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TRPV1 activation is involved in the cardioprotection of remote limb ischemic postconditioning in ischemia-reperfusion injury rats

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ABSTRACT

Limb remote ischemic postconditioning (RIPostC) has been proved to be a safe and effective measurement of cardioprotection against ischemia-reperfusion injury. But what bridges the remote organ insult and the cardioprotective effect in heart remains to be elucidated. This study aimed to found that whether TRPV1 may mediate the cardioprotective effect from remote organ to heart and the role of CGRP and SP in this process. We found that RIPostC effectively ameliorated cardiac ischemia/reperfusion injury in terms of limiting infarct size, lowering CK and cTnI release and improving cardiac function. In addition, these cardioprotective effects could be significantly abolished by inhibition of either CGRP or SP receptors with corresponding antagonists (CGRP8-37 for CGRP and RP-67580 for SP) injected before reperfusion. Besides, RIPostC resulted in significantly increase in the levels of CGRP and SP in plasma and hearts, as well as the levels and mRNA expression of CGRP and SP in DRG. The increase in CGRP and SP levels in plasma and hearts were markedly inhibited by TRPV1 receptor antagonist capsazepine. These findings indicate that limb remote ischemic postconditioning could attenuate cardiac ischemia/reperfusion injury in rats, and the cardioprotective mechanism is via TRPV1-mediated upregulation of CGRP and SP, which could subsequently act on their corresponding receptors in heart tissue.

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1. Introduction

Limb remote ischemic postconditioning (RIPostC), consisting of repeated occlusion/release cycles in the bilateral femoral arteries immediately after prolonged myocardial ischemia, is a powerful non-drug cardioprotective strategy [1,2]. RIPostC is implemented on arteries lying remotely from heart tolerance of relatively more severe insult than arteries in heart, which has big advantages like noninvasive and high feasibility in nature [3]. In addition, it could be carried out even after ischemia occurred, which is the case in most patients with acute coronary events. Since the concept of postconditioning was first introduced in 2003 [4], initial clinical studies in human have confirmed its cardioprotective effect [5,6]. In addition, the underlying mechanism of RIPostC has been explained partially both in animal models and humans back in 2007 [7]. Following investigations further reported possible cardioprotective mechanisms of RIPostC regarding the involvement of mitochondrial permeability transition pore (mPTP) [8], protein kinase C (PKC) [9], autophagy [10], to name a few, in animal I/R models. But how the ischemia insulting on remote organ act on heart tissue or, put it another way, what mediates the transition from limb insult to cardioprotection is still uncharted. Fortunately, studies on Limb remote ischemic preconditioning (RIPC) have shed light on the possibility of "bridging effects" between remote ischemia and heart through neural pathways and/or endogenic hormones [5,11–13].

Transient receptor potential vanilloid 1 (TRPV1) is a nonselective cation channel that widely expressed in primary sensory nerves [14]. Physical and/or chemical stimuli could activate TRPV1 and subsequently trigger the release of some neuropeptides, calcitonin gene-related peptide (CGRP) and substance P (SP), in particular, from peripheral nerve terminals [15]. Previous studies have witnessed that blockage of TRPV1 in mice would induce a more severe heart injury and significant reduction of heart function, which could be rescued in part by exogenous CGRP and SP supplement [14], and selectively TRPV1 activation [16]. Besides, our recent study has demonstrated that Ischemic postconditioning effectively protected non-diabetic hearts against ischemia/reperfusion injury by CGRP and SP release, which could be abolished by inhibiting TRPV1, CGRP receptor or SP receptor [17]. In addition, the synthesis and secretion of CGRP and SP have been proven to play a

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major role in the afferent process of noxious stimulation [15,18]. So we hypothesis that TRPV1 may mediate the cardioprotective effect from remote organ to heart by receiving the peripheral injury stimulation and upregulating the CGRP and SP levels that target corresponding receptors in heart tissue.

2. Materials and methods

2.1. Experimental animals

All procedures were performed with the approval of our Institutional Council for Animal Research and in accordance 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 1996).

