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Protective Effects of Hydrogen Peroxide Against Ischemia/Reperfusion Injury in Perfused Rat Hearts

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Among the several mechanisms proposed for ischemic preconditioning (IPC), generation of reactive oxygen species (ROS) is reported to be involved in the cardioprotective effects of IPC. The present study was designed to investigate whether repetitive exposure to hydrogen peroxide (H_2O_2) can protect the myocardium against subsequent ischemia/reperfusion injury, and whether the H_2O_2-induced cardioprotection is related to the preservation of energy metabolism. Langendorff-perfused rat hearts were exposed to two, 5 min episodes of IPC or to various concentrations of H_2O_2 twice and then to 35 min global ischemia and 40 min reperfusion. Using ^{31}P nuclear magnetic resonance (^{31}P-NMR) spectroscopy, cardiac phosphocreatine (PCr) and ATP and intracellular pH (pH_i) were monitored. IPC and the treatment with 2 μmol/L H_2O_2 significantly improved the post-ischemic recovery of left ventricular developed pressure (LVDP) and the PCr and ATP compared with those of the control ischemia/reperfusion (LVDP: 36.9±7.4% of baseline in control hearts, 84.0±3.5% in IPC, 65.4±3.8% in H_2O_2; PCr: 51.1±5.3% in control hearts, 81.4±5.5% in IPC, 81.7±5.2% in H_2O_2; ATP: 12.3±1.6% in control hearts; 30.0±2.8% in IPC, 28.6±2.3% in H_2O_2, mean ± SE, $p<0.05$). However, lower (0.5 μmol/L) or higher (10 μmol/L) concentration of H_2O_2 had no effect. There were significant linear correlations between mean LVDP and high-energy metabolites after 40 min reperfusion in H_2O_2-treated hearts. In IPC-treated hearts, the mean LVDP was greater than that in the 2 μmol/L H_2O_2-treated hearts under similar levels of high-energy metabolites. IPC also ameliorated intracellular acidification (6.38±0.03 in control hearts, 6.65±0.04 in IPC, $p<0.05$), but treatment with H_2O_2 did not affect pH_i during ischemia (6.40±0.05 in H_2O_2). In conclusion, H_2O_2 had protective effects against ischemia/reperfusion injury and the effects were related to the preservation of energy metabolism. IPC could have additional protective mechanisms that are associated with the amelioration of intracelluar acidosis during ischemia. (*Circ J* 2003; **67:** 253–258)

Key Words: Energy metabolism; Ischemic preconditioning; Oxygen species; Perfused hearts; NMR spectroscopy

Ischemic preconditioning (IPC) is a cardioprotective phenomenon that was proposed initially by Murry et al[1] Single or multiple brief episodes of ischemia/reperfusion protect the myocardium from the injury caused by subsequent prolonged ischemia/reperfusion. Various agonists such as adenosine, bradykinin and opioids are involved in the mechanism of IPC through intracellular signaling pathways[2]

The generation of reactive oxygen species (ROS) is known to be a major cause of ischemia/reperfusion injury[3–5] and recent studies have reported that ROS can also exert IPC-like protective effects in ischemic/reperfused myocardium[6,7] In those cases, the brief exposure to exogenous oxygen species induced beneficial effects against subsequent ischemia/reperfusion injury. Therefore, it is suggested that the generation of ROS may be involved in the protective effects of IPC, although their source and concentrations remain unknown.

Although ROS affect many membrane proteins, such as sarcolemmal Na^+ and Ca^{2+} channels, Na^+/Ca^{2+} exchange, the sarcolemmal and sarcoplasmic reticulum (SR) Ca^{2+}-ATPase and ryanodine receptors[8–13] recent reports have shown that ROS can also alter mitochondrial function through the mitochondrial ATP-sensitive potassium (mitoK$_{ATP}$) channels and the mitochondrial permeability transition pore (mPTP)[14–19] Therefore, it is expected that the protective effects of ROS against ischemia/reperfusion injury are dependent on the preservation of energy metabolism, but unfortunately, there have not been any reports of the effects of ROS in view of the correlation between contractile function and energy metabolism.

