The pH Hypothesis of Postconditioning
Staccato Reperfusion Reintroduces Oxygen and Perpetuates Myocardial Acidosis

Michael V. Cohen, MD; Xi-Ming Yang, MD, PhD; James M. Downey, PhD

Background—It is unclear how reperfusion of infarcting hearts with alternating cycles of coronary reperfusion/occlusion attenuates infarction, but prevention of mitochondrial permeability transition pore (MPTP) formation is crucial. Acidosis also suppresses MPTP formation. We tested whether postconditioning protects by maintaining acidosis during early reoxygenation.

Methods and Results—After 30-minute regional ischemia in isolated rabbit hearts, reperfusion with buffer (pH 7.4) caused 34.4% of the risk zone to infarct, whereas 2 minutes of postconditioning (6 cycles of 10-second reperfusion/10-second occlusion) at reperfusion resulted in 10.7% infarction. One minute (3 cycles) of postconditioning was not protective. Hypercapnic buffer (pH 6.9) for the first 2 minutes of reperfusion in lieu of postconditioning caused equivalent cardioprotection (15.0% infarction), whereas 1 minute of acidosis did not protect. Delaying postconditioning (6 cycles) or 2 minutes of acidosis for 1 minute aborted protection. Reperfusion with buffer (pH 7.7) blocked postconditioning protection, but addition of the MPTP closer cyclosporin A restored protection. Reactive oxygen species scavenger N-2-mercaptopropionyl glycine, protein kinase C antagonist chelerythrine, and mitochondrial KATP channel closer 5-hydroxydecanoate each blocked protection from 2 minutes of acidosis as they did for postconditioning.

Conclusion—Thus, postconditioning prevents MPTP formation by maintaining acidosis during the first minutes of reperfusion as reoxygenated myocardium produces reactive oxygen species that activate protective signaling to inhibit MPTP formation after pH normalization. (Circulation. 2007;115:1895-1903.)

Key Words: acidosis, free radicals, mitochondrial permeability transition pore, myocardial infarction, reperfusion

Ischemic preconditioning is cardioprotective and depends on cell signaling but has only limited clinical utility because of the requirement it be instituted prior to onset of myocardial ischemia. Several very brief coronary occlusions immediately after relief of a prolonged occlusion are nearly as protective as preconditioning. Postconditioning protects in situ and in vitro rabbit hearts with 4 cycles of 30-second reperfusion/30-second occlusion and 6 cycles of 10-second reperfusion/10-second occlusion, respectively. Many of the signaling molecules and messengers involved in preconditioning (protein kinase C [PKC], adenosine receptors, phosphatidylinositol 3-kinase, extracellular signal-regulated kinase, mitochondrial ATP-sensitive potassium channels [mitoKATP]) are also important in postconditioning, which suggests common mechanisms. Nonetheless, the means by which postconditioning protects against infarction remains obscure.

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Mitochondrial permeability transition pore (MPTP) formation leads to catastrophic consequences for reperfused cells, such as necrosis and apoptosis. Preconditioning suppresses MPTP formation early in reperfusion, as does postconditioning. Additionally, cyclosporin A (CsA), which is a closer of MPTP, infused at reperfusion is cardioprotective, whereas atracyloside, which opens MPTP, aborts protection of preconditioning. Because acidosis prevents MPTP formation by blocking Ca" binding to adenine nucleotide translocase (a component of MPTP) and displacing cyclophilin from it, we speculated that postconditioning might prevent MPTP formation by maintaining acidosis during the first minutes of reoxygenation.

Reperfusion of isolated hearts and ventricular tissue for the initial 5 to 10 minutes with acidified perfusate or blood improves posts ischemic function, but relief of stunning cannot be separated from infarct reduction. Kitakaze et al reported 50% less infarction in dog hearts after respiratory or metabolic acidosis maintained for an hour after the lethal ischemic insult. If acidosis during initial reoxygenation is the mechanism of postconditioning, then just 2 minutes of acidosis should be sufficient to protect. If repetitive coronary occlusions of postconditioning prevented normalization of...
The present study was performed in accordance with the Guide for the Care and Use of Laboratory Animals, and approved by the Institutional Animal Care and Use Committee.

