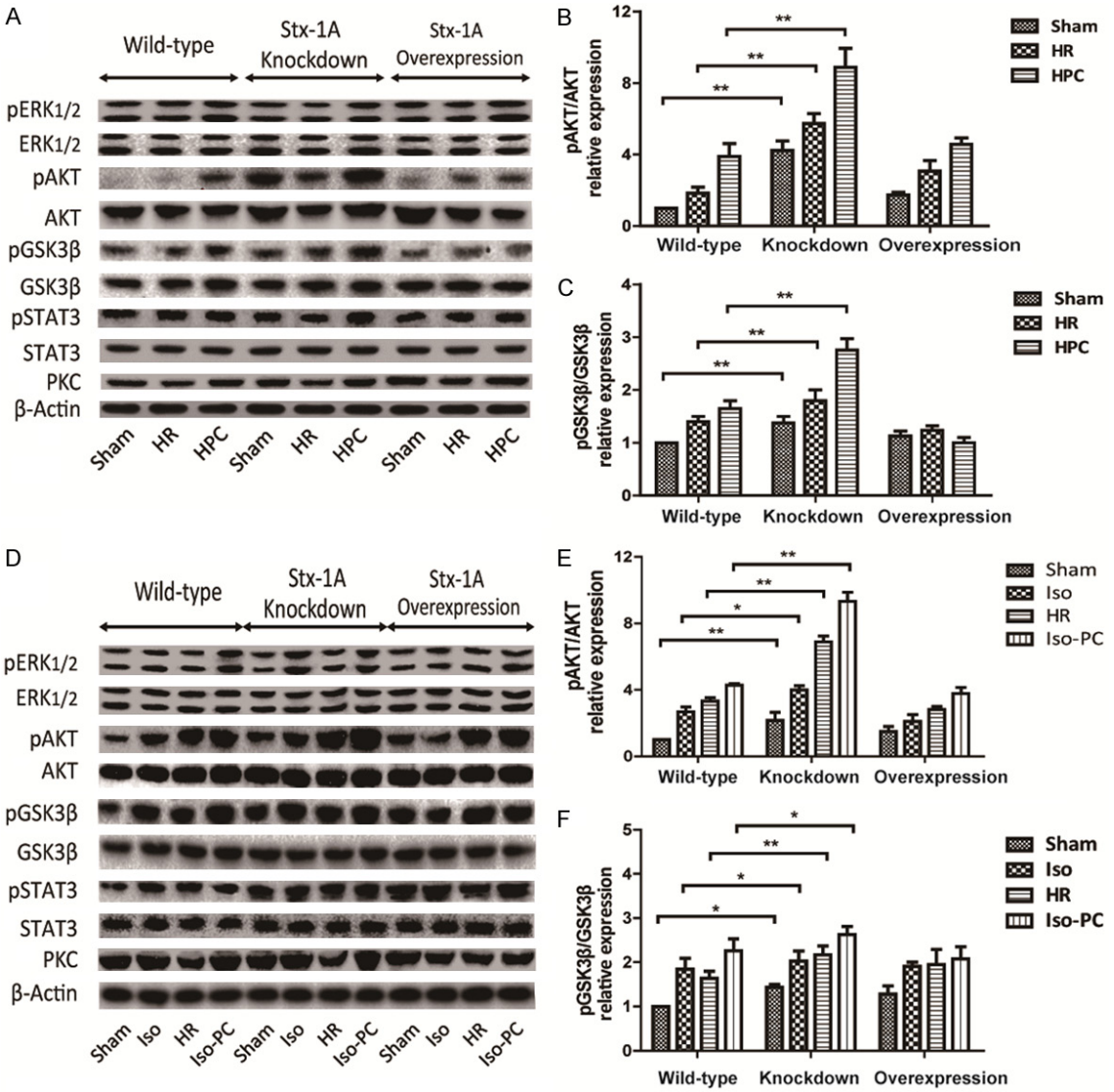



**Figure 6.** Cardioprotective signaling pathway activities. A. Representative strips of ERK1/2, AKT/GSK3β, JNK/STAT3 and PKC during HPC. β-actin was used as internal control. B. Histograms showing relative AKT activities under different circumstances. C. Histograms showing relative GSK3β activities under different circumstances. D. Representative strips of ERK1/2, AKT/GSK3β, JNK/STAT3 and PKC during Iso-PC. β-actin was used as internal control. E. Histograms showing relative AKT activities under different circumstances. F. Histograms showing relative GSK3β activities under different circumstances. *, $P<0.05$ and **, $P<0.01$.