The purpose of this study was to investigate whether repeated exposure to hydrogen peroxide (H_2O_2) can protect the myocardium against subsequent ischemia/reperfusion injury, and whether the IPC- and H_2O_2-induced cardioprotection relates to the preservation of energy metabolism. To clarify these issues, we used ^{31}P nuclear magnetic resonance (^{31}P-NMR) spectroscopy to investigate the time courses of the contractile parameters and high-energy meta-

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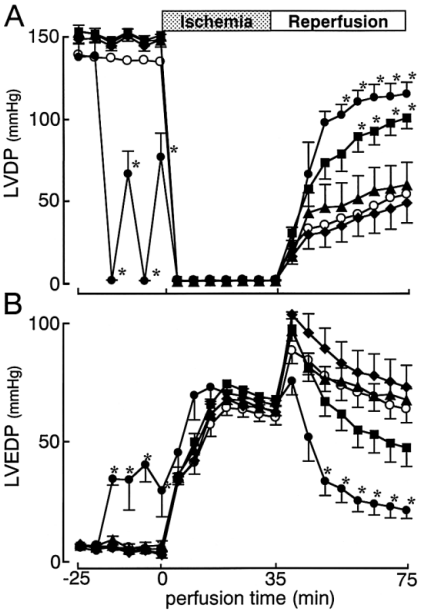

Fig 1. Time course of LVDP (A) and LVEDP (B) during ischemia/reperfusion. (A) In control hearts (open circles), LVDP decreased rapidly and then almost disappeared during ischemia. After reperfusion, LVDP recovered gradually. IPC (closed circles) decreased the basaline LVDP, but improved the recovery of LVDP at reperfusion. The treatment with 2 μmol/L H_2O_2 (closed squares) also improved the recovery of LVDP, but lower (0.5 μmol/L; closed triangles) and higher (10 μmol/L; closed diamonds) concentrations of H_2O_2 induced no significant improvement. (B) In control hearts, LVEDP increased gradually and reached a plateau during ischemia. After reperfusion, LVEDP elevated transiently and thereafter decreased gradually, but did not fall below the end-ischemic level. IPC increased LVEDP, but accelerated the recovery of LVEDP after reperfusion. The treatment with H_2O_2 at any concentrations had no significant effects on LVEDP. Values are means ± SEM. *p<0.05 vs control hearts.

bolites during global ischemia/reperfusion in Langendorff-perfused rat hearts.

Methods

Isolated Rat Heart Preparations

Male Sprague-Dawley rats weighing 320–400 g were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital. After intravenous injection of 400 U/kg heparin sodium, the hearts were removed and perfused retrogradely through the aorta in a non-circulating Langendorff apparatus with modified Krebs-Henseleit (K-H) buffer consisted of (mmol/L) NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 1.25, $NaHCO_3$ 25, and glucose 11. The buffer was saturated with 95% O_2–5% CO_2 (pH 7.4, 37°C) for 40 min. Hearts were perfused at a constant pressure of 90 cm H_2O. The investigation conformed to 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).

Measurement of Cardiac Performance

A water-filled latex balloon-tipped catheter was inserted into the left ventricle through the left atrium and was adjusted to a left ventricular end-diastolic pressure (LVEDP) of 5–10 mmHg during the initial equilibration. The distal end of the catheter was connected to a pressure transducer (Nihon Kohden Co, Japan) via a polyethylene tube for continuous measurement of left ventricular (LV) pressure. The heart was electrically paced at a rate of 5 Hz through 3 mol/L KCl-agar salt bridges. Cardiac function was determined using LV developed pressure (LVDP), which was calculated from the difference between LV peak systolic pressure and LVEDP. LV peak systolic pressure, LVEDP and heart rate were monitored on a polygraph. The coronary effluent was collected in a beaker, and coronary flow (CF) was determined volumetrically at the end of equilibration during the treatment and reperfusion periods.