**Methods**

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**Isolated Rabbit Heart Model**

New Zealand White rabbits (Steven and Adrienne Weaver, Mobile, Ala) were anesthetized with sodium pentobarbital, intubated, and ventilated with 100% oxygen. A branch of the left coronary artery was clamped off and perfused with Krebs-Henseleit bicarbonate buffer that contained (in mM) 118.5 NaCl, 25 NaHCO₃, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 2.5 CaCl₂, and 10.0 glucose. A fluid-filled latex balloon inserted into the left ventricle was inflated to set an end-diastolic pressure of 5 mm Hg.

Perfusion buffer was normally bubbled with 95% O₂/5% CO₂ just before the coronary occlusion and lasted for 2 hours. After 2 hours of reperfusion, the coronary artery was reoccluded, and perfusion was switched to acidic buffer equilibrated with 5% CO₂. In additional hearts postconditioned with 6 cycles, buffer saturated with 100% O₂ was perfused for the last minute of coronary occlusion and the initial 3 minutes of reperfusion, followed by perfusion with buffer equilibrated with 95% O₂/5% CO₂. Because alkalotic pH precipitated Ca⁺⁺ salt in the buffer, CaCl₂ concentration was lowered to 1.5 mmol/L during perfusion with alkalotic buffer. To test whether the lowered calcium influenced infarction, hearts were also postconditioned in the presence of 1.5 mmol/L CaCl₂ and 5% CO₂. Finally, CsA (0.75 μmol/L) was added to the alkalotic perfusate and infused into the risk region during only the reperfusion phases of postconditioning cycles. In the 1- and 2-minute acidic reperfusion groups, perfusion was switched to buffer saturated with 20% CO₂ just before the coronary occlusion was removed, and after the initial 1 or 2 minutes of reperfusion, buffer equilibrated with 5% CO₂ was resumed, respectively. No postconditioning was performed. In the group with 1-minute delay acidic reperfusion, hearts were reperfused with acidic buffer for 2 minutes, but the switch to high-CO₂ perfusate was not started until 1 minute after release of the coronary occlusion. In the 1- and 2-minute acidic and alkalotic reperfusion control groups, coronary effluent was sampled every 2 seconds from the commencement of reperfusion for 10 seconds, and then every 10 seconds for the next 2 to 4 minutes. In the 1-minute delay acidic reperfusion group, effluent was sampled 2 and 10 seconds after release of the coronary occlusion, then every 10 seconds for 50 seconds, then 2 and 10 seconds after the switch to acidic buffer, and finally every 10 to 30 seconds for 3 minutes. Infarct size was quantitated in each heart in which pH was measured. The pH, pCO₂, and pO₂ were measured in coronary effluent with an ABL-5 blood gas analyzer (Radiometer, Copenhagen, Denmark).

In the ten, eleventh, and twelfth groups, 20-minute infusions of either 300 μmol/L of the free radical scavenger N-2-mercaptopropionyl glycine (MPG), 2.8 μmol/L of the PKC antagonist chelerythrine, or 200 μmol/L of the mitoK ATP closer 5-hydroxydecanoate (5-HD), started 5 minutes before reperfusion, were superimposed on 2-minute acidic reperfusion. Control studies were performed in which MPG, chelerythrine, or 5-HD was administered as above but without acidosis during reperfusion. In all hearts reperfusion lasted for 2 hours.

In 2 groups, effluent pH was sequentially measured after 30 minutes of global ischemia and during and after 6 cycles of 10-second reperfusion/10-second global ischemia. The perfusate during reperfusion phases of the cycles was equilibrated with either 95% O₂/5% CO₂ or 100% O₂. In a control group without postconditioning, reperfusion was accomplished with standard 5% CO₂ buffer.

