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Prolonged transient acidosis during early reperfusion contributes to the cardioprotective effects of postconditioning

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Fujita M, Asanuma H, Hirata A, Wakeno M, Takahama H, Sasaki H, Kim J, Takashima S, Tsukamoto O, Minamino T, Shinozaki Y, Tomoike H, Hori M, Kitakaze M. Prolonged transient acidosis during early reperfusion contributes to the cardioprotective effects of postconditioning. *Am J Physiol Heart Circ Physiol* 292: H2004–H2008, 2007. First published January 5, 2006; doi:10.1152/ajpheart.01051.2006.—We have previously reported that the prolonged transient acidosis during early reperfusion mediates the cardioprotective effects in canine hearts. Recently, postconditioning has been shown to be one of the novel strategies to mediate cardioprotection. We tested the contribution of the prolonged transient acidosis to the cardioprotection of postconditioning. Open-chest anesthetized dogs subjected to 90-min occlusion of the left anterior descending coronary artery and 6-h reperfusion were divided into four groups: *1*) control group; no intervention after reperfusion ($n = 6$); *2*) postconditioning (Postcon) group; four cycles of 1-min reperfusion and 1-min reocclusion ($n = 7$); *3*) Postcon + sodium bicarbonate ($NaHCO_3$) group; four cycles of 1-min reperfusion and 1-min reocclusion with the administration of $NaHCO_3$ ($n = 8$); and *4*) $NaHCO_3$ group; administration of $NaHCO_3$ without postconditioning ($n = 6$). Infarct size, the area at risk (AAR), collateral blood flow during ischemia, and pH in coronary venous blood were measured. The phosphorylation of Akt and extracellular signal-regulated kinase (ERK) in ischemic myocardium was assessed by Western blot analysis. Systemic hemodynamic parameters, AAR, and collateral blood flow were not different among the four groups. Postconditioning induced prolonged transient acidosis during the early reperfusion phase. Administration of $NaHCO_3$ completely abolished the infarct size-limiting effects of postconditioning. Furthermore, the phosphorylation of Akt and ERK in ischemic myocardium induced by postconditioning was also blunted by the cotreatment of $NaHCO_3$. In conclusion, postconditioning mediates its cardioprotective effects possibly via prolonged transient acidosis during the early reperfusion phase with the activation of Akt and ERK.

acidosis; reperfusion; postconditioning; reperfusion injury salvage kinase

POSTCONDITIONING, defined as brief periods of ischemia immediately after the onset of reperfusion, has been recently shown to be one of the novel strategies of cardioprotection targeting the early reperfusion phase, which protects the myocardium against ischemia and reperfusion injury (12, 19, 23). Recent studies have revealed that one promising explanation for this cardioprotection is the upregulation of prosurvival kinase named the reperfusion injury salvage kinase (RISK), consisting of phosphatidylinositol 3-kinase-Akt and extracellular signal-regulated kinase (ERK) (20, 22). On the other hand, we have previously reported that the prolonged transient acidosis during the early reperfusion phase induced by the staged reperfusion, inhalation of carbon dioxide (CO_2), or infusion of hydrochloric acid (HCl) attenuated the severity of myocardial stunning or size of infarction in canine hearts (9, 14). Considering these results, we developed the hypothesis that postconditioning causes the prolonged transient acidosis during the early reperfusion phase, and, if so, we further tested the idea that this acidosis, evoked by the procedure of brief ischemia, activates RISK pathways, contributing to the beneficial effects of postconditioning.

To test our hypothesis, this study was conducted to investigate whether *1*) postconditioning induces the prolonged transient acidosis during the early reperfusion phase, *2*) cardioprotective effects of postconditioning are blunted by the infusion of sodium bicarbonate ($NaHCO_3$), and finally, *3*) the activation of RISK pathways induced by postconditioning is also abolished by the infusion of $NaHCO_3$ in canine hearts.

MATERIALS AND METHODS

The investigation conformed with the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996). Experimental protocols were approved by the Osaka University Ethical Committee for Laboratory Animal Use.

Materials

Antibodies against phospho-Akt and Akt were obtained from Cell Signaling Technologies (Beverly, MA), and antibodies against phospho-ERK and ERK were from Promega (Madison, WI).

