Abstract. Vagus nerve activation may have important therapeutic significance for myocardial ischemia-reperfusion (IR) injury. Nitric oxide (NO) plays a vital role in the cardioprotective effects of anesthetic preconditioning (APC). Moreover, acetylcholine (ACh) prevents cardiomyocyte damage by activating AMP-activated protein kinase (AMPK) and increasing the phosphorylation of Ca\(^{2+}\)/calmodulin-dependent protein kinase β (CaMKKβ). The aim of the present study was to determine whether APC could protect heart function by antagonizing IR damage via the cholinergic system. It was hypothesized that the NO synthase (NOS)/CaMKKβ/AMPK pathway might be involved in the cardioprotective effects induced by cholinergic receptor activation. Isolated rat hearts were subjected to ischemia for 30 min followed by 120 min of reperfusion. Volatile anesthetic sevoflurane (3.5%) was administered for 15 min before ischemia, then rinsed for 15 min. The muscarinic acetylcholine receptor (mAChR) antagonist atropine (ATR; 100 nM) and the nicotinic acetylcholine receptor (nAChR) antagonist hexamethonium (HEM; 50 µM) were administered 10 min before APC. Both mAChR and nAChR were involved in APC-induced cardioprotection. ATR and HEM treatment both abolished the protective effects of APC on IR damage in isolated hearts, demonstrating the importance of cholinergic receptors in the protection mechanism of APC. The present study thus suggests that APC plays a cardioprotective role, in part, by regulating neurohumoral pathways. In addition, there may be functional coupling between the two cholinergic receptors, and the NOS and CaMKKβ/AMPK pathways may play roles in shared pathways that mediate the cardioprotective effects of APC. These findings may provide insight into potential new mechanisms of APC-induced cardioprotection against IR injury.

Introduction

Anesthetic preconditioning (APC) refers to exposure of the heart to a volatile anesthetic followed by its washout (1,2). We previously demonstrated that APC protects the heart against subsequent ischemia-reperfusion (IR) injury (1,2). Although APC is an effective strategy to reduce myocardial injury, its exact underlying mechanism remains poorly understood. The autonomic balance between the sympathetic and parasympathetic nervous systems plays an important role in the regulation of the cardiovascular system (3). Acetylcholine (ACh) is the main neurotransmitter of the vagus nerve. It may mimic the effects of myocardial ischemic preconditioning (IPC) and protect the heart against myocardial IR injury (4). This indicates that the activation of ACh receptors is involved in cardioprotective signaling pathways (4-6). There are two main types of cholinergic receptors in the heart, namely, the muscarinic (mAChR) and the nicotinic receptors (nAChR) (4,7,8). Evidence has shown that through pharmacological or direct-current electrical stimulation, both mAChRs and nAChRs can trigger signaling pathways that protect the heart against IR injury (9,10).

Nitric oxide (NO) is an endogenous regulatory molecule involved in various physiological processes (11). The endogenous NO synthase (eNOS) pathway is expressed in isolated cardiomyocytes and regulates the negative chronotropic effects of cholinergic receptor stimulation (12). In addition, activation of the neuronal NOS (nNOS)/AMP-activated protein kinase (AMPK)/mTOR pathway has been linked to the cardioprotective effects caused by IPC (13). ACh prevents cardiomyocyte damage by activating AMPK
and inhibiting reactive oxygen species (ROS) formation (14). Recently, it was reported that during myocardial ischemia, vagus nerve stimulation (VNS) may activate AMPK, leading to the phosphorylation of calcium/calmodulin-dependent protein kinase β (CaMKKβ) (15). This suggested that the CaMKKβ/AMPK signaling pathway is involved in VNS-mediated protective effects. However, whether and how APC regulates cholinergic receptors to prevent IR injury and improve cardiac function remains unclear. ACh initiates downstream signaling by activating G-protein-coupled mACHRs or by binding to nAChRs, which are ligand-gated ion channels (16). Complex neural processing occurs within the heart, not only in response to central efferent input, but also to sensory afferent information from the myocardium (17). Nevertheless, whether APC exerts cardioprotection through the upregulation of cholinergic receptors in the isolated heart remains to be determined. Therefore, the aim of the present study was to determine the role of cholinergic receptors in APC-induced cardioprotection against IR injury in an isolated rat heart model. In addition, whether the NOS and CaMKKβ/AMPK pathways are involved in the beneficial effects of cholinergic receptor activation was also examined.