**Infarct Size Measurement**

After 2 hours of reperfusion, the coronary artery was reoccluded, and 2- to 9-μm diameter fluorescent microspheres (Duke Scientific, Palo Alto, Calif) were injected into the perfusate. The risk zone was nonfluorescent. Hearts were weighed, frozen, and sliced. Slices were incubated for 8 minutes at 37°C in buffered 1% triphenyltetrazolium chloride, which stains noninfarcted myocardium brick red. Slices were fixed in 10% formalin, areas of infarct and risk zone were determined by planimetry, and volumes were calculated by multiplying areas by slice thickness and summing them for each heart. Infarct size was expressed as a percentage of risk zone.

**Chemicals**

MPG, chelerythrine, and 5-HD were purchased from Sigma Aldrich Chemical Co. (St. Louis, Mo), dissolved in 0.9% saline, and diluted in Krebs-Henseleit buffer.

**Statistics**

Data are presented as mean±SEM. One-way ANOVA with Student-Newman-Keuls post hoc test tested for differences in baseline...
hemodynamics and infarct size between groups. ANOVA for repeated measures with the Tukey post hoc test examined temporal differences in hemodynamics in any given group. The difference was significant if $P \leq 0.05$.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Coronary Effluent pH**

The pH of buffer bubbled with 5% CO$_2$ varied from 7.41 to 7.44. At the end of 30-minute regional coronary occlusion, the pH of coronary effluent ranged from 7.4 to 7.5. In the first seconds after resumption of flow to ischemic myocardium in control hearts, washout of acidic metabolites mixed with effluent from normally perfused myocardium, which caused pH to fall to 7.3 (Figure 2). The pH increased to 7.4 to 7.5 over the next minute. In hearts reperfused with acidic buffer (pH 6.85 to 6.93), coronary effluent pH fell within seconds to a range of 7.0 to 7.1 and remained at this level for the duration of acidic perfusion. Resumption of perfusion with pH 7.4 buffer increased effluent pH to a range of 7.4 to 7.5 over the next minute. The pH changes in the group in which acidic perfusion was delayed for 1 minute of reperfusion mirrored changes in control hearts for the first minute and in other acidic perfusate groups thereafter. In hearts perfused with alkalotic coronary effluent (pH 7.70 to 7.84), pH fell from 7.62 to 7.40 within 2 seconds of reperfusion and then rapidly rose to 7.6 by 20 seconds. The pH plateaued at 7.69 before a decrease to 7.45 after resumption of perfusion with buffer saturated with 5% CO$_2$. No effect on pH of coronary effluent was produced by MPG, chelerythrine, or 5-HD.

Coronary effluent pH in hearts postconditioned with regional ischemia is influenced by effluent that issues from normally perfused myocardium. In global ischemia, the entire heart is ischemic, which thus eliminates different zones created with regional ischemia. Coronary effluent pH fell to a range of 6.6 to 6.7 within 10 seconds after 30 minutes of global ischemia, which indicated extrusion of hydrogen ions from ischemic tissue into the perfusate (Figure 3). In control hearts, effluent pH recovered with a time constant of about 40 seconds. The pH during reperfusion phases of postconditioning with pH 7.4 buffer remained low for 120 seconds, which indicated continuing extrusion of H$^+$ into the perfusate and thus a continued state of intracellular acidosis. Effluent pH quickly returned to normal when postconditioning was performed with alkalotic buffer (pH 7.8), which indicated effective neutralization of H$^+$ extrusion.

**Hemodynamics**

Baseline left ventricular developed pressure tended to be higher in hearts destined to undergo postconditioning (6 cycles), although there was no difference during coronary occlusion (Table 1). Developed pressure and coronary flow fell in all groups during coronary occlusion with partial rebound during reperfusion. Acidic perfusion had no independent hemodynamic effects.