Instrumentation

Beagle dogs (Oriental Yeast, Tokyo, Japan) weighing 8–12 kg were prepared as described previously (17). Briefly, the trachea was intubated, and the dog was ventilated with room air mixed with oxygen. The chest was opened through the left fifth intercostal space, and the heart was suspended in a pericardial cradle. After heparinization (500 U/kg), the proximal portion of the left anterior descending coronary artery (LAD) was cannulated and perfused with blood via the carotid artery through an extracorporeal bypass tube. A small

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collecting tube was inserted into a small coronary vein near the perfused area to sample coronary venous blood. The drained venous blood was collected in a reservoir placed at the level of the left atrium. We selectively infused $NaHCO_3$ into the LAD-perfused area through this bypass tube. The left atrium was catheterized for the microsphere injection to measure myocardial collateral blood flow at 80 min of sustained ischemia. Hydration was maintained by a slow normal saline infusion. The femoral artery was also cannulated to measure the systemic mean arterial blood pressure (MAP). Both MAP and heart rate (HR) were monitored continuously during the study.

Experimental Protocols

Protocol I: effects of postconditioning on pH in coronary venous blood during early reperfusion. First, to confirm that postconditioning induces the prolonged transient acidosis during the early reperfusion phase, coronary venous blood was sampled for gas analysis in dogs with or without four cycles of 1-min coronary occlusion and a subsequent 1-min reperfusion (postconditioning) at baseline, 90 min of ischemia, and the end of postconditioning procedures ($n = 3$ each) (Fig. 1).

Protocol II: effects of the infusion of $NaHCO_3$ on the postconditioning-induced infarct size-limiting effects. Next, we investigated whether cardioprotective effects of postconditioning are blunted by the correction of prolonged transient acidosis during early reperfusion. Figure 2 indicates all details of the schedules of this protocol. Six dogs were subjected to 90 min of ischemia and subsequent 6-h reperfusion without any intervention (control group, $n = 6$). In other dogs, after 90 min of ischemia, the postconditioning procedure was performed by four cycles of 1-min coronary occlusion separated by 1-min reperfusion by occluding the bypass tube just after reperfusion [postconditioning (Postcon) group, $n = 7$]. To examine the effects of adjustment of acidosis during early reperfusion, we infused 10 μmol/kg of $NaHCO_3$ intravenously just before the end of 90 min of ischemia and continuously infused $NaHCO_3$ into the LAD at 100 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ during postconditioning procedures (Postcon + $NaHCO_3$ group, $n = 8$). Furthermore, to elucidate the sole effects of $NaHCO_3$ on infarct size, we infused $NaHCO_3$ into six dogs in the same maneuver as with the Postcon + $NaHCO_3$ group without the postconditioning ($NaHCO_3$ group, $n = 6$). All dogs were subjected to 6-h reperfusion, and infarct size was assessed at 6-h reperfusion.

Protocol III: effects of the infusion of $NaHCO_3$ on postconditioning-induced activation of RISK pathways. To investigate the effects of the postconditioning procedure on the activation of RISK in the heart, we performed Western blot analysis using four dogs in this protocol. At 15 min after the end of 90-min ischemia or brief ischemia of postconditioning with and without the infusion of $NaHCO_3$, hearts were excised and the myocardial tissue from the endocardial to epicardial portion at risk, which was identified as the edge of the region showing necrosis, was quickly placed into liquid nitrogen and

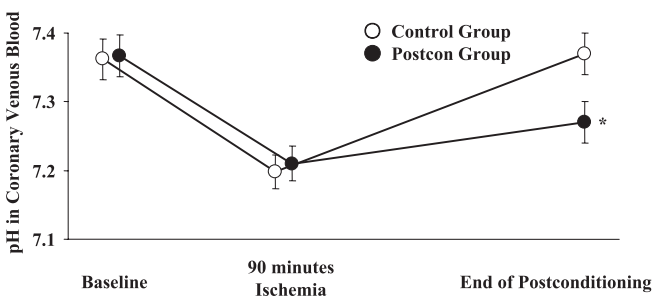

Fig. 1. The serial changes in pH in coronary venous blood before and during the early reperfusion phase with or without the postconditioning procedure performed by 4 cycles of 1 min of coronary occlusion and a subsequent 1 min of reperfusion after 90 min of ischemia. Postcon group, postconditioning group. *$P < 0.05$ vs. control group.

