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Effect of myocardial ischemic preconditioning on ischemia-reperfusion stimulation-induced activation in rat thoracic spinal cord with functional MRI☆

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ABSTRACT

Background: Myocardial ischemia and reperfusion-evoked spinal reflexes involve nociceptive signals that trigger neuronal excitation through cardiac afferents, projecting into the thoracic spinal cord. Ischemic preconditioning (IPC) involves brief episodes of sublethal ischemia and reperfusion enhances the resistance of the myocardium to subsequent ischemic insults. This study investigated the effects of IPC on ischemia-reperfusion (I/R) stimulation-induced activation in the thoracic spinal cord of rats.

Methods: A new remotely controlled I/R model was established. The infarct size was determined as a percentage of area at risk (IS/AAR) and arrhythmia scores were evaluated. Non-invasive *in vivo* fMRI was used for signal quantitative analysis of thoracic spinal spatiotemporal. The role of IPC on the excitability of substantia gelatinosa (SG) neurons was assessed by spinal patch clamp recording technique. The altered expressions of *c*-Fos, SP, and CGRP in T_4 segment were detected by immunohistochemical staining.

Results: The novel I/R model was induced successfully and reliably utilized, and IPC treatment markedly reduced the myocardial infarct size. fMRI analysis revealed that IPC reduced the increased BOLD signals induced by prolonged ischemia-reperfusion. Patch clamp recording showed that IPC treatment reversed the enhanced excitability of SG neurons during I/R treatment. The results of immunofluorescent staining indicated that IPC mitigated the I/R-induced dramatic increase of *c*-Fos, and reduced the release of SP and CGRP in dorsal horns of spinal cord.

Conclusions: These results suggested that IPC suppressed neuronal activation induced by I/R stimuli in rat thoracic spinal cord using spinal cord fMRI and patch clamp recording techniques.

1. Introduction

Ischemic preconditioning (IPC) is an interventional nonlethal ischemic stress to heart, which protects against subsequent lethal myocardial ischemia-reperfusion (I/R) injury. During cardiac ischemia and reperfusion, an array of chemical mediators [1,2] activate cardiac ischemia-sensitive afferent neurons (CISAN) and release neuropeptides present within the myocardium and spinal cord [3–5]. Sensory signals associated with sympathoexcitatory responses are transmitted to the dorsal horn of the thoracic spinal cord [5–7], resulting in the release of neurotransmitters including substance P (SP) and calcitonin gene-related peptide (CGRP) [8–11]. However, the augmentation of cardiac sympathetic tone during a prolonged I/R course aggravated the imbalance of myocardial oxygen supply-demand and the occurrence of arrhythmias [12–14]. Despite these recognized autonomic responses, the spinal mechanisms that enable the spatiotemporal organization and the differences during these processes remain largely elusive.

Immunohistochemistry evidence revealed that the presence of nociceptive afferent neurons was associated with myocardial I/R containing

☆ We take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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both C and Aδ afferent fibers [4,6]. A majority of these neurons are associated with spinal segments T_1–T_4, and their axons are terminated mainly in the laminae I–V, VII, and X. In the dorsal horn of spinal grey matter, lamina II (substantia gelatinosa; SG) is critical in the modulation of cardiac nociceptive transmissions [15–17]. In addition, several brief periods of coronary artery occlusion and reperfusion that reverse further degradation of myocardial performance prior to sustained ischemia elicits excitatory neuronal activity in the thoracic spinal segments [2,7,10,12,18,19]. Consequently, we hypothesized that IPC attenuates excitatory neuronal activity in thoracic segments in response to ischemia-reperfusion stimuli, contributing to its cardiac protection.

Functional magnetic resonance imaging (fMRI) could non-invasively detect these neuronal activations [20]. This has been used in the detection of varied nociceptive stimuli-induced neuronal responses in the spinal cord [21]. In principle, the techniques could be applied to investigate the neuronal activity of spinal cord induced by myocardial I/R stimulus. Therefore, this study aimed to observe the spatiotemporal neuronal activity of the thoracic spinal cord in different conditions and to investigate whether IPC suppress the cardiac nociceptive transmission in response to I/R stimulus.

2. Materials and methods

The experiments conducted in this study comply with the ARRIVE guidelines and are carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). A detailed description of the materials and methods used in this study has been provided in the supplemental materials. Only the group information and the description of new technique were briefly provided here.