**Infarct Size**

Risk zone volume was equivalent in all groups (Table 2). Timing and required duration of acidosis needed to trigger protection were identical to those for ischemic postconditioning. Postconditioning with 6 cycles (2 minutes duration) decreased infarct size from 34.4±2.2% of risk zone in control hearts to...
### TABLE 1. Hemodynamics

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>25- to 29-Minute Occlusion*</th>
<th>30-Minute Occlusion</th>
<th>1- to 3-Minute Reperfusion†</th>
<th>30-Minute Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR, bpm</td>
<td>LVDP, mm Hg</td>
<td>CF, mL/min per g</td>
<td>HR, bpm</td>
<td>LVDP, mm Hg</td>
</tr>
<tr>
<td>Control (n=9)</td>
<td>231 ± 6</td>
<td>104 ± 3</td>
<td>9.8 ± 0.4</td>
<td>209 ± 8</td>
<td>46 ± 4§</td>
</tr>
<tr>
<td>Postconditioned, 6 cycles (n=7)</td>
<td>212 ± 3</td>
<td>121 ± 4‡</td>
<td>10.1 ± 0.2</td>
<td>193 ± 8</td>
<td>54 ± 7§</td>
</tr>
<tr>
<td>Postconditioned, 6 cycles, alkalosis + ↓ Ca$^{2+}$ (n=6)</td>
<td>208 ± 8</td>
<td>111 ± 2</td>
<td>9.6 ± 0.2</td>
<td>206 ± 10</td>
<td>44 ± 9§</td>
</tr>
<tr>
<td>Postconditioned, 6 cycles, alkalosis + ↓ Ca$^{2+}$ + CaA (n=6)</td>
<td>213 ± 7</td>
<td>106 ± 3</td>
<td>9.5 ± 0.3</td>
<td>210 ± 15</td>
<td>42 ± 4§</td>
</tr>
<tr>
<td>Postconditioned, 6 cycles, ↓ Ca$^{2+}$ (n=4)</td>
<td>213 ± 8</td>
<td>114 ± 1</td>
<td>10.2 ± 0.2</td>
<td>184 ± 20</td>
<td>40 ± 5§</td>
</tr>
<tr>
<td>Postconditioned, 3 cycles (n=6)</td>
<td>235 ± 6</td>
<td>111 ± 4</td>
<td>10.4 ± 0.2</td>
<td>220 ± 9</td>
<td>49 ± 5§</td>
</tr>
<tr>
<td>2-Minute acidosis (n=6)</td>
<td>227 ± 15</td>
<td>118 ± 2</td>
<td>10.7 ± 0.2</td>
<td>223 ± 8</td>
<td>43 ± 5§</td>
</tr>
<tr>
<td>1-Minute acidosis (n=6)</td>
<td>212 ± 8</td>
<td>115 ± 3</td>
<td>10.3 ± 0.3</td>
<td>213 ± 7</td>
<td>48 ± 3§</td>
</tr>
<tr>
<td>1-Minute delay acidosis (n=6)</td>
<td>230 ± 11</td>
<td>113 ± 2</td>
<td>10.5 ± 0.3</td>
<td>227 ± 12</td>
<td>54 ± 7§</td>
</tr>
<tr>
<td>2-Minute acidosis + MPG (n=6)</td>
<td>211 ± 6</td>
<td>115 ± 3</td>
<td>9.5 ± 0.2</td>
<td>205 ± 8</td>
<td>51 ± 7§</td>
</tr>
<tr>
<td>MPG (n=4)</td>
<td>223 ± 5</td>
<td>108 ± 6</td>
<td>10.0 ± 0.2</td>
<td>208 ± 13</td>
<td>64 ± 6§</td>
</tr>
<tr>
<td>2-Minute acidosis + Che1 (n=6)</td>
<td>231 ± 7</td>
<td>111 ± 4</td>
<td>9.6 ± 0.2</td>
<td>227 ± 13</td>
<td>50 ± 8§</td>
</tr>
<tr>
<td>Che1 (n=5)</td>
<td>230 ± 4</td>
<td>108 ± 2</td>
<td>9.3 ± 0.1</td>
<td>220 ± 8</td>
<td>45 ± 3§</td>
</tr>
<tr>
<td>2-Minute acidosis + 5-HD (n=6)</td>
<td>217 ± 9</td>
<td>115 ± 1</td>
<td>10.1 ± 0.3</td>
<td>196 ± 5</td>
<td>51 ± 6§</td>
</tr>
<tr>
<td>5-HD (n=4)</td>
<td>224 ± 11</td>
<td>100 ± 7</td>
<td>9.3 ± 0.1</td>
<td>231 ± 10</td>
<td>38 ± 3§</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. CF indicates coronary flow; Che1, charrythine; LVDP, left ventricular-developed pressure; HR, heart rate; and n, number of hearts.