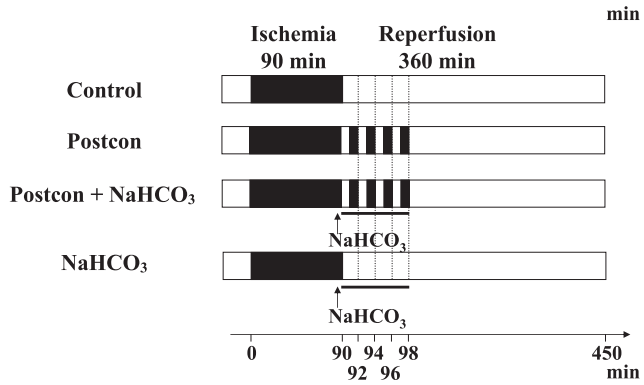

Fig. 2. The time course of the experimental *protocol II*.

stored at −80°C. Phosphorylation of Akt and ERK and total content of Akt and ERK were evaluated by Western blot analysis as reported previously (8). The immunoreactive bands were quantified by densitometry (Molecular Dynamics).

Criteria for Exclusion

To ensure that all animals included in the data analysis of infarct size were healthy and exposed to similar extents of ischemia, exclusion criteria described previously were used (1, 17). Briefly, the following standards were employed for the exclusion of unsatisfactory dogs: *1*) subendocardial collateral blood flow >15 $ml \cdot 100\ g^{-1} \cdot min^{-1}$; *2*) HR >170 beats/min; and *3*) more than two consecutive attempts required to terminate ventricular fibrillation using low-energy DC pulses applied directly to the heart.

Measurements of Infarct Size and Collateral Blood Flow

Six hours after the onset of reperfusion, while the LAD was reoccluded and perfused with autologous blood, Evans blue dye was injected into a systemic vein to identify the area at risk and the nonischemic area in the hearts. The heart was then immediately removed and sliced into serial transverse sections that were 6–7 mm in width. The nonischemic area was defined as the tissue showing blue staining. The ischemic region was harvested and incubated at 37°C for 20–30 min in 1% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma Chemical) in 0.1 mol/l phosphate buffer adjusted to pH 7.4. TTC stains the noninfarcted myocardium a brick-red color, indicating the presence of a formazan product created through the reduction of TTC by dehydrogenases in viable tissues. In *protocol II*, the area of myocardial necrosis and the area at risk were measured in all of the dogs on completion of the protocol by an operator who had no knowledge of the treatment given to each animal. Infarct size was expressed as a percentage of the area at risk. Regional myocardial blood flow was determined as described previously (16). Nonradioactive microspheres (Sekisui Plastic, Tokyo, Japan) made of inert plastic were labeled with bromine (Br). The microspheres were administered 80 min after the start of coronary occlusion. The radio fluorescence of the stable heavy elements was measured with a wavelength dispersive spectrometer (PW 1480, Phillips, Almelo, The Netherlands). Because the level of energy emitted is characteristic for specific elements, it was possible to quantify the radio fluorescence of the heavy element with which the microspheres were labeled. Myocardial blood flow was calculated according to the following formula: time flow = (tissue count) × (reference flow)/(reference count) and was expressed in milliliters per minute per gram wet weight. Endomyocardial blood flow was measured at the inner half of the LV wall. For randomization of the study, all measurements were done at the completion of protocol without knowledge of the treatment in each heart.

Table 1. *Heart rate and mean arterial pressure for experimental groups*

Group	Baseline	90-min Ischemia	Reperfusion, min 60	180	360
Heart rate, beats/min					
Control	126±3	122±9	125±6	129±4	120±7
Postcon	125±5	122±9	123±7	124±7	121±7
Postcon + $NaHCO_3$	129±7	125±6	123±5	122±5	121±5
$NaHCO_3$	127±12	120±10	127±12	119±10	127±14
Mean arterial pressure, mmHg					
Control	102±9	97±4	93±7	96±6	99±5
Postcon	99±6	95±4	94±3	95±5	93±3
Postcon + $NaHCO_3$	102±3	97±5	93±9	90±9	92±5
$NaHCO_3$	100±2	98±6	93±3	93±7	94±9

Values are means ± SE. Postcon, postconditioning.

Statistical Analysis

Value are expressed as means ± SE. Comparisons of the time course of the change in HR, MAP, and pH in coronary venous blood between groups were performed using two-way repeated-measures ANOVA. Comparisons of other data between groups were performed using one-way factional ANOVA with the Fisher post hoc test. Analysis of covariance by regional collateral flow in the inner half LV wall as the covariate was used to account for the effect of collateral blood flow on infarct size. A P value <0.05 was considered to represent statistical significance.