In order to meet the needs of real-time imaging and remote control in fMRI measurements, the novel method of inducing I/R injury by inflating and deflating the fixed saccule has been invented and the efficiency of it has been determined. The remote-controlled quantitative saccule that consisted of a saccule connected to a pressure pump with a distensible extension tube was placed around the left coronary artery of the heart to allow induction of I/R by inflating and deflating the fixed saccule (Supplemental Fig. 1A). The classical approach for inducing I/R was utilized in the control groups.

Here, the SD rats were randomly divided into six groups, with 6 rats in each group: classical sham operation group (cSHAM group), classical ischemia-reperfusion group (cIR group), classical ischemic preconditioning group (cIPC group), and the saccules groups: saccule sham operation groups (SHAM group), saccule ischemia-reperfusion group (IR group) and saccule ischemic preconditioning group (IPC group).

For fMRI axial data analysis, a common ROI, including the whole grey and white matter of spinal cord of the slice 5 corresponding to a T_4 segment of the spinal cord was selected in every animal. The signals from all fMRI were run for every animal among the three groups, under the same conditions, and were averaged. The statistical *t*-value maps were computed by comparing the experimental fMRI data that was acquired during the baseline and stimulation periods on a pixel-by-pixel basis. To detect the activation, statistical thresholds were set at a *p*-value of 0.001. The color-encoded fMRI activation maps (*t*-values) generated for data from all ~5 min fMRI runs from one representative animal in each group were overlaid on the original EPI images using customized software developed in Matlab. The increase in MRI signal intensities was displayed with colors that varied from blue to red.

For changes in the BOLD signals, the rest condition (baseline) data were subtracted from the stimulated condition data. The signal intensity was illustrated in a time-dependent manner and the data were averaged for each animal and then for the three groups. All the data were reported as mean ± SEM using Prism v5.0 (GraphPad Software, USA). BOLD fMRI and hemodynamic parameters were analyzed by using two-way ANOVA for repeated measures. A one-way ANOVA followed by Tukey's *post hoc* tests was used to assess the differences in other data. Statistical differences were considered to be significant if the *p*-values were <0.05.

3. Results

3.1. Hemodynamic response, morphometrics, and arrhythmias among different models

A total of 41 rats were included in this study. Five rats were omitted for further analysis due to mean arterial blood pressure (MAP) of <30 mm Hg ($n = 2$) or intractable ventricular fibrillation ($n = 3$) during the ischemic period. The hemodynamic responses were comparable with their specific baseline values among different periods. As expected, there was a significant drop of MAP after 30 min of ischemia and 2 h of reperfusion in all the groups, confirming the successful induction of I/R injury model, except in cSHAM and SHAM groups (Supplemental Table 1). Comparison with cIR and IR models showed that the infarct size/ area at risk (IS/AAR) values in cIPC and IPC groups were markedly reduced to 25.6 ± 3.4% and 28.5 ± 4.1%, respectively ($p < 0.05$; Fig. 1B and C), but the difference showed no significant difference between these two