Table 1 Changes in Coronary Flow During Ischemia/Reperfusion

(ml/min)	*Baseline*	*Pre-ischemia*	*Reperfusion 40 min*
Control	*15.2±0.7*	*14.9±0.7*	*7.3±0.8†*
IPC	*15.3±0.7*	*14.4±1.5*	*11.3±1.3*†*
H_2O_2			
0.5 μmol/L	*15.6±0.2*	*15.9±0.4*	*8.9±1.1†*
2 μmol/L	*14.9±0.4*	*15.4±0.8*	*10.4±0.5†*
10 μmol/L	*14.4±0.8*	*15.3±0.9*	*7.8±0.7†*

*Values are means±SE. †p<0.05 vs Baseline, *p<0.05 vs Control by ANOVA.*

^{31}P NMR Spectroscopy

The details of the measurement of high-energy metabolites by ^{31}P-NMR have been described previously[20] Briefly, the heart was placed in a sample tube (17 mm in diameter) that was mounted in a single-turn Helmholz coil. The temperature around the heart was maintained at 37°C by warming the sample tube with a heat-controlled water jacket. We used a JEOL GSX 270 wide-bore NMR spectrometer (6.35 tesla; Nihon Denshi, Japan) and operated at 109.16 MHz. We signal-averaged 296 scans of a 45° broadband pulse with a 1-s interpulse delay, and 5 min was required to obtain each spectrum. The intracellular contents of phosphocreatine (PCr), ATP and inorganic phosphate (P_i) were determined from their respective peak areas compared with the external standard peak area of methylene diphosphonate (MDP), which was enclosed in a capillary tube and fixed inside the sample tube. The intracellular pH (pH_i) was calculated from the difference of the chemical shifts between the P_i and PCr peaks.

Experimental Protocols

After the hearts were excised and cannulated, they were allowed to stabilize for a period of at least 20 min before treatment. Five groups of hearts were used for the ^{31}P-NMR and LV pressure measurements. The treatment groups consisted of perfusion with either modified K-H buffer alone (control) (n=8), ischemic preconditioning (IPC, n=6), 0.5 μmol/L H_2O_2 (n=6), 2 μmol/L H_2O_2 (n=8), and 10 μmol/L H_2O_2 (n=6). IPC was obtained with 2 episodes of 5 min global ischemia, and H_2O_2 at each concentration was perfused twice for 5 min. After the treatment period, hearts were subjected to 35 min of global ischemia followed by 40 min of reperfusion. There was no H_2O_2 in the buffer during reperfusion.

Statistical Analysis

All values are expressed as means ± SEM. Group comparisons were performed by analysis of variance (ANOVA), followed by Bonferroni's post-hoc test. A value of $p<0.05$ was considered significant.