RESULTS

Mortality and Exclusions

Of 35 dogs that were randomly assigned to four groups for assessment of infarct size in *protocol II*, five dogs developed lethal arrhythmia (ventricular tachycardia or fibrillation) at least once. Among these five dogs, lethal arrhythmia to match the exclusion criteria occurred in two dogs during 90 min of ischemia and in three dogs during reperfusion after 90 min of ischemia. These five animals were excluded from the assessment of infarct size. In the remaining 30 dogs, three were excluded from the data analysis because myocardial collateral blood flow was >15 ml·100 g^{-1}·min^{-1}. Therefore, 27 dogs completed *protocol II* satisfactorily and were used for data analysis.

Protocol I: Serial Changes in pH in Coronary Venous Blood During Early Reperfusion

Figure 1 shows the serial changes in pH in coronary venous blood at baseline, 90-min ischemia, and the end of the postconditioning procedure (8 min after the end of 90-min ischemia). The pH in coronary venous blood at baseline and 90 min ischemia was similar between two groups (7.36 ± 0.03 and 7.20 ± 0.02 in control group and 7.37 ± 0.03 and 7.21 ± 0.03 in Postcon group, respectively). The pH in coronary venous blood at the end of postconditioning procedures was significantly lower compared with that of the control group at the corresponding time (7.37 ± 0.03 in control group and 7.27 ± 0.03 in Postcon group, respectively). These results suggested that postconditioning procedures induced the prolonged transient acidosis during the early reperfusion phase.

Protocol II: Changes in Hemodynamic Parameters, Area at Risk, and Collateral Blood Flow Among All Groups

There were no significant differences in both HR and MAP among all groups in each experiment (Table 1). Both area at risk and collateral blood flow at 80 min of sustained ischemia were comparable in all groups (Fig. 3, *A* and *B*, respectively).

Infarct Size Normalized by Area at Risk Among All Groups

Figure 4*A* shows the infarct size normalized by the area at risk in the four groups in *protocol I*, and Fig. 4*B* illustrates the regression plots of the infarct size as a percentage of the area at risk against collateral blood flow in the four groups. Postconditioning procedure (Postcon group) markedly attenuated infarct size compared with the control group (11.8 ± 3.1% vs. 36.7 ± 5.0%, respectively, $P < 0.05$). The infusion of $NaHCO_3$ (Postcon + $NaHCO_3$ group) completely abolished the infarct size-limiting effects of postconditioning (35.3 ± 5.3%). The infusion of $NaHCO_3$ alone slightly increased the infarct size (38.8 ± 4.9%) compared the control group, but the difference did not reach the significant level.

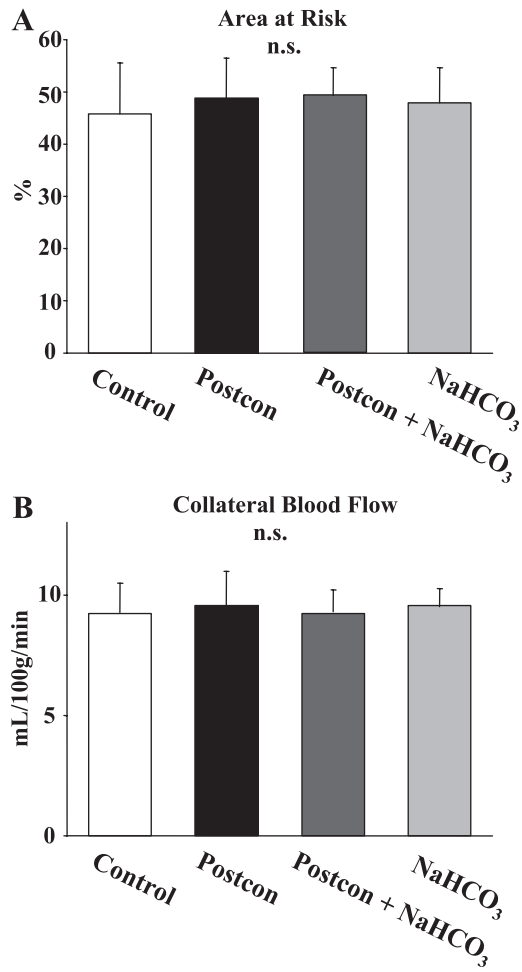

Fig. 3. The area at risk (*A*) and collateral blood flow (*B*) among all groups. There were no differences in the risk area and collateral blood flow among all groups; ns, not significant.