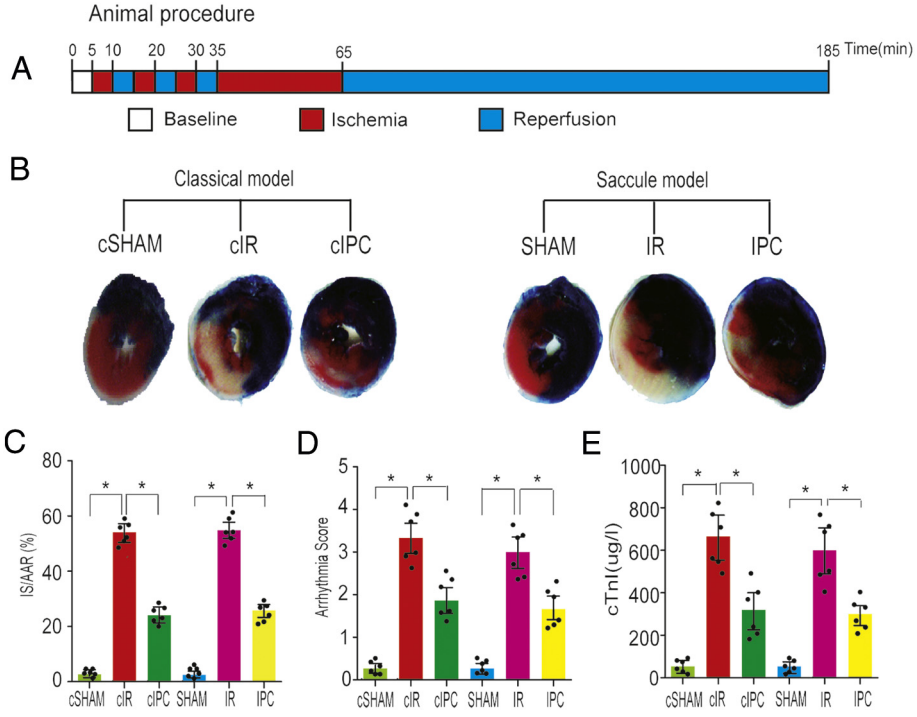

Fig. 1. Morphometrics (B) and arrhythmia characterization (C, D, and E) in the rat hearts in different groups. *Note: A-schedule of the animal experimental procedures in the IPC group. B- Blue, Non-ischemic area. Red: area at risk (AAR). White: infarct size (IS). Data were expressed as mean ± SEM (n = 6) and analyzed by one-way ANOVA with Tukey's test. *p < 0.05.*

groups. Similarly, the two approaches of IPC treatments resulted in a substantial drop in the arrhythmias scores and serum troponin levels (cTnI) as compared to cIR and IR groups ($p < 0.05$; Fig. 1D and E).

3.2. Nociceptive I/R-evoked fMRI spatiotemporal response

The positive BOLD spatial responses were compared in I/R stimuli conditions (Fig. 2B). The results revealed that there were only weak signal fluctuations in some non-specific regions during the whole scanning sessions in the SHAM group. Compared with the SHAM group, the moderate activations were roughly observed in the dorsal and ventral layers of the spinal cord during ischemic sessions in IR animals. Furthermore, myocardial reperfusion elicited robust fMRI signal changes in IR animals along with significantly stronger activations than during ischemia. However, the long-lasting reperfusion stimulus evoked by IPC elicited weak functional activations compared to the IR group.

Furthermore, the time courses of fMRI signals in slice 5 were calculated and were obtained during the I/R stimuli periods. Changes in the BOLD signals in slice 5 of the spinal cord were ranged between 1.8% and 3.7% across all the ischemic sessions in both the IR and IPC groups, with no significant difference observed between these two groups ($p > 0.05$, Fig. 2C). However, there was an increasing trend as compared to the SHAM group during the reperfusion sessions ($p < 0.05$; Fig. 2C). The time-course of the average fMRI signals during the nociceptive reperfusion stimuli periods was higher than that during ischemia. Compared to the IR group, IPC treatment reduced the BOLD signals ($p < 0.05$; Fig. 2C).

3.3. Neuronal activation marked by c-Fos response to cardiac I/R and IPC

Similar to the BOLD-fMRI analysis, the site of T_4 in the spinal cord was used for analysis. The *c*-Fos baseline expression was observed in animals that did not receive any treatment after primary surgery (Fig. 3A and B). The *c*-Fos-positive neurons were significantly increased after cardiac I/R in the IR group ($p < 0.05$). These neurons were predominantly dispersed throughout the left and right dorsal and ventral areas of the spinal cord, especially across the dorsal regions (laminae I–V) (Fig. 3C and D). However, the IPC method significantly reduced the number of *c*-Fos-positive neurons in the grey matter and laminae I–V as compared to the IR group (Fig. 3B and D).

3.4. Modulation of SP and CGRP release response to cardiac I/R

The basal release of substance P (SP) and calcitonin gene-related peptide (CGRP) in the T_4 segment of spinal cord in the sham operation conditions was observed. Double immunofluorescent evidence of spinal cord indicated that SP and CGRP were abundant and co-localized in the laminae I–III of the dorsal horn bilaterally in the IR group. However, the IPC treatment significantly decreased the release of SP and CGRP above basal levels when compared to the animals in the IR group (Fig. 3E).