Male Sprague–Dawley (SD) rats weighing 250–300 g were obtained from the Laboratory Animal Center of the Academy of Military Medical Sciences. Animals were housed in specific pathogen free animal facilities (maintained at 20–25 °C, 55% relative humidity, and an automatic 12-h light/dark cycle) and received standard laboratory diet and tap water ad libitum. All animals were allowed to acclimate for 1 week prior to experiments.

2.2. Treatments of SD rats

2.2.1. Ischemia/reperfusion model

Sprague–Dawley rats were subjected to 30 min of myocardial ischemia (ligation of left anterior descending coronary artery) followed by 3 h of reperfusion (release of the ligation) to simulate ischemia/reperfusion (I/R).

2.2.2. Inhibition of CGRP, SP and TRPV1 receptors in I/R rats [19]

CGRP antagonist CGRP8-37 (2 mg/kg, 2 min before reperfusion), SP antagonist RP-67580 (5 mg/kg, 5 min before reperfusion) and TRPV1 antagonist capsazepine (CAPZ, 3 mg/kg, 10 min before reperfusion) were intravenously injected in the adult SD rats in proper sequence to inhibit the corresponding receptors.

2.2.3. RIPostC

After the establishment of I/R model, limb RIPostC was carried out by three cycles of 5 min occlusion and 5 min release of the bilateral femoral artery using clamps immediately after reperfusion begins. The experimental design for limb RIPostC in each group is diagrammatic represented in Fig. 1. RIPostC were implemented at the setting of no process (I/R + RIPostC group), pretreatment with CAPZ (RIPostC + CAPZ group), CGRP antagonist CGRP8-37 (RIPostC + CGRP8-37 group), and SP antagonist RP-67580 (RIPostC + RP-67580 group), while the control group received no RIPostC (I/R group). There were 7 rats for each group (n = 7).

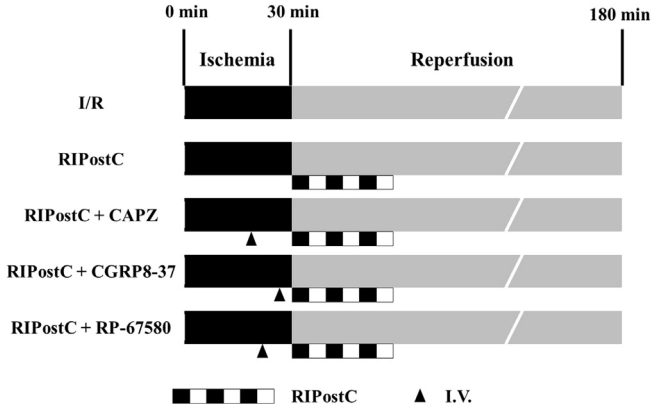

Fig. 1. The diagrammatic representation of experimental design. Ischemia and reperfusion were carried out for 30 min and 180 min respectively, while RIPostC was carried out by three cycles of 5 min occlusion and 5 min; I.V. denotes intravenous injection with CAPZ, CGRP-37 or RP67580 before reperfusion. I/R, ischemia/reperfusion; RIPostC, remote ischemic postconditioning; CAPZ, TRPV1 antagonist capsazepin; CGRP8-37, CGRP antagonist; RP-67580, SP antagonist.

2.3. Measurement of CGRP and SP levels [20]

We introduced radioimmunoassay for quantitative analysis of CGRP and SP level. Living tissue milled with 1 ml normal saline as well as 2 ml plasma were utilized to extract CGRP and SP protein. Commercially available rabbit anti-rat CGRP and SP radioimmunoas-say kits (Phoenix Pharmaceuticals) were employed to measure CGRP and SP release, respectively.

2.4. mRNA expression of CGRP and SP in DRG [20]

We utilized Realtime PCR to quantify mRNA expression. The DRG tissue sample was milled with 1 ml Trizol followed by homogenation in iced bath. Then total RNA was extracted by the Qiangen kit follow the instruction. Then reverse transcribed into cDNA with M-MLV reverse transcriptase with random primer (promega). Real-time PCR was performed with SYBR-green dye and Taq polymerase in the 7Dx realtime system (Applied biosystem). Gene expression was quantified by using the comparative CT method, normalized to GAPDH and expressed as fold induction of control.