Materials and methods

Animals. The present study was approved by The Institutional Animal Care and Use Committee of the Affiliated Suzhou Science & Technology Town Hospital of Nanjing Medical University. Male, 8-10-week-old Sprague-Dawley rats (250±50 g) were purchased from the Animal Center of Suzhou University and housed under a 12-h light/dark cycle at 25°C and 60% humidity. All rats were provided with food and water ad libitum. All animals were treated in accordance with the National Institutes of Health's Guidelines for the Care and Use of Experimental Animals.

Isolated heart preparation. Preparation of isolated heart was performed as described previously (2,18). Briefly, rats were intraperitoneally anesthetized with 50 mg/kg pentobarbital sodium, then decapitated when they did not respond to a noxious stimulus to the hind limb. The heart was excised and perfused using the Langendorff method at a perfusion pressure of 80 mmHg. A thermostatically controlled water circulator (Lauda E100; Lauda) was used to maintain the temperature of 80 mmHg. Left ventricular pressure (LVP) was measured using a volume equalizer of a high-pressure liquid chromatography to measure myocardial ATP and CP.

Measurement of hemodynamic function. Hemodynamic parameters were monitored throughout the experiment. After 30 min of ischemia, hemodynamic function was assessed by determining the left ventricular developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP) and aortic cannula to measure sevoflurane concentration by gas chromatography. Inflow sevoflurane concentration was 0.64±0.02 mM, which is equivalent to 3.34±0.22% atmosphere and a minimal alveolar concentration of 1.5±0.4% (1). The ATR and HEM groups were used to evaluate the effects of cholinergic receptors. ATR and HEM doses were selected according to a previous study (17).

Myocardial high-energy phosphate analysis. At the end of reperfusion, the heart was frozen using aluminum forceps pre-cooled in liquid nitrogen to measure myocardial ATP and creatine phosphate (CP) levels, as described previously (19). Cardiac CP is converted to ATP by the enzymatic reaction of creatine kinase (20). Briefly, frozen ventricles were ground and mixed with 0.3 M HClO4 and 0.25 mM EDTA under liquid nitrogen cooling. The extract was centrifuged at 8,000 x g for 15 min at 4°C, and the resulting supernatant was sampled using high-pressure liquid chromatography to measure myocardial ATP and CP.

Determination of NOS activity and NO levels. At the end of the experiment, the heart was removed and homogenized in a 0.9% ice-cold saline solution, then centrifuged at 600 x g for 10 min at 4°C. NOS activity and NO levels were measured using a diagnostic assay kit (Nanjing Jiancheng Bioengineering Institute). The absorbance at a wavelength of 530 nm was measured using a DU-640 spectrophotometer (Beckman Coulter, Inc.), and normalized to the control according to the manufacturer's instructions (11).

Western blot analysis. Western blot analysis was performed as described previously (2). Hearts were homogenized using RIPA lysis buffer (cat. no. 20-188; EMD Millipore) and a complete mammalian proteinase inhibitor cocktail (cat. no. PI101; Roche Diagnostics GmbH) and then
Centrifuged at 13,200 x g for 20 min at 4°C. A BCA assay kit (cat. no. P0010; Beyotime Institute of Biotechnology) was used to determine the protein concentration. After denaturation, 20 µg of each sample was dissolved in Laemmli sample buffer (cat. no. S3401; Sigma-Aldrich; Merck KGaA) and separated using SDS-PAGE on a 10% gel. The samples were then transferred to a nitrocellulose membrane, which was blocked with 5% skim milk in PBS for 1 h at room temperature. The membranes were subsequently incubated with primary antibody at 4°C overnight, and then incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:10,000; cat. no. ab6721; Abbam) or HRP-conjugated goat anti-mouse secondary antibody (1:10,000; cat. no. sc-2031; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. Protein bands were visualized using a SuperSignal West Pico kit (cat. no. 34577; Pierce; Thermo Fisher Scientific, Inc.). Band density was quantified using UN-SCAN-IT software (v.7.0; Silk Scientific, Inc.). The primary antibodies were specific for eNOS (cat. no. sc-376751; 1:1,000; Santa Cruz Biotechnology, Inc.), nNOS (cat. no. sc-5302; 1:1,000; Santa Cruz Biotechnology, Inc.); AMPK (cat. no. 2352; 1:1,000; Cell Signaling Technology, Inc.); phosphorylated (p)-AMPK (Thr172; cat. no. 5884; 1:1,000; Cell signaling Technology, Inc.); p-CaMKK2 (Ser511; cat. no. AF4487; 1:1,000; Affinity Biosciences) and CAMKK2 (polyclonal antibody; cat. no. DF4793; 1:1,000; Affinity Biosciences); as well as the housekeeping protein GAPDH (cat. no. AG019; 1:1,000; Shanghai Biyuntian Biotechnology Co., Ltd.).