*Hemodynamics recorded at beginning of drug infusion: 29 min for CaA, and 25 min for all others.

†Hemodynamics recorded at end of postconditioning cycles or at end of perfusion with acidic buffer.

Statistical significance of difference between control and experimental groups at baseline: §P<0.01.

Statistical significance of difference between baseline and other time points in a given group: $P<0.001$, $||P<0.01$, $|||P<0.05$. 
10.7±2.9% (P<0.001) (Figure 4). This protection was mimicked in the 2-minute acidic reperfusion group. When only 3 cycles of postconditioning (1 minute duration) were applied, no protection was seen (37.9±1.5% infarction). Similarly, 1 minute of acidic reperfusion was not protective. Delay of the onset of postconditioning by only 1 minute aborts protection in rabbit hearts.23 Protection was also lost when onset of 2 minutes of acidic reperfusion was delayed for 1 minute. When postconditioning was performed with alkalotic buffer, it was no longer protective (34.8±2.5% infarction) (Figure 5). Low Ca²⁺ buffer equilibrated with 5% CO² had no effect on the protection of postconditioning (Figure 5). When CsA was added to alkalotic perfusate during reperfusion phases of 6 postconditioning cycles, the protection of postconditioning was restored (Figure 5).

Finally, as previously seen in postconditioning, coadministration of either MPG, chelerythrine, or 5-HD aborted protection of 2 minutes of acidic reperfusion (Figure 6), which indicated that both postconditioning and acidosis use the same mechanism for protection. Neither MPG, 5-HD, nor chelerythrine had any independent effect on infarction (Table 2).

**Discussion**

Postconditioning is protective in animals3,4 and has had beneficial functional effects in patients who underwent coronary angioplasty for acute coronary occlusion.26 Until we understand its mechanism, however, it will be impossible to