2.5. Myocardial injury, infarction size and cardiac function evaluation

2.5.1. Myocardial injury

Myocardial injury was evaluated by creatine kinase (CK) and cardiac troponin I (TnI) release as previously described [17]. Two milliliters blood was extracted through external jugular vein, followed by 2500r/min centrifugation for 10 min at 4 °C. Creatine kinase (CK) and cardiac troponin I (cTnI) levels were determined using the colorimetric assay kit (BioAssay Systems, detection range: 5–300 U/L) and high sensitivity ELISA kit (Life Diagnostics, detection range: 0.156–10 ng/mL), respectively.

2.5.2. Infarction size

To determine the area at risk (AAR), Left Anterior Descending (LAD) was ligated immediately after reperfusion, followed by injecting 2 ml of 3% Evans blue dye via the external jugular vein. Heart tissue was eviscerated 1 min later before left ventricle (LV) been isolated and sliced (2 mm thick) serially along Left Ventricular Long Axis (LVLA). Then the normal heart tissue could be identified as blue, while the ischemia area as pink. Afterwards, the ischemia myocardial tissue was further incubated in a 37 °C 1% solution of buffered (pH 7.4) triphenyltetrazolium chloride (TTC) for 15 min, which could distinguish the area of necrosis (AN) as grey white. The slices were then fixed in 10% methanol before photographing. Image-Pro Plus 6.0 was employed to determine the areas of LV, AAR and AN. The percentages of AAR and AN that accounting for the area of LV (AAR/LV% and AN/AAR%, respectively) were calculated as the surrogate of AAR and AN [21].

2.5.3. Cardiac function

The microtip catheter transducer was installed to the LV through the right carotid artery with the other end connected to Medlab biological signal collection and disposal system. The heart rate (HR), LV systolic pressure (LVSP), LV end-diastolic surediastolic pressure

(LVEDP), Maximum rise/fall rate of LV pressure (±dP/dtmax) were recorded for the comparison of cardiac function in different conditions.

2.6. Statistical analysis

All statistical results were analyzed by GraphPad Prism 5.01. All variables are presented as mean ± standard deviation (SD). Comparisons between two groups were conducted using the independent Student's t-test. Comparisons among more groups were conducted via one-way ANOVA followed by the Student–Newman–Keuls test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. RIPostC attenuated myocardial damage and rescued the cardiac function

Limb RIPostC was performed immediately after myocardial ischemia. We subsequently evaluated the cardioprotective effect of limb RIPostC by measuring myocardial infarct area in I/R model rats, as well as plasma levels of myocardial injury biomarkers, including CK and TnI, after ischemia. As is shown in Fig. 2A, limb RIPostC significantly ($P < 0.05$) rescued myocardial infarct area comparing to I/R group. In addition, the plasma CK and TnI levels were also significantly ($P < 0.05$) reduced by limb RIPostC (Fig. 2B).

Besides, the cardiac function indexes (HR, LVEDP, LVSP and ±dP/dtmax included) showed no significant differences between each group as baseline. However, RIPostC significantly improved cardiac function comparing to the I/R group by means of LVEDP reduction and LVSP, ±dP/dtmax elevation 3 h after reperfusion. (Fig. 3).

3.2. Mechanism of RIPostC-induced-cardioprotection: role of TRPV1

However, pretreatment with TRPV1 antagonist capsazepine (3 mg/kg 10 min before reperfusion) significantly abolished such cardioprotective effect above (Fig. 2), evidencing the key role of TRPV1 in RIPostC-induced myocardial protection. The influence of limb RIPostC on CK and TnI plasma levels before and after capsazepine pretreatment showed the same tendency (see in Fig. 2B), which further consolidated the significance of TRPV1 in the cardioprotective effect of RIPostC.