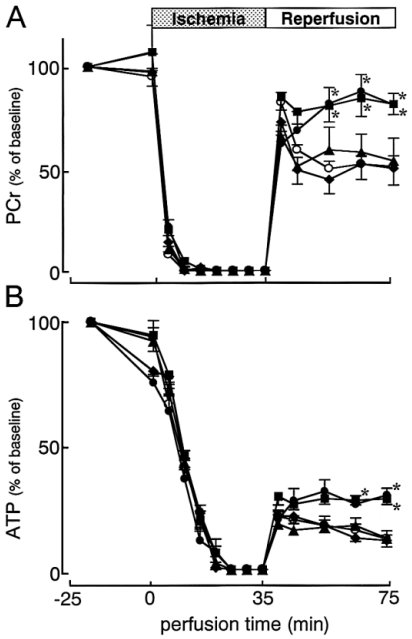

Fig 2. Time courses of PCr (A), ATP (B) during ischemia/reperfusion. (A, B) In control hearts (open circles), both PCr and ATP decreased during ischemia and became undetectable. After reperfusion, they recovered transiently, but then decreased again and the levels became plateau. IPC (closed circles) and the treatment with 10 μmol/L H_2O_2 (closed diamonds) tended to decrease the baseline ATP. Both IPC and the treatment with 2 μmol/L H_2O_2 (closed squares) significantly improved the recovery of them at reperfusion to similar extent. However, lower (0.5 μmol/L; closed triangles) and higher (10 μmol/L) concentrations of H_2O_2 induced no significant improvement in PCr or ATP. Values are means ± SEM. *p<0.05 vs control hearts.

Results

H_2O_2-Induced Improvement in Post-Ischemic Contractile Function

Fig 1 shows the time courses of the various contractile parameters during ischemia/reperfusion in the 5 experimental groups. There were no significant differences among the groups in basal LVDP and LVEDP. IPC decreased LVDP and increased LVEDP, because the subsequent global ischemia was applied before they completely recovered. Treatment with H_2O_2 in the range of 0.5–10 μmol/L did not induce any changes in LVDP or LVEDP. In the ischemic period, LVDP decreased rapidly during the initial 5 min and then almost disappeared. LVEDP increased gradually and reached a plateau around 20 min of ischemia. IPC and the treatment with H_2O_2 did not alter contractile function during ischemia. After reperfusion, LVDP recovered gradually and reached 52.0±11.2 mmHg at 40 min (36.9±7.4% of the pre-ischemic level). LVEDP elevated transiently and thereafter decreased gradually, but did not fall below the end-ischemic levels. IPC and the treatment with 2 μmol/L H_2O_2 significantly improved the recovery of LVDP to 106.2±5.8 mmHg and 97.5±6.4 mmHg, respectively (84.0±3.5% and 65.4±3.8% of the pre-ischemic levels, p<0.05 vs control hearts). IPC also tended to reduce the transient increase in LVEDP and accelerated the following recovery. The treatment with 2 μmol/L H_2O_2 did not affect the transient increase in LVEDP, but tended to stimulate the following reduction. Lower (0.5 μmol/L) and higher (10 μmol/L) concentrations of H_2O_2 induced no significant improvement in the contractile parameters.

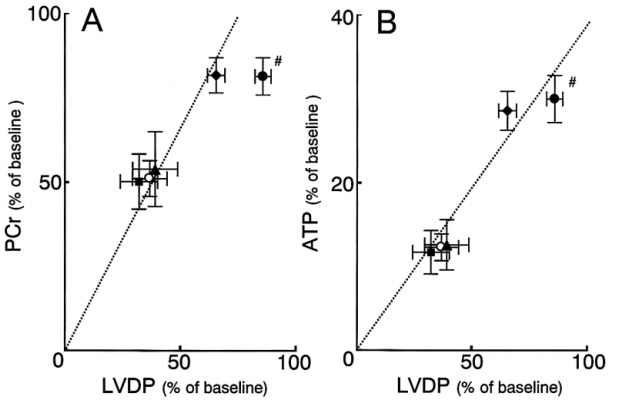

Fig 3. The relationships between mean LVDP and PCr (A) and ATP (B) after reperfusion. The mean values of PCr (A) and ATP (B) at 40 min of reperfusion were plotted against comparable LVDP in control hearts (open circles), IPC (closed circles), and H_2O_2-treated hearts (0.5 μmol/L: closed triangles; 2 μmol/L: closed square; and 10 μmol/L: closed diamonds). There were significant positive correlations between LVDP and PCr (r=0.93), and between LVDP and ATP levels (r=0.94) in control and H_2O_2-treated hearts (p<0.05). In IPC-treated hearts, the mean LVDP was significantly higher than that in 2 μM H_2O_2-treated hearts even under the similar PCr and ATP concentrations. #p<0.05 vs 2 μmol/L H_2O_2 in LVDP.