**TABLE 2. Risk Zone and Infarct Sizes**

<table>
<thead>
<tr>
<th>Condition</th>
<th>BW, kg</th>
<th>HW, g</th>
<th>Risk Zone, cm³</th>
<th>Infarct Size, cm³</th>
<th>I/R, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.1±0.1</td>
<td>7.0±0.1</td>
<td>1.24±0.10</td>
<td>0.43±0.04</td>
<td>34.4±2.2</td>
</tr>
<tr>
<td>Postconditioned, 6 cycles</td>
<td>2.0±0.0</td>
<td>6.9±0.1</td>
<td>1.18±0.06</td>
<td>0.13±0.03</td>
<td>10.5±2.9</td>
</tr>
<tr>
<td>Postconditioned, 6 cycles, alkalosis + ↓ Ca²⁺</td>
<td>2.2±0.0</td>
<td>6.9±0.1</td>
<td>1.07±0.08</td>
<td>0.38±0.05</td>
<td>34.8±2.5</td>
</tr>
<tr>
<td>Postconditioned, 6 cycles, alkalosis + ↓ Ca²⁺ + CsA</td>
<td>2.0±0.1</td>
<td>6.8±0.1</td>
<td>1.06±0.08</td>
<td>0.16±0.02</td>
<td>15.1±1.95</td>
</tr>
<tr>
<td>Postconditioned, 6 cycles, ↓ Ca²⁺</td>
<td>2.3±0.1</td>
<td>6.9±0.1</td>
<td>1.05±0.08</td>
<td>0.12±0.02</td>
<td>11.0±1.95</td>
</tr>
<tr>
<td>Postconditioned, 3 cycles</td>
<td>2.3±0.0</td>
<td>7.0±0.1</td>
<td>1.21±0.12</td>
<td>0.46±0.05</td>
<td>37.9±1.5</td>
</tr>
<tr>
<td>2-Minute acidosis</td>
<td>2.1±0.0</td>
<td>6.8±0.1</td>
<td>1.30±0.07</td>
<td>0.20±0.04</td>
<td>15.0±2.6</td>
</tr>
<tr>
<td>1-Minute acidosis</td>
<td>2.2±0.0</td>
<td>6.9±0.1</td>
<td>1.21±0.06</td>
<td>0.34±0.04</td>
<td>28.3±2.6</td>
</tr>
<tr>
<td>1-Minute delay acidosis</td>
<td>2.2±0.0</td>
<td>6.9±0.2</td>
<td>1.21±0.10</td>
<td>0.44±0.06</td>
<td>36.4±4.2</td>
</tr>
<tr>
<td>2-Minute acidosis + MPG</td>
<td>2.1±0.0</td>
<td>7.0±0.1</td>
<td>1.28±0.09</td>
<td>0.49±0.07</td>
<td>37.5±3.9</td>
</tr>
<tr>
<td>MPG</td>
<td>2.3±0.1</td>
<td>6.9±0.2</td>
<td>1.07±0.10</td>
<td>0.40±0.04</td>
<td>37.0±2.3</td>
</tr>
<tr>
<td>2-minute acidosis + Chel</td>
<td>2.1±0.0</td>
<td>6.8±0.2</td>
<td>1.26±0.14</td>
<td>0.48±0.07</td>
<td>37.1±2.0</td>
</tr>
<tr>
<td>Chel</td>
<td>2.1±0.1</td>
<td>6.4±0.1</td>
<td>1.16±0.07</td>
<td>0.37±0.03</td>
<td>31.5±1.0</td>
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<tr>
<td>2-Minute acidosis + 5-HD</td>
<td>2.1±0.0</td>
<td>6.8±0.1</td>
<td>1.03±0.09</td>
<td>0.37±0.05</td>
<td>35.2±2.4</td>
</tr>
<tr>
<td>5-HD</td>
<td>1.8±0.1</td>
<td>6.9±0.1</td>
<td>1.23±0.16</td>
<td>0.44±0.09</td>
<td>35.1±3.9</td>
</tr>
</tbody>
</table>

Mean±SEM. BW indicates body weight; HW, heart weight; and I/R, ratio of infarct volume to risk zone volume.

Statistical significance of difference between control and experimental groups (which includes “Postconditioned, 6 cycles” but not other “Postconditioned” groups): *P<0.001, †P<0.05.

Statistical significance of difference between “Postconditioned, 6 cycles” and other “Postconditioned” groups: ‡P<0.001, §P<0.001 versus control. **P<0.001 versus Postcond-6 cycles.

**Figure 4.** Infarct size as a percentage of risk zone in individual hearts (○) and groups (●). Postcond indicates postconditioning. *P<0.001 versus control.

**Figure 5.** Infarct size as a percentage of risk zone in individual hearts (○) and groups (●). *P<0.001 versus control. **P<0.001 versus Postcond-6 cycles. †P was not significant versus Postcond-6 cycles and P<0.001 versus Postcond-6 cycles + alkalosis + low Ca²⁺.