3.5. Inhibitory effect of IPC on the excitability of SG neurons

To investigate the influence of myocardial I/R stimuli, and verify the modulatory effects of IPC method, the excitability of SG neurons was detected by the patch-clamp method. Compared to the SHAM group,

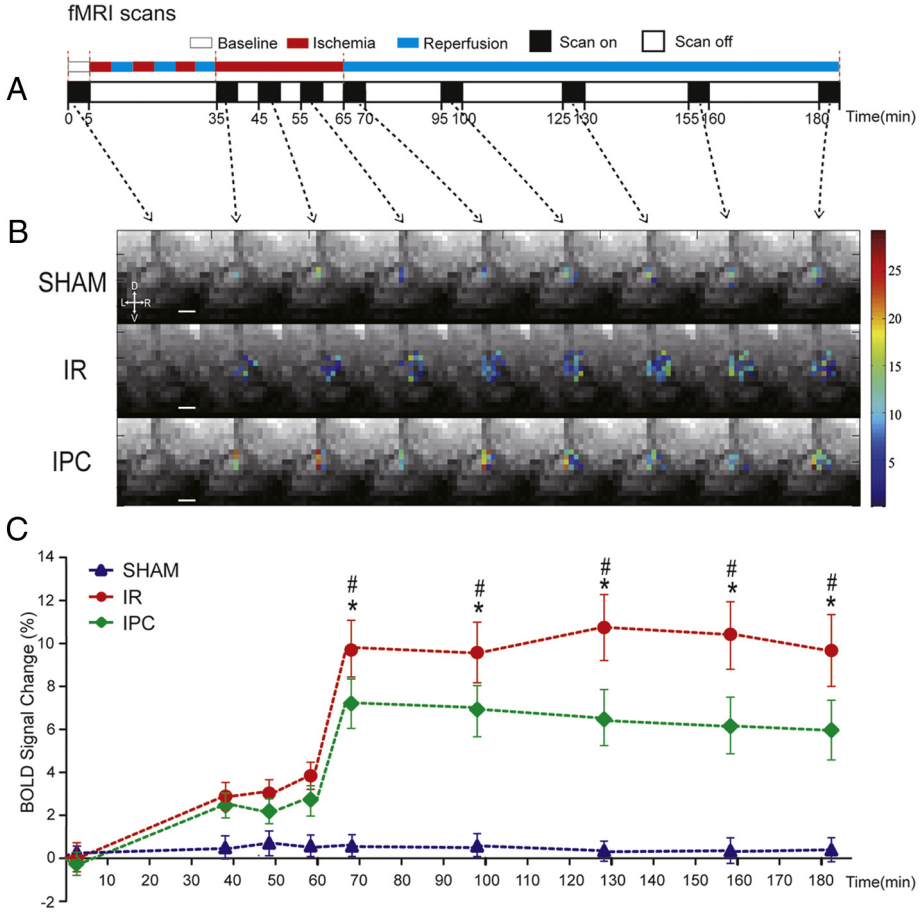

Fig. 2. Quantitative BOLD signal analysis of fMRI in the axial slice and average temporal profiles of the modulations of IPC. *Note: A-schedule of fMRI scans during myocardial I/R sessions; B-spatial distributions of BOLD activations in the spinal cord (axial slice 5) during different periods of cardiac nociceptive stimulation for SHAM, IR and IPC groups; Dorsal; V: Ventral; L: Left; R: Right; scale bars: 2 mm. C-time course of the average BOLD signals in response to myocardial ischemia and reperfusion stimulus in these three groups. Results were expressed as mean ± SEM (n = 6) and analyzed by two-way ANOVA for repeated measures. *p < 0.05 IR vs. SHAM; #p < 0.05 IPC vs. IR.*