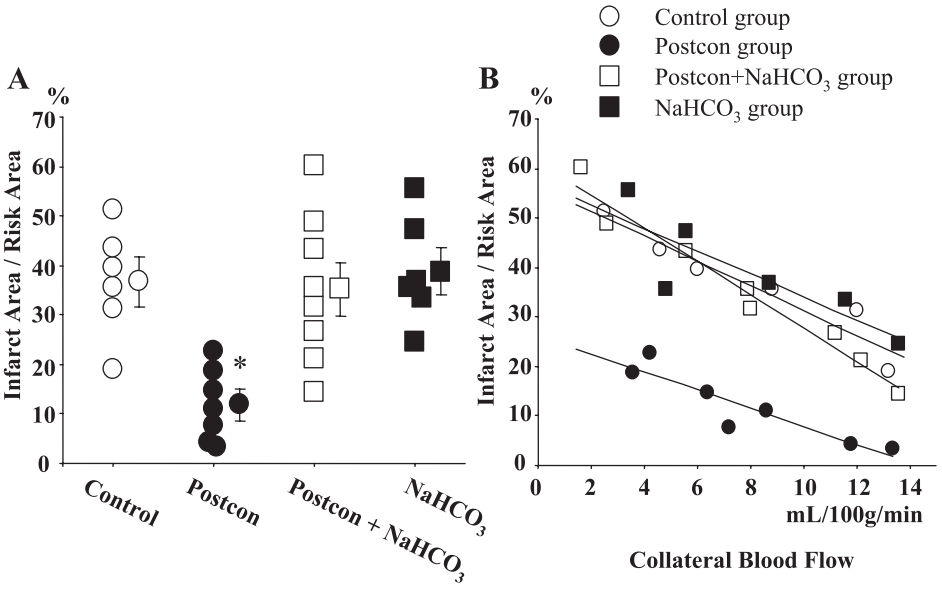

Fig. 4. *A*: infarct size expressed as a percentage of risk area. *B*: plots of infarct size expressed as percentage of risk area and regional collateral blood flow during ischemia. Infarct size decreased in Postcon group compared with the control group. This size-limiting effect was blunted by the concomitant infusion of $NaHCO_3$ (Postcon + $NaHCO_3$ group). $*P < 0.05$ vs. control group.

Protocol III: Abrogation of Phosphorylation of Akt and ERK in Ischemic Myocardium with the Infusion of $NaHCO_3$

As shown in Fig. 5, postconditioning induced the phosphorylation of Akt and ERK compared with that of control, and this phosphorylation was abrogated by the cotreatment of $NaHCO_3$. The infusion of $NaHCO_3$ alone did not affect the phosphorylation of Akt and ERK.

DISCUSSION

We have demonstrated here that postconditioning procedures limited infarct size and that these effects were blunted by an infusion of $NaHCO_3$ in canine hearts. Furthermore, the phosphorylation of either Akt or ERK induced by postconditioning was abolished by the cotreatment of $NaHCO_3$. These results suggest that postconditioning exerts cardioprotective effects via the prolonged transient acidosis activating RISK pathways during the early reperfusion phase in canine hearts.

Effects of the Infusion of $NaHCO_3$ on Postconditioning-Induced Activation of RISK Pathways

Recent studies have demonstrated that postconditioning procedures protect the heart against reperfusion injury by activating prosurvival kinase termed RISK pathways through the activation of Akt and ERK (20, 21), and the activation of RISK phosphorylates downstream targets such as glycogen synthase kinase-3β, BAD/Bax, and endothelial nitric oxide (NO) synthase, leading to the inhibition of the mitochondrial permeability transition pore opening (2, 21). The inhibition of the mitochondrial permeability transition pore may be a key end-effector of cell death and cardioprotection (4–6). We have previously shown that either metabolic or respiratory acidosis evoked by the infusion of HCl or the incubation with 30% CO_2, respectively, during reperfusion limits the infarct size resulting from myocardial ischemia in dogs (14). In this study, we have further shown that correction of the prolonged tran-