3.3. Effect of CGRP and SP in the transmission of limb ischemia insult

After the implement of limb RIPostC, plasma and heart tissues were collected. It turned out that the CGRP and SP level elevated significantly ($P < 0.05$, Fig. 4A) in plasma and heart tissue. This suggested that the elevation of CGRP and SP levels could have exerted their effects by targeting the heart tissue. To validate this possibility, we utilized CGRP antagonist CGRP8-37 (2 mg/kg, 2 min before reperfusion) and SP antagonist RP-67580 (5 mg/kg, 5 min before reperfusion) before reperfusion. It showed that they significantly ($P < 0.05$, Figs. 2 and 3) abolished the RIPostC-induced cardioprotective effect, while the plasma and heart levels of CGRP and SP showed no significant reduction ($P > 0.05$, Fig. 4A). This indicated that CGRP and SP may have exerted their effects by targeting the corresponding receptors in heart tissue.

3.4. TRPV1 mediates the cardioprotective effect of RIPostC by upregulation of CGRP and SP

In addition, the TRPV1 antagonists capsazepine (3 mg/kg 10 min before reperfusion) could abolish the shared elevation of CGRP and SP in plasma, heart tissue and DRG ($P < 0.05$, Fig. 4A), while pretreatment with CGRP8-37 (2 mg/kg, 2 min before reperfusion) and RP-67580 (5 mg/kg, 5 min before reperfusion) showed no such impact ($P > 0.05$, Fig. 4A). Further investigation showed that the mRNA expression of CGRP and SP also increased significantly ($P < 0.05$, Fig. 4B) in DRG.

This suggested that RIPostC may promote the level of CGRP and SP in plasma, DRG and heart tissue via TRPV1 activation.

4. Discussion

The present study aims to investigate the possible “bridging role” of TRPV1 in mediating the RIPostC-induced cardioprotection