**Statistical analysis.** Data are presented as the mean ± SD. Each experiment was repeated at least three times. SPSS 19.0 (IBM Corp.) was used to conduct statistical analyses. One-way ANOVA was used to compare the differences among five groups, followed by Tukey’s post-hoc test to determine the differences between groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Cardiac performance.** The LVDP significantly decreased following IR compared with the sham group (Fig. 1A). Moreover, the LVDP significantly increased in the APC group compared with the IR group. By contrast, APC-induced improvement of LVDP was significantly inhibited by ATR or HEM treatment (P<0.05). Hearts subjected to IR displayed a significant increase in LVDP (Fig. 1B). However, a significant reduction in LVEDP was observed after APC treatment. The effect of APC treatment was inhibited by ATR or HEM treatment. Cardiac contractility (+dP/dt) and relaxation (-dP/dt) (Fig. 1C and D) were reduced during ischemia in all groups. Following reperfusion, contractility increased but still remained lower than that recorded before ischemia throughout reperfusion in each group. APC significantly improved contractile recovery in IR hearts, which was also abolished by ATR or HEM treatment.

**ATP and CP content.** At the end of reperfusion, the ATP and CP content were determined in trans-mural sections obtained...
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from the left ventricular free wall (Fig. 2). In the IR groups, the ATP content was significantly reduced (7.7±1.6 µmol/g) compared with the sham group (24.5±2.8 µmol/g; Fig. 2A). In addition, the CP content was also significantly decreased in the IR group compared with the sham group (19.2±3.5 µmol/g; Fig. 2B). However, the levels of ATP and CP were better preserved in the APC-treated group (15.6±1.8 and 17.5±2.1 µmol/g) compared with the IR group. Both the mAChR antagonist ATR and nAChR antagonist HEM abolished the preserving effects of APC.

Effects of APC on NOS activity and NO content. Following IR, both NOS activity and NO levels were significantly decreased in the IR group compared with the sham group (Fig. 3). Moreover, NOS activity and NO levels significantly increased in the APC group compared with the IR group (Fig. 3A and B). However, ATR and HEM treatment abolished the APC-induced increase in NOS activity and NO levels. These results indicated that both NOS activity and NO levels were decreased following IR injury; but increased after APC treatment. However, the effects of APC were reversed by ATR and HEM administration.

Effects of APC on eNOS and nNOS phosphorylation. Western blotting bands of eNOS, p-eNOS, nNOS and phosphorylated nNOS (p-nNOS) protein in Fig. 4A. The percentile ratio of p-eNOS/total eNOS and p-nNOS/total nNOS are displayed in Fig. 4B and C. There was a significant increase in eNOS and nNOS phosphorylation in the IR group compared with the sham group. Treatment with APC significantly increased the phosphorylation of eNOS and nNOS (APC group vs. IR group). However, this effect was significantly attenuated following ATR and HEM administration (APC+ATR group or APC+HEM group vs. APC group).