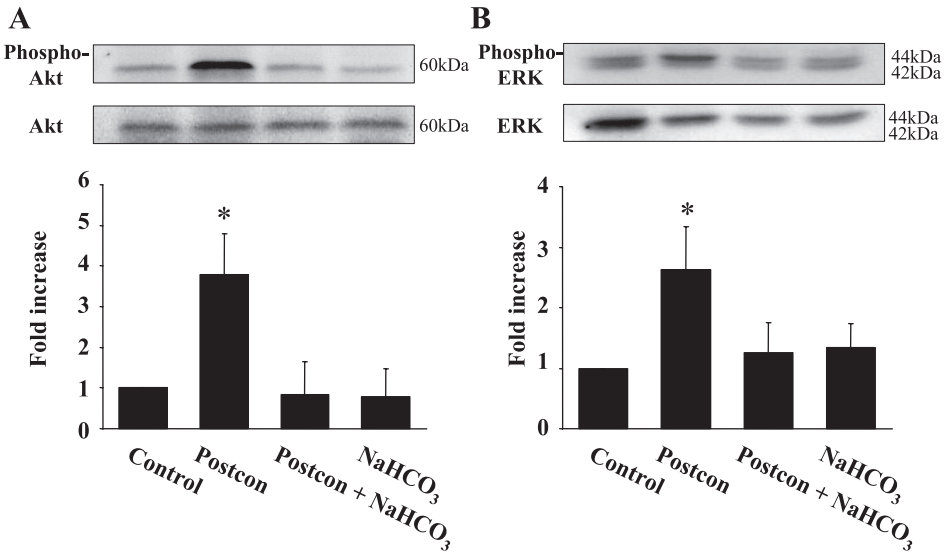

Fig. 5. Effects of postconditioning on the activation of Akt and ERK in ischemic myocardium. *Top*: the representative cases in Western blot analysis against phosphorylated and total Akt (*A*) and phosphorylated and total ERK (*B*) in ischemic myocardium. *Bottom*: densitometry graphs indicating fold expression over control for total Akt (*A*) and ERK (*B*). $n = 3$ Each. $*P < 0.05$ vs. control group.

sient acidosis with the infusion of $NaHCO_3$ blunts the cardioprotection of the postconditioning, indicating that prolonged acidosis during early reperfusion contributes to the postconditioning-induced cardioprotection. Our data showed that the phosphorylation of either Akt or ERK was blunted by the cotreatment of $NaHCO_3$. Although further investigation will be needed, we postulated that acidosis leads to the activation of RISK pathways, thus leading to the attenuation of ischemia-reperfusion injury, possibly via the inhibition of the mitochondrial permeability transition pore opening.

Other Potential Cellular Mechanisms of Acidosis-Induced Attenuation of Ischemia and Reperfusion Injury

Acidosis during reperfusion has been reported to have various cardiac beneficial effects. First, acidosis is reported to attenuate myocardial consumption via the inhibition of Ca^{2+} overload (18). Postconditioning treatment was associated with a decrease in intracellular and mitochondrial Ca^{2+} concentrations, suggesting that postconditioning reduces reperfusion injury in cardiomyocytes mediated by the attenuation of Ca^{2+} overload (19). Second, H^+ also attenuates the activation of neutrophils and attenuates free radical generation, which lead to the myocardial damage from reperfusion injury (3). Third, intriguingly, acidosis elicits release of NO and augments the effects of adenosine (7, 13, 15), both of which are believed to have various cardioprotective effects. In previous reports, the infarct size-limiting effects of postconditioning were blunted by the concomitant treatment of either the inhibition of NO synthase or adenosine (11, 22). These results may suggest that the postconditioning-induced cardioprotective effects were mediated by the increase in NO and adenosine levels induced by acidosis. Moreover, glibenclamide, the ATP-sensitive potassium channel inhibitor, attenuated the acidosis-induced vasodilatation in isolated porcine coronary arterioles, suggesting that the opening of ATP-sensitive potassium channels exerts the cardioprotective effects during acidosis (10). Glibenclamide also blunted the infarct size-limiting effects of postconditioning (22). These results potentially suggest that postconditioning leads to the opening of ATP-sensitive potassium channels via acidosis, thus leading to the attenuation of infarct size.

Taken all together, we can conclude that the prolonged transient acidosis induced by postconditioning procedures was attributable to the attenuation of reperfusion injury.

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GRANTS

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