With respect to CF, there were no significant differences among the groups in either basal or pre-ischemic values. However, CF after 40 min of reperfusion was significantly higher in IPC compared with that in control hearts, whereas the treatment with H_2O_2 did not affect it (Table 1).

H_2O_2-Induced Improvement in the Metabolic Parameters

Next, we investigated the effects of IPC and the treatment with H_2O_2 on high-energy metabolites using ^{31}P-NMR spectroscopy (Fig 2). IPC and 10 μmol/L H_2O_2 tended to decrease ATP, whereas H_2O_2 at 0.5 μmol/L and 2 μmol/L did not induce any changes in their basal levels. In the ischemic period, PCr decreased rapidly and could not be detected at 10 min. The reduction in ATP was slower than that in PCr, but ATP also became undetectable after approximately 25 min of ischemia. IPC and the treatment with H_2O_2 had no effects on the levels of PCr and ATP during ischemia. After reperfusion, both PCr and ATP recovered rapidly at the initial 5 min, but then decreased again and their levels reached a plateau until 40 min of reperfusion (51.1±5.3% and 12.3±1.6% of pre-ischemic levels). Both IPC and the treatment with 2 μmol/L H_2O_2 significantly improved the recovery of high-energy metabolites to similar extent (83.5±5.4% and 29.3±3.3% in IPC, 81.7±5.2% and 28.6±2.3% in H_2O_2, respectively, p<0.05 vs control hearts). However, lower (0.5 μmol/L) and higher (10 μmol/L) concentrations of H_2O_2 induced no significant improvement in high-energy metabolites.

Relationships Between LVDP and High-Energy Metabolites After Reperfusion

In order to clarify the relationship between the recovery of contractile function and the levels of the high-energy metabolites after reperfusion, the mean values of PCr (Fig 3A) and ATP (Fig 3B) at 40 min of reperfusion were plotted against comparable LVDP. When the regression lines included zero, there were significant positive correlations between LVDP and PCr (r=0.93) and between LVDP and ATP levels (r=0.94) in control hearts, 0.5 μmol/L,

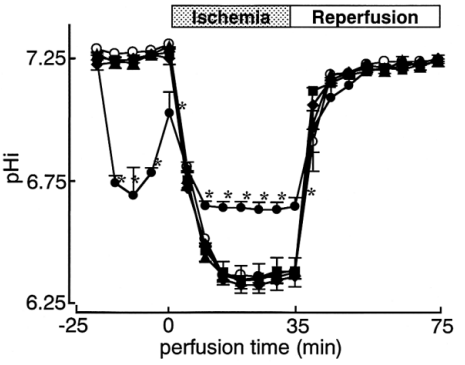

Fig 4. Time course of pH_i during ischemia/reperfusion. In control hearts (open circles), pH_i fell rapidly after starting ischemia and remained constant throughout the ischemic periods. After reperfusion, pH_i recovered rapidly and almost completely until 40 min of reperfusion. IPC (closed circles) significantly reduced pH_i and the value at the onset of ischemia was significantly lower than that in control hearts. However, IPC ameliorated the decrease in pH_i during ischemia, while the treatment with H_2O_2 at any concentrations (closed triangles, squares and diamonds) did not affect the basal pH_i and the changes during ischemia/reperfusion. Values are means ± SEM. *$p<0.05$ vs control hearts.

2 μmol/L and 10 μmol/L H_2O_2-treated hearts ($p<0.05$). In IPC-treated hearts, the mean LVDP was significantly higher than that in 2 μmol/L H_2O_2-treated hearts, even with similar levels of PCr and ATP. The relationship between LVDP and ATP apparently altered after reperfusion compared with that in the pre-ischemic period. Because LVDP also relies on the level of LVEDP, a large increase in LVEDP after reperfusion might augment LVDP at a relatively small recovery of ATP. The mean LVEDP at 40 min of reperfusion was also smaller in IPC-treated hearts than that in 2 μmol/L H_2O_2-treated hearts at comparable PCr and ATP levels (data not shown).