Effects of SPC on AMPK and CaMKK β phosphorylation. Western blotting bands of AMPK and p-AMPK(Thr172) protein and the ratio of p-AMPK(Thr172)/total AMPK are presented in Fig. 5A. Western blotting bands of CAMKK β and p-CAMKK β (Ser511) protein and the ratio of p-CAMKK β (Ser511)/total CAMKK β are displayed in Fig. 5B. There was a significant decrease in AMPK and CAMKK β phosphorylation in the IR group compared with the sham group. Treatment with APC significantly increased the phosphorylation of AMPK and CAMKK β (APC group vs. IR group). Following ATR and HEM administration, this effect was significantly reduced (APC+ATR group or APC+HEM group, vs. APC group).
Discussion

Our recent study demonstrated that APC reduced myocardial enzyme release and infarct size by enhancing the recovery of cardiac function, thereby reducing myocardial damage after IR (2). Possible mechanisms underlying the role of cholinergic receptors in alleviating IR injury have been proposed in several previous studies, including NOS and ROS-mediated CaMKII pathways (14,15,21,22). However, whether and how APC regulates the intrinsic cardiac nervous system to improve cardiac function remains unknown. The current study demonstrated that both mAChRs and nAChRs participate in APC-induced cardioprotection in isolated rat hearts following IR. ATR and HEM attenuated the protective effects of APC against IR injury, highlighting the importance of cholinergic receptors in the mechanism of APC-induced cardioprotection. Thus, the present findings indicated that APC plays a cardioprotective role, in part, by regulating neurohumoral pathways. In addition, NOS and CaMKKβ/AMPK may be involved in shared pathways that mediate the cardioprotective mechanisms of APC.

Previous studies suggested that increased vagal nerve activity could reduce myocardial IR injury (3,4,7,23). The main vagal neurotransmitter ACh can replicate the cardioprotective effects of IPC (17,24,25). Upon pharmacological or direct-current stimulation, both mAChRs and nAChRs can trigger cardioprotective signaling cascades, which are effective against I/R injury (21). In addition, previous studies have demonstrated that cardiomyocytes synthesized and secreted ACh, which provided further evidence for the importance of non-neurocholinergic signaling cascades in the maintenance of myocardial function in physiological and pathological states (26-28). A recent study examined the role of the intrinsic cardiac nervous system in the classic myocardial IPC mechanism and demonstrated that intrinsic cardiac ganglia remain intact in isolated hearts subjected to IR injury (17). In addition, IPC activated the intrinsic cardiac nerve reflex, leading to the release of ACh in the ventricle and inducing protective effects through the activation of cholinergic receptors. Treatment with ATR and HEM also blocked the protective effects of IPC (17). The present study was consistent with these previous findings. APC reduced IR injury by activating intrinsic cardiac cholinergic receptors, and this protective effect was abrogated by ATR and HEM, indicating that APC protected the heart against IR injury via intrinsic neuronal mechanisms.

NO plays a number of beneficial roles during myocardial reperfusion, including regulating myocardial contractility, opening K<sub>Atp</sub> channels to the sarcolemma and mitochondria, antioxidant effects and oxygen free radical production (29,30). Our previous study indicated that NO played a vital role in the cardioprotective effects of APC (11). APC alleviated cardiac dysfunction caused by IR, reduced the area of infarction after ischemia, and led to higher levels of eNOS and nNOS.
phosphorylation, NOS content and NO production. The present study is consistent with our previous reports (31,32). NO, a neurosensor of parasympathetic nerves, had a significant effect on the promotion of vagus nerves by increasing the release of ACh and reducing the downstream effects of catecholamines on heart rate and contractility (33). Thus, control of cardiac contraction through eNOS activation may represent an important function of cholinergic receptor activation (34). In the present study, the mAChR antagonist ATR and the nAChR antagonist HEM eliminated the effects of APC on eNOS and nNOS phosphorylation, increased NOS content and NO production and antagonized the protective effects of APC on the heart. These results indicated that eNOS and nNOS phosphorylation is one of the downstream pathways of APC-induced cholinergic receptor activation and cardioprotection.