dioprotection. Consistent with our findings, previous researches had found a role of Stx-1A in maintaining cell viability [29, 30]. Peng *et al* found that botulinum neurotoxins, which cleaved and incapacitate Stx-1A and SNAP25 could cause neuronal death [29]. They also found that only a small percentage of endogenous Stx-1A was needed for supporting neuron survival.

The common signaling pathways utilized by both HPC and Iso-PC encouraged us to try to find a common molecule. As Stx-1A was an important target of volatile anesthetics [18-20, 31] and could interact with cardioprotective pathways such as $K_{ATP}$ channels [12, 14-16], we proposed that Stx-1A might be the potential target and tested the relationship between Stx-1A levels and cardioprotection induced by HPC and Iso-PC. Interestingly, when Stx-1A was knocked down by siRNA, the protective effect of Iso-PC but not HPC was blocked. Besides, no difference in Iso-PC and HPC induced cardioprotection was found when Stx-1A was artifi-

cially up-regulated (**Figures 4** and **5**). These data demonstrated that Iso-PC but not HPC target Stx-1A to exert cardioprotection and implied that Iso-PC and HPC might utilize similar pathways to induce different candidate proteins expression. Another possible explanation might by the times of preconditioning cycles. Previously studies have demonstrated that three cycles of IPC would produce a greater protection against myocardial injury than one cycle of IPC and repetitive IPC may activate more pathways than a single IPC stimulus [32, 33]. In our present study, we performed one-cycle Iso-PC and three-cycle HPC. Three cycles of HPC may elicited greater protective effect and activate more pathways comparing with one cycle of Iso-PC.

When exploring the signaling pathways participating in Iso-PC cardioprotection mediated by Stx-1A, we found AKT/GSK3β signaling was elevated by Iso-PC, HPC and Stx-1A knockdown (**Figures 2** and **6**) while no change was found when Stx-1A was overexpressed (**Figure 6**). Considering that enhanced phosphorylation of AKT and GSK3β was cardioprotective, these data suggested Stx-1A a downstreaming but not an upstreaming molecule of AKT/GSK3β phosphorylation and the activation of AKT/GSK3β pathway could be attributed to the feedback regulation of Stx-1A knockdown. This was identical with previous study by Cheng et al [34] in which they found that insulin induced AKT activation could robustly enhance Stx-1A expression in pancreatic β cells. These data also suggested a cardioprotective role of Stx-1A.

Earlier studies have documented that various pathological conditions may render the myocardium more susceptible to IR injury, such as diabetes (hyperglycemia), obesity [35] and hyperlipidemia [36]. And GSK-3β over-expression has been shown to play critical roles in diabetes-induced myocardial oxidative damage and remodeling. Furthermore, patients with diabetes mellitus showed defects in Stx-1A expression [37, 38]. Along with our finding, these results suggested a potential role of Stx-1A in ischemia-susceptible myocardium of patients undergoing metabolic disorders.

There were several limitations in our study. Firstly, we did not conduct *in vivo* experiments to confirm the cardioprotective role of Stx-1A. Further studies using Stx-1A knockout mice might be helpful. Secondly, our data suggested that Stx-1A expression in cardiomyocytes was regulated by AKT/GSK3β signaling but we did not use corresponding pharmacological strategies to identify it. Thirdly, the downstreaming pathways of Stx-1A in cardioprotection remain elusive although several potential mechanisms might responsible which included plasma membrane repair, autocrine/paracrine mechanism or $K_{ATP}$ channels [16, 26, 27].

In conclusion, our study found that syntaxin-1A is cardioprotective and a potential target of isoflurane induced cardioprotection. Further studies are needed to test whether syntaxin-1A was regulated by AKT/GSK3β signaling.

## Acknowledgements

This work received financial support from the Program for Outstanding Academic Leader of Shanghai Municipality, the 12th Five-Year Key Project of PLA (No. BWS12J027), National Natural Science Foundation of China (No. 81372103 & No. 81400893).

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