IPC-Induced Amelioration in Intracellular Acidosis During Ischemia

During ischemia, pH_i fell rapidly and remained constant throughout the ischemic period. After reperfusion, pH_i recovered rapidly and almost completely until 40 min of reperfusion (Fig 4). IPC significantly reduced pH_i and the value at the onset of ischemia was significantly lower than that in control hearts. However, IPC ameliorated the decrease in pH_i during ischemia, whereas treatment with H_2O_2 at any concentration did not affect the basal pH_i or the changes in pH_i during ischemia/reperfusion.

Discussion

By using ^{31}P-NMR spectroscopy in perfused rat hearts, we could clearly demonstrate that (1) IPC and repeated exposure to 2 μmol/L H_2O_2 significantly improved contractile function after reperfusion without changing CF, whereas lower (0.5 μmol/L) and higher (10 μmol/L) concentrations of H_2O_2 had no effect; (2) both the IPC- and H_2O_2-induced contractile recovery related to the preservation of high-energy metabolites; and (3) IPC ameliorated the intracellular acidification during ischemia, whereas treatment with H_2O_2 did not affect it. These findings suggest that the generation of ROS during brief episodes of ischemia/reperfusion may preserve energy metabolism against subsequent ischemia/reperfusion, thereby improving contractile function after reperfusion. This scenario at least partly explains the protective effects of IPC, but it may have additional protective mechanisms, because in the present study there was a clear difference between IPC and H_2O_2 in the effect on pH_i during ischemia.

ROS, such as superoxide anion (O_2^-), H_2O_2 and hydroxyl radical (OH^-), are generated during ischemia/reperfusion, and have toxic effects in ischemic/reperfused myocardium;[3–5] such as shortening of action potential duration (APD), a decrease in developed force, and a rise in diastolic tension (contracture). Although the details of how ROS worsen cardiac function are complex and are not entirely understood, cellular Ca^{2+} mishandling appears to be a common endpoint. Actually, many Ca^{2+} regulatory proteins can be affected by ROS, including the SR Ca^{2+} ATPase, ryanodine receptors, Na^+/Ca^{2+} exchange, the Na^+/K^+ ATPase, and the sarcolemmal Ca^{2+} ATPase[8–13] Because Ca^{2+} leak and Na^+ window current may also be increased by ROS, cellular Na^+ and Ca^{2+} gain may mediate Ca^{2+} overload.

On the other hand, ROS generated during a brief period of ischemia/reperfusion can induce cardioprotection against subsequent ischemia/reperfusion injury. Previous studies have suggested that exogenous oxygen species induce IPC-like effects. Tritte et al showed that a low flux of oxygen radicals generated by purine/xanthine oxidase reproduced the beneficial effects of IPC both on the infarct size and on the post-ischemic recovery of myocardial contractile function in isolated rabbit hearts[21] Vanden Hoek et al reported that the exposure of chick embryonic ventricular myocytes to 15 μmol/L H_2O_2 decreased subsequent hypoxia/reoxygenation-induced cell death[16] Additionally, it has been indicated that free radical scavengers when given prior to IPC attenuate the response to IPC. Vanden Hoek et al showed that free radical scavengers abrogated the protection induced by hypoxic preconditioning;[16] and Tanaka et al also reported that treatment with superoxide dismutase and N-2-mercaptopropionyl glycine attenuated the limiting effect of IPC for infarct size in rabbit hearts[22] These results suggest that the cardioprotection induced by IPC may be mediated, at least in part, by ROS generated during brief ischemia/reperfusion.