10.7±2.9% (P<0.001) (Figure 4). This protection was mimicked in the 2-minute acidic reperfusion group. When only 3 cycles of postconditioning (1 minute duration) were applied, no protection was seen (37.9±1.5% infarction). Similarly, 1 minute of acidic reperfusion was not protective. Delay of the onset of postconditioning by only 1 minute aborts protection in rabbit hearts.23 Protection was also lost when onset of 2 minutes of acidic reperfusion was delayed for 1 minute. When postconditioning was performed with alkalotic buffer, it was no longer protective (34.8±2.5% infarction) (Figure 5). Low Ca²⁺ buffer equilibrated with 5% CO₂ had no effect on the protection of postconditioning (Figure 5). When CsA was added to alkalotic perfusate during reperfusion phases of 6 postconditioning cycles, the protection of postconditioning was restored (Figure 5).
design an optimal postconditioning protocol. Acidic perfusion of myocardium immediately after release of a prolonged coronary occlusion mimics infarct reduction seen with postconditioning. Both acidic reperfusion and postconditioning had to last for 2 minutes to be effective. Only 1 minute of either was not protective, and, similar to postconditioning, acidic perfusion had to be commenced immediately after release of the coronary occlusion. Alkaline perfusate aborted the protection of postconditioning. Finally both postconditioning and acidic reperfusion depend on the same signal transduction pathways for protection.

This effect of pH suggests involvement of MPTP, because acidosis is known to prevent MPTP formation, a protective intervention, whereas alkalosis promotes its formation. Indeed suppression of MPTP with CsA for 2 minutes rescued hearts in which the protection of postconditioning was blocked by alkalotic perfusate. Our results parallel those of Qian et al in cultured rat hepatocytes. During anoxia, pH was 6.3, and MPTP remained closed but formed with superfusion at pH 7.4, which led to massive cell necrosis. If cells were superfused at pH 6.2, MPTP stayed closed and >80% of cells remained viable. If superfusion was done at pH 7.4 in the presence of CsA, MPTP did not form, and again >80% of cells survived despite pH 7.2. The MPTP hypothesis is supported by other investigations in both preconditioning and postconditioning, which indicate that MPTP plays an important role in the mechanism of protection. Inhibition of MPTP with either of the immunosuppressants sanglifehrin or CsA administered just after release of a coronary occlusion protects otherwise untreated hearts, whereas the opening of MPTP at reperfusion with atracyloside aborts protection of ischemic preconditioning. Sanglifehrin infused during the first 15 minutes of reperfusion protected hearts, whereas protection was lost if infusion was commenced 15 minutes after onset of reperfusion. Argaud et al reported that mitochondria from postconditioned hearts resisted Ca++-induced MPTP opening at neutral pH, but in those samples protective kinases would already have been activated and a low pH would no longer be needed.

Figure 7 illustrates our hypothesis. Myocardium of the naïve heart (Figure 7, top panel) becomes acidotic during ischemia, but this acidosis is quickly relieved after reperfusion. MPTP that could not open in acidic milieu during ischemia quickly opens as pH rises back to a neutral level. MPTP opening leads to collapse of the mitochondrial transmembrane potential, cessation of ATP production, and subsequent cell death.

In the preconditioned heart (Figure 7, middle panel), brief ischemia prior to prolonged coronary occlusion releases agonists to G-protein coupled receptors such as bradykinin and opioids, which trigger a signal cascade that involves phosphatidylinositol 3-kinase, nitric oxide, protein kinase G,
and opening of mitoK\textsubscript{ATP}. Restoration of oxygenation during brief reperfusion causes mitochondria to produce reactive oxygen species (ROS) that act as second messengers, which culminates in activation of PKC. PKC initiates a second signaling cascade at the onset of reperfusion by increasing the heart’s sensitivity to adenosine agonists such that adenosine that had been previously