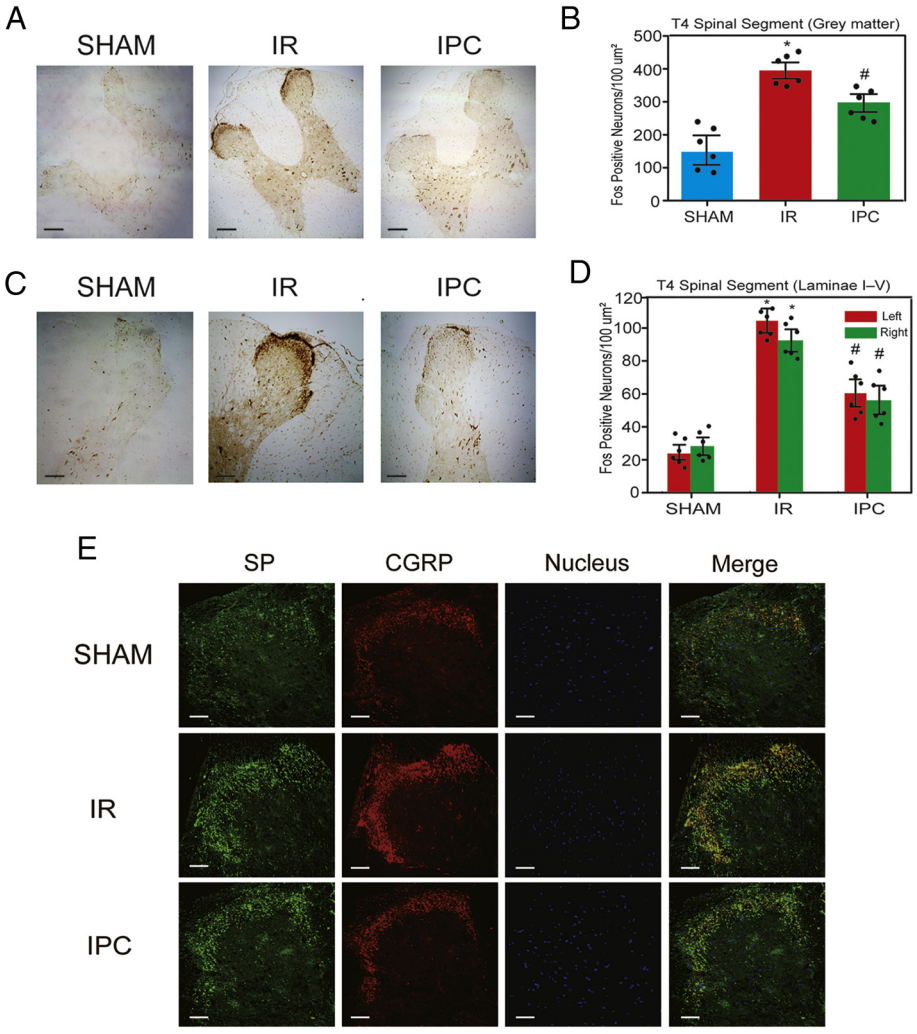

Fig. 3. Neuronal activations, SP and CGRP release at T_4 spinal level after reperfusion. *Note: A, C- c-Fos immunoreactivity in rat spinal cord in response to I/R stimulus. Scale bars represent 500 μm and 100 μm, respectively. B, D- Quantification of c-Fos-positive neurons in the grey matter and dorsal horn (laminae I–V) of T_4 spinal segment. n = 6. One-way ANOVA with Tukey's test, $^{*}p < 0.05$ IR vs. SHAM; $^{\#}p < 0.05$IPC vs. IR. E-Localization of SP and CGRP release in response to I/R stimulus in SHAM, IR, and IPC groups. Scale bars: 100 μm.*

the action potential (AP) firing rates for SG neurons in the IR and IPC groups were significantly increased (Fig. 4A). However, IPC treatment could inhibit the firing rate as compared to the IR group with the same depolarizing current. The average AP evoked by the same currents in the IR group was about 3-fold that of in the SHAM group ($^{*}p < 0.05$ *vs.* SHAM, Fig. 4B). Furthermore, the average AP was significantly increased in the IPC group as compared to the SHAM group, but was much lower than that in the IR group during the microinjection period ($^{\#}p < 0.05$ *vs.* IR, Fig. 4B).

Additionally, the absolute values of the threshold of AP (APT) and the peak of AP (APP) were calculated. The values of both parameters were significantly increased in the IR group when compared to the SHAM group. The IPC treatment decreased the neuronal excitability by reducing the absolute values of APT and APP ($^{\#}p < 0.05$ *vs.* IR, Fig. 4C and D). However, no significant differences were detected in the resting membrane potential (RMP) and in the AP half-width of the SG neurons among IR, IPC and SHAM groups (Fig. 4E and F).

4. Discussion

In the current study, a novel cardiac I/R animal model has been proposed, and its efficiency was compared with the traditional approach. The spinal fMRI method was utilized to detect the changes in BOLD signals in the thoracic spinal cord of anesthetized rats in the constructed models. Compared with the conventional methods, the novel IPC method demonstrated a cardioprotective effect, involving attenuated neuronal activation and modulation of excitability in SG neurons potentially.

