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, Tomaïke 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 (NaHCO3) group; four cycles of 1-min reperfusion and 1-min reocclusion with the administration of NaHCO3 (n = 8); and 4) NaHCO3 group; administration of NaHCO3 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 NaHCO3 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 NaHCO3. In conclusion, postconditioning mediates its cardioprotective effects possibly via prolonged transient acidosis during the early reperfusion phase with the activation of Akt and ERK.

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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 NaHCO₃ 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 NaHCO₃ 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 NaHCO₃ intravenously just before the end of 90 min of ischemia and continuously infused NaHCO₃ into the LAD at 100 μmol·kg⁻¹·min⁻¹ during postconditioning procedures (Postcon + NaHCO₃ group, n = 8). Furthermore, to elucidate the sole effects of NaHCO₃ on infarct size, we infused NaHCO₃ into six dogs in the same manner as with the Postcon + NaHCO₃ group without the postconditioning (NaHCO₃ 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 NaHCO₃ 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 the epicardial portion at risk, which was identified as the edge of the region showing necrosis, was quickly placed into liquid nitrogen and 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·100 g⁻¹·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, Philips, 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.

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**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.
POSTCONDITIONING VIA ACIDOSIS

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>90-min Ischemia</th>
<th>Reperfusion, min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart rate, beats/min</td>
<td>Mean arterial pressure, mmHg</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>126±3</td>
<td>122±9</td>
<td>125±6</td>
</tr>
<tr>
<td></td>
<td>Postcon</td>
<td>125±5</td>
<td>122±9</td>
</tr>
<tr>
<td>Postcon + NaHCO₃</td>
<td>129±7</td>
<td>125±6</td>
<td>123±5</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>127±12</td>
<td>120±10</td>
<td>127±12</td>
</tr>
</tbody>
</table>

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 4A shows the infarct size normalized by the area at risk in the four groups in protocol I, and Fig. 4B 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 NaHCO₃ (Postcon + NaHCO₃ group) completely abolished the infarct size-limiting effects of postconditioning (35.3 ± 5.3%). The infusion of NaHCO₃ alone slightly increased the infarct size (38.8 ± 4.9%) compared the control group, but the difference did not reach the significant level.
Protocol III: Abrogation of Phosphorylation of Akt and ERK in Ischemic Myocardium with the Infusion of NaHCO₃

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 NaHCO₃. The infusion of NaHCO₃ 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 NaHCO₃ in canine hearts. Furthermore, the phosphorylation of either Akt or ERK induced by postconditioning was abolished by the cotreatment of NaHCO₃. 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 NaHCO₃ 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 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% CO₂, 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-
sient acidosis with the infusion of NaHCO₃ blunts the cardio-

protection of the postconditioning, indicating that prolonged

acidosis during early reperfusion contributes to the postcondi-
tioning-induced cardioprotection. Our data showed that the

phosphorylation of either Akt or ERK was blunted by the
cotreatment of NaHCO₃. 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 mitochon-
drial 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 Ca²⁺
overload (18). Postconditioning treatment was associated with
a decrease in intracellular and mitochondrial Ca²⁺ concentra-
tions, suggesting that postconditioning reduces reperfusion
injury in cardiomyocytes mediated by the attenuation of Ca²⁺
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 po-
tassium channel inhibitor, attenuated the acidosis-induced va-
sodilatation 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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