Recent reports have shown that ROS can also affect mitochondrial function through mitoK$_{ATP}$ channels and mPTP[14–19] It is now widely accepted that mitoK$_{ATP}$ channels are an important end effector of IPC or a mediator of the signal transduction pathways[2,23–25] The irreversible opening of mPTP has been recognized as an early event in lethal cell damage (eg, apoptosis);[17,26] however, recent studies have revealed the existence of the flickering of mPTP (low-conductance state) and this brief and reversible opening of mPTP may relieve mitochondria of the excessive load of metabolites or ions (eg, Ca^{2+})[14,15,27,28] We recently reported that diazoxide, a selective mitoK$_{ATP}$ channels opener, may accelerate the low-conductance state of mPTP[29]

In the present findings, a lower concentration (2 μmol/L) of H_2O_2 improved the recovery of both cardiac contractile function and energy metabolism after reperfusion, whereas a higher concentration (10 μmol/L) did not. Although why the action of H_2O_2 changed drastically between 0.5 and 10 μmol/L is unclear, Takeshima et al showed that 20 μmol/L H_2O_2 prior to sustained ischemia completely abolished severe reperfusion arrhythmias in rat hearts, but all hearts treated with 160 μmol/L H_2O_2 had severe arrhythmias throughout reperfusion[30] Therefore, at higher concentra-

tions, H_2O_2 may itself induce deleterious effects on ischemic/reperfused myocardium, by the hydroxyl radical and by lipid peroxidation[30,31] In fact, treatment with 10 μmol/L H_2O_2 tended to reduce the baseline ATP whereas H_2O_2 at lower concentrations did not affect it. In the present study, the concentration of H_2O_2 that produced protective effects was somewhat lower and limited to a relatively narrow range compared with those reported previously. The reason for this discrepancy is unknown, but may be ascribed to differences in animal species or in duration and/or time of exposure to H_2O_2. Furthermore, the source and exact concentrations of oxygen species produced by various lengths of ischemia/reperfusion remain unknown.

In the present study, IPC decreased the basal contractile function and ameliorated the intracellular acidification during prolonged ischemia. Furthermore, the mean LVDP at 40 min of reperfusion was higher than that in 2 μmol/L H_2O_2-treated hearts, even with similar levels of PCr and ATP (see Fig 3). These findings suggest that IPC could have exerted a protective action through additional mechanisms to that of preservation of high-energy metabolites. A previous report has shown that IPC may decrease H^+ production from glycolysis and activate extrusion mechanisms during subsequent ischemia[32] The intracellular acidosis during ischemia causes cellular Na^+ loading by activating Na^+/H^+ exchange and inhibiting the Na^+/K^+ pump and this Na^+ loading in turn leads to Ca^{2+} overload via Na^+/Ca^{2+} exchange on reperfusion[33] Furthermore, the rapid return of pH_i also results in the pH paradox by activating Ca^{2+}-dependent degradative enzymes. The amelioration of intracellular acidosis by IPC may therefore prevent the irreversible cellular damage caused by Ca^{2+} overload[34] Additionally, IPC induces the opening of sarcolemmal K^+_{ATP} channels via activation of protein kinase C and it has been reported that the opening of sarcolemmal K^+_{ATP} channels enhances membrane repolarization and a shortening of APD, leading to the reduction of Ca^{2+} entry into cardiac cells through voltage-gated Ca^{2+} channels and Na^+/Ca^{2+} exchange[35–37] Such reduction in Ca^{2+} entry may also prevent Ca^{2+} overload, although the shortening of APD per se could not protect the myocardium against ischemia/reperfusion injury[38] In the present study, IPC tended to reduce the transient increase in LVEDP at reperfusion, and accelerated the following recovery, suggesting a reduction in cellular Ca^{2+} overload.

In conclusion, the generation of ROS during brief periods of ischemia can improve cardiac contractile function after reperfusion. The preservation of energy metabolism is suggested as a possible mechanism of the protective action, but further studies are needed to clarify the precise mechanism of IPC.

Acknowledgments

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