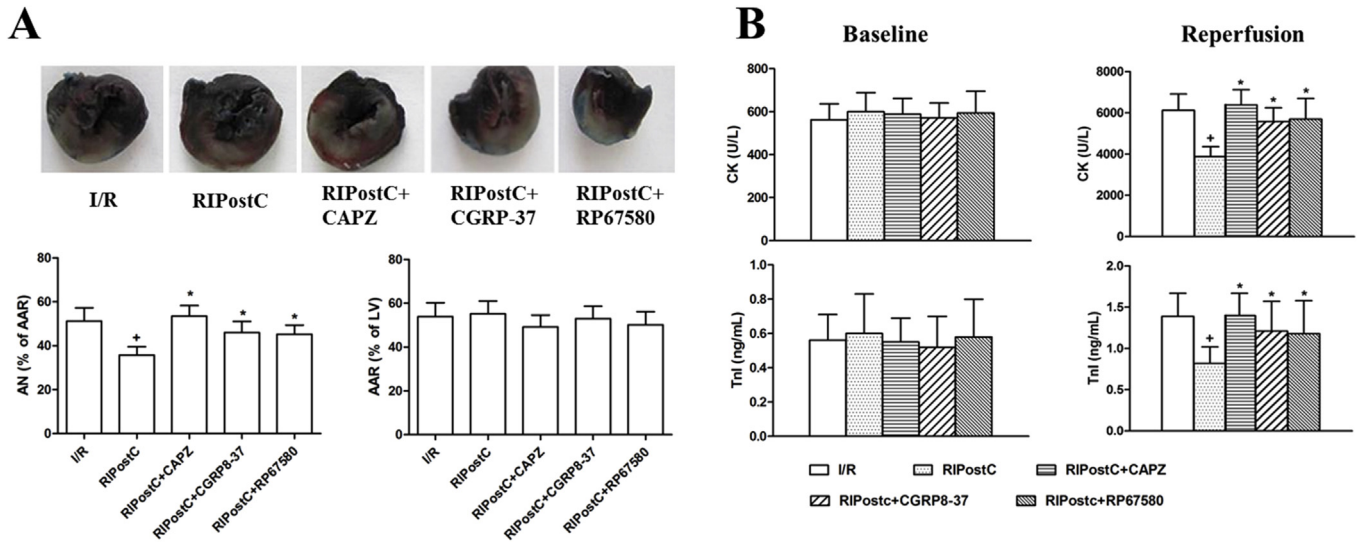

Fig. 2. Heart injury comparison among different groups by means of Myocardial infarct area and Plasma CK and TnI levels. (A) The images of infarcted heart tissues under different treatments and calculated AAR (area under risk)/AN (area of necrosis) of each groups. The AAR of different treatments manifested as dark blue colour, while the AN as grey white. In particular, the grey white area of RIPostC group is significantly smaller than other groups both in the image and calculated result. (B) Comparison of CK and TnI levels among each group (n = 7). $^{+}$ denotes $P < 0.05$ versus I/R group; * denotes $P < 0.05$ versus RIPostC group.