4.1. Approaches of I/R models

The classical method of I/R experiments involved pulling the snare, securing the threads with a mosquito hemostat and loosening the snare, which can easily damage the myocardium and coronary blood vessels. Furthermore, this method cannot achieve the remote control in the fMRI machine for real-time tomography imaging during myocardial perfusion. To resolve this issue, a remote-controlled hydraulic occluder from a previous work has been utilized, which consisted of a distensible silicone tube to a syringe *via* an indistensible extension tube to allow the induction of renal ischemia and reperfusion [25]. This approach enabled the non-invasive *in vivo* renal imaging. In this study, a quantitative inflatable saccule was utilized for the induction of myocardial I/R. The I/R injury was evidenced by increased levels of IS/AAR and cTnI, accompanied by higher arrhythmia scores, which thus confirmed feasibility and steadiness of this novel method for ischemia-reperfusion or pretreatment administration. Also it achieved the requirement of real-time imaging and remote control during MRI scanning in the animal I/R experiments.

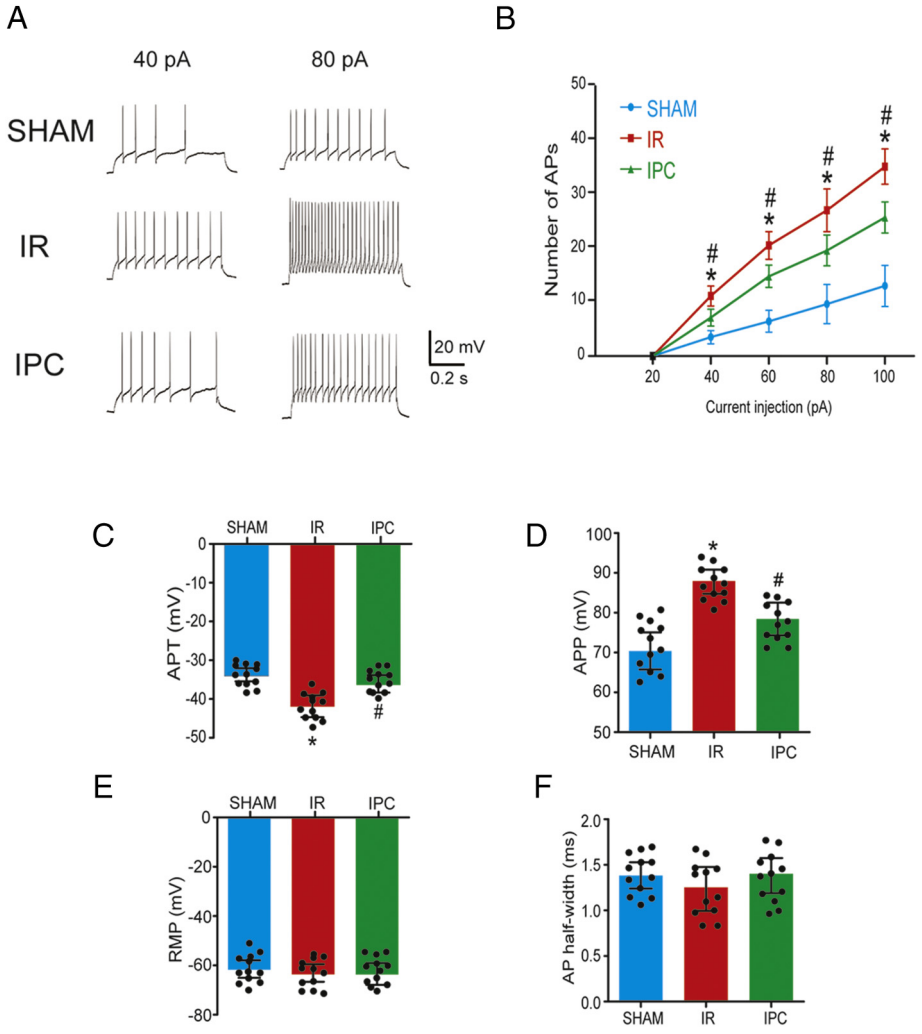

Fig. 4. Reversal of IR-induced increased excitability of SG neurons by IPC. *Note: A-representative traces of neuronal discharges at a given depolarization currents (+40 pA and +80 pA) in SHAM, IR, and IPC groups; B-suppression effects of IPC on SG neurons; absolute average values of APT (C) and APP (D), RMP (E) and AP half-width (F) in SG neurons in the three different groups. One-way ANOVA with Tukey's test.* $^{*}p < 0.05$ *IR vs. SHAM;* $^{\#}p < 0.05$ *IPC vs. IR.*