AMPK is a key cellular energy sensor and regulator of metabolic homeostasis (15). Increasing evidence suggested that AMPK dysfunction is associated with the occurrence and development of a number of cardiovascular diseases, including atherosclerosis, myocardial IR injury and cardiac remodeling (35-37). Activation of AMPK protected the myocardium from IR injury by regulating mitochondrial function (38) and preventing myocardial necrosis and systolic dysfunction (39). Other studies reported that VNS activates AMPK and is accompanied by CaMKKβ phosphorylation during myocardial ischemia (15). These findings suggested that the intrinsic cardiac nervous system may be involved in APC-mediated cardioprotective effects against IR injury through the cholinergic receptor and CaMKKβ/AMPK signaling during myocardial IR. The present study also demonstrated that APC significantly increased the phosphorylation of AMPK and CaMKKβ, and that the administration of ATR and HEM could suppress this increase in phosphorylation. This indicated that the activation of the CaMKKβ/AMPK signaling pathway by cholinergic receptors was involved in APC-mediated cardioprotection. Taken together, these results represented an important complement to the understanding of the role of cholinergic receptors in APC-induced cardioprotection against IR injury. Nonetheless, the myocardial protective properties of volatile anesthetics, including sevoflurane, may also be due to their cardiosuppressive nature. These cardiac depressant effects decrease myocardial oxygen demand and may thus improve the myocardial oxygen balance during ischemia (40). A previous study demonstrated that both isoflurane and sevoflurane increased coronary blood flow and decreased coronary vascular resistance, including resistance through the collateral circulation (41). Therefore, further research is required to ascertain the effects of APC on the microcirculation under myocardium ischemia conditions.

The present study has its limitations. Although HEM is widely used as a nAChR antagonist, it also has low affinity to M2 receptors (17). Therefore, in the present study, 50 µM was used in order to achieve high specificity to nicotinic receptors. Nevertheless, the potential nonspecific effects of HEM still remains a possibility. Moreover, the interaction between NO

Figure 5. Western blot analysis of AMPK, CaMKKβ, AMPK phosphorylation at Thr-172 and CaMKKβ phosphorylation at Ser-511 in the homogenates of myocardial tissue from rat hearts. (A) Western blot bands of AMPK and p-AMPK(Thr172) protein and the ratio of p-AMPK(Thr172)/total AMPK. (B) Western blot bands of CaMKKβ and p-CaMKKβ(Ser511) protein and the ratio of p-CaMKKβ(Ser511)/total CaMKKβ. APC-induced increases of AMPK and CaMKKβ phosphorylation were reduced by the muscarinic acetylcholine receptor antagonist atropine (ATR, 100 nM) and nicotinic acetylcholine receptor antagonist hexamethonium (HEM, 50 µM). The data are presented as the mean ± standard deviation. n=5 hearts/group. *P<0.05 vs. Sham group; &P<0.05 vs. IR group; #P<0.05 vs. APC group. CaMKKβ, calcium/calmodulin-dependent protein kinase kinase β; p, phosphorylated; IR, ischemia-reperfusion; APC, anesthetic preconditioning; ATR, atropine; HEM, hexamethonium; CTL, control.
and the CaMKβ/AMPK signaling pathway is still unclear. In addition, it was reported that cholinergic receptor activation also plays a cardioprotective role through other pathways, such as, phosphoinositide 3-kinase and Erk1/2 signaling (42), Bcl-2 family proteins and caspase-3-related pathways (42), as well as Akt and GSK-3β enzyme activity (43). The interaction between these signal pathways is still poorly understand. Lastly, cholinergic receptor activation has previously been reported to mediate apoptosis and oxidative stress during I/R-induced cell injury (21). However, how apoptosis, oxidative stress and CaMKβ/AMPK pathways regulate cholinergic receptors to resist IR injury through APC was not evaluated in the present study.

In summary, the present study demonstrated that APC could protect cardiac function against IR injury through the activation of cholinergic receptor-mediated eNOS, nNOS and CaMKβ/AMPK phosphorylation. The present findings may provide insight into novel mechanisms of APC-induced cardioprotection against IR injury.

Acknowledgements
The authors acknowledge Dr Tuanjie Che (Precision Medicine and Translational Medicine Laboratory, Suzhou Science & Technology Town Hospital, China) for his technical assistance in the study.

Funding
The present study was supported by the Suzhou Science and Technology Development Plan (grant nos. SS201756 and SS201613); the Suzhou New District Science and Technology Project (grant no. 2017Z004) and the National Science and Technology Development Plan (grant no. NSFC 81703501).

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
YY, YL, CW and JA were responsible for experimental design, data collection, data analysis and manuscript writing. YY, YL, JW, LH and SQ performed the experiments. JA revised manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The present study was approved by The Institutional Animal Care and Use Committee of the Affiliated Suzhou Science & Technology Town Hospital of Nanjing Medical University (grant no. IRB2018032).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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