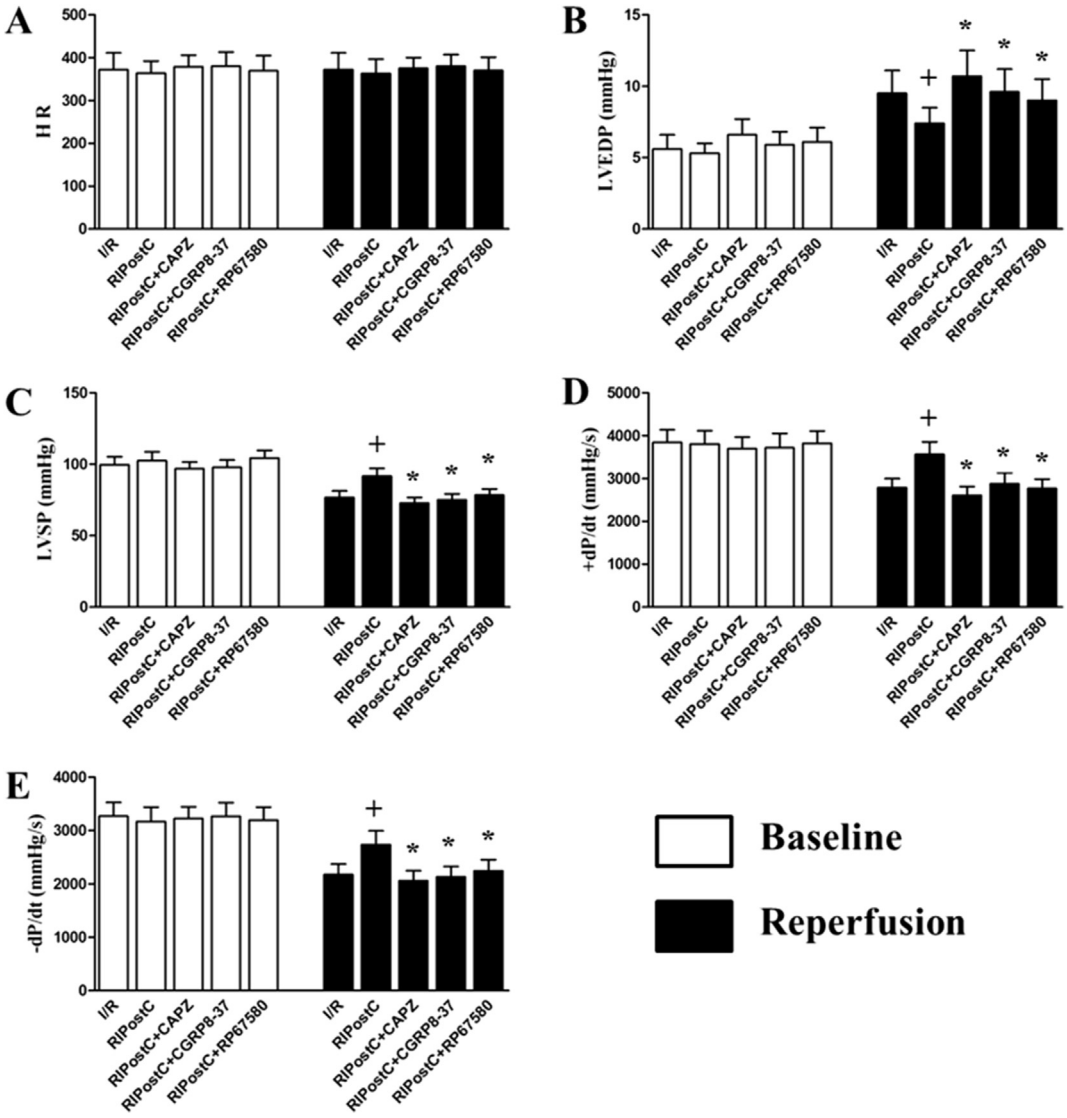

Fig. 3. Comparison of cardiac functions among groups under different treatments. (A) HR; (B) LVEDP; (C) LVSP; (D) +dP/dt; (E) -dP/dt (n = 7 for each group). $^{+}$ denotes $P < 0.05$ versus I/R group; * denotes $P < 0.05$ versus RIPostC group.

from remote organ to heart and the potential mechanisms. We utilized I/R rat model to observe the cardioprotective effect of RIPostC in different conditions, involving the presence or absence of TRPV1 receptor antagonist capsazepine, CGRP receptor antagonist CGRP8-37 or SP receptor antagonist RP-67580. The findings are mainly three fold: firstly, RIPostC significantly rescues the myocardial infarction size and heart function from ischemia-reperfusion injury, in which TRPV1 plays a vital role; secondly, CGRP and SP are upregulated when TRPV1 received "injury signal" elicited by RIPostC and subsequently act on the corresponding receptors in heart tissue to exert cardioprotection; thirdly, the TRPV1 located in the heart tissue and DRG may transitionally convert the "injury signal" from remote organ to generate effective factors. These results confirmed our "neurohumoral-mediation" hypothesis that RIPostC activates TRPV1, raising the concentration of neurotransmitter CGRP and SP in plasma, DRG and heart tissue, which could further target their corresponding receptors in heart to bring out the cardioprotective effect. They also confirmed our hypothesis of the neurohumoral mechanism which transmits the protective effect of RIPostC from remote organ to heart.

TRPV1 could be activated by multiple physical and/or chemical stimuli which further upregulates the level of CGRP and SP [15,18]. The ischemia injury of distal limb from limb RIPostC could give rise to the local release of hydrogen ion (H^{+}), bradykinin and reactive oxygen, which may activate TRPV1 directly or indirectly [22,23]. In fact, pretreatment with capsaicin, which serves as a TRPV1 inhibitor, has been demonstrated to be fatal in remote postconditioning's protection against cerebral ischemia [24]. Moreover, our recent study also indicated that decreased TRPV1 expression could abolish the cardioprotection of IPostC [17]. As expected, our current study demonstrated that the inhibiting of TRPV1 by capsazepin significantly reduced the cardioprotective effect of remote RIPostC, suggesting the "sensor role" of TRPV1 in the protective effect of RIPostC. However, we did not utilize TRPV1 agonist to investigate whether the protective effect of RIPostC could recover from TRPV1 blockage in the present study, there did exist report that selectively activate TRPV1 could exert a significant cardioprotective effect against reperfusion (I/R) injury via activation of the TRPV1 in wild-type (WT) but not in TRPV1 gene knock-out mice [16,25].

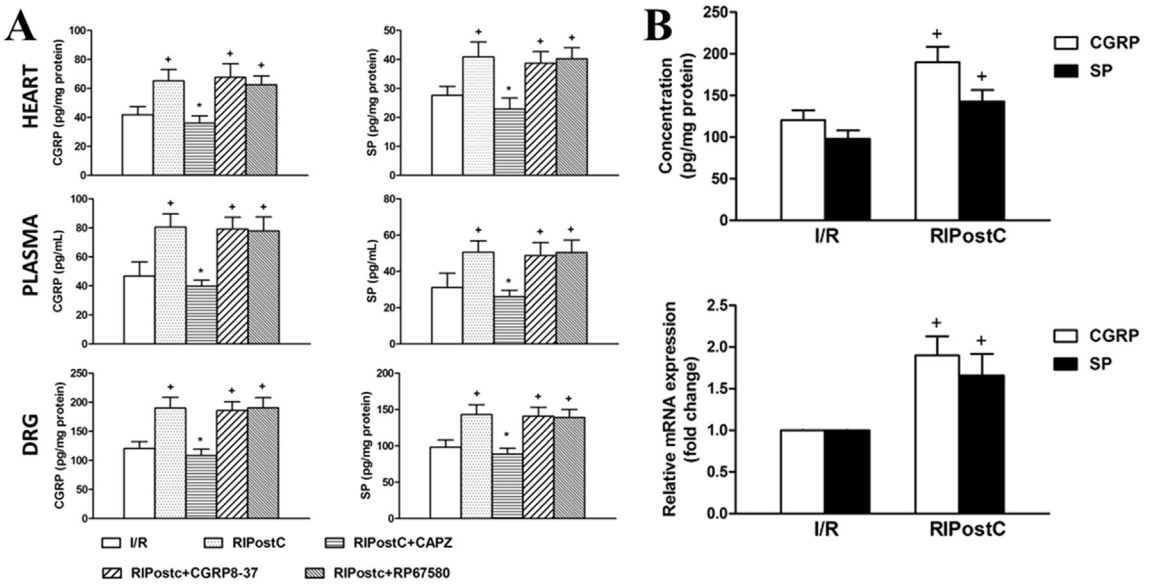

Fig. 4. The expression levels of CGRP and SP in heart tissue, plasma and DRG. (A) Protein levels of CGRP and SP under different treatments (n = 7 for each group) before reperfusion in heart, plasma and DRG. $^{+}$ denotes $P < 0.05$ versus I/R group; * denotes $P < 0.05$ versus RIPostC group. (B) Protein and mRNA levels of CGRP and SP between I/R and RIPostC group in DRG. $^{+}$ denotes $P < 0.05$ versus I/R group.

In addition, we also found that the employment of CGRP antagonist CGRP8-37 and SP antagonist RP-67580 substantially hindered the cardioprotective effect of RIPostC. This highlighted the imperative role of CGRP and SP in RIPostC-induced cardioprotection and the necessity of their binding to corresponding receptors in heart. CGRP and SP are crucial neurotransmitters that acknowledged as beneficial molecules for cardiovascular system. In addition, they have been demonstrated to exert cardioprotective effect against ischemia-reperfusion injury [26–29], which further confirmed their effectiveness in cardioprotection during RIPostC.

Neurons in DRG are the main source of CGRP and SP in peripheral tissue and circulatory system, which makes DRG the major mediator of noxious stimulation from periphery to afferent pathway [15,18]. In our study, paralleled with the elevated level of CGRP and SP in plasma and heart tissue, we observed a significant ($P < 0.05$) increase in mRNA level in DRG. So we infer that DRG receives the “injury signal” from RIPostC and converted them into cardioprotective effect by upregulating the level of CGRP and SP, which would subsequently be secreted to plasma and target the corresponding receptors in heart. However, we did not evaluate the mRNA level of CGRP and SP in other tissues, like heart tissue, after RIPostC. Further study may be in need to sort out the different contribution of TRPV1 between DRG and other TRPV1 innervation tissues.

Presently, we suggested that neural-hormone pathway was involved in remote postconditioning-induced cardioprotection. However, we did not test whether there exist other factors released from local area of limb insult that may have exerted cardioprotective effects directly to the heart tissue. In fact former research had indicated that release of mediators such as opioids after postconditioning may account for the cardioprotective effect [30], but this does not contradict with findings of our study.

To sum up, the present study confirmed our hypothesis that neural-hormone pathways mediated the cardioprotective effect of RIPostC, in which TRPV1 played a “bridging role” by upregulating the CGRP and SP which targeted the corresponding receptors in heart tissue.

Conflict of interest

None.

Acknowledgment

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