4.2. Application of spinal BOLD fMRI on myocardial ischemia and reperfusion

BOLD fMRI technique could evaluate the neuronal activity in living animals noninvasively by measuring the local hemodynamic responses to the increased cellular activity. Previous studies have demonstrated that fMRI could reliably detect the spatiotemporal neuronal changes in the lumbar and cervical spinal cord of rats during noxious stimulation [26–29]. Spin-echo approach has been used to acquire the fMRI data due to its high sensitivity in detecting BOLD contrast of neuronal activation [30]. However, the application of fMRI technique to the rodent thoracic spinal cord has not been reported before, attributing to the effect of the technique by motion artifacts, strong signal noise and susceptibility artifacts [31]. The current protocol of fMRI was utilized for studies related to I/R stimuli-evoked signal changes in the thoracic spinal segments. The mean percentage signal changes detected in the spinal cord of IR rats during I/R stimuli was $2.61 \pm 0.76\%$ and $10.09 \pm 1.35\%$, respectively as compared to those in the SHAM group. However, the prominent BOLD signals from reperfusion stimuli in a single spinal segment from slice 5 could be effectively attenuated by IPC in advance. Interestingly, robust average percentage signal changes did not occur during the sustained ischemic periods between IR and IPC animals, indicating that the protective effects of IPC were exerted during the course of myocardial reperfusion.

Although the temporal BOLD response of rat thoracic spinal cord was clearly demonstrated, the spatial localization of the neuronal activation in the cross-section of the spinal cord could not be verified due to low-resolution activation maps. Previously, the nociceptive stimulation induced by ischemia-excited neurons was determined by *c*-Fos in laminae I–V in the cervical and thoracic spinal cord and brain stem [32]. The presence of neuronal activation in the imaging region of spinal cord was further verified by *c*-Fos, which was considered as an excitability-dependent and useful marker of neuronal activation [33]. In addition, previous studies showed a good agreement between the sites of neuronal activity as determined by *c*-Fos expression and fMRI in the lumbar spinal cord during noxious stimulation of rat paws [27,28]. In the present study, IPC effectively diminished the high levels of neuronal activation, mainly in the superficial laminae. Thus, a decrease in the number of active neurons at the superficial laminae of the thoracic spinal level underlies the modification of input signals from cardiac nociception by IPC. However, these results showed no close association with neuronal activation regions as determined by fMRI. The maximal translation of *c*-Fos into the protein occurs at approximately 2 h following noxious stimulation in the sensory neurons of spinal cord [33], whereas the changes in blood oxygenation level occur in a few seconds in BOLD fMRI. Despite this temporal difference, incomplete accordance of this spatial variation with histology and functional imaging of the spinal cord, the hypothesis at least partially supported that the

preservative effects elicited by IPC involves the modification of neuronal activation at thoracic spinal segments.

Myocardial ischemia and reperfusion alter the chemical milieu of the heart, thereby altering the activity induced by the associated cardiac sensory neurons. Electrophysiological studies previously showed that intrapericardial infusion containing one of these chemical mediators can lead to increased discharge rate of spinal neurons in segments T_2–T_6 and C_1–C_2 [2,6,7,34,35]. This indicated that changes in neuronal excitability in the dorsal horn contribute to the integrated and modulative cardiovascular reflex in response to cardiac I/R. In addition, SP and CGRP served as excitatory neurotransmitters in cardiac ischemic signaling [9–11]. Thus, the excitability of SG neurons determined the processing of nociceptive information from heart to the higher brain. Interestingly, IPC resulted in significant inhibition of IR-enhanced SG neuronal AP firing by reversing the lowered APT and APP to the basal level and attenuated the IR-induced spinal release of SP and CGRP of the superficial laminae. These observations were considered as at least partial initial evidences, suggesting that the changes of neuronal excitability in one category of SG neurons may be responsible for the cardioprotective effects mediated by IPC.

Taken together, our results revealed that BOLD fMRI can be utilized to understand the cardiac nociception-evoked signal responses in thoracic spinal cord. The present study fingings partially emphasized the inhibitory effects of IPC on neuronal activation of the spinal cord and proposed an additional pathway that might attenuate myocardial I/R injury. Since SG neurons are known to play a pivotal role in the regulation of input-output of cardiac nociceptive information, the neuronal activity manipulation by IPC implies a novel and potential target for treatment of ischemic heart diseases.

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Conflict of interest

None declared.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2019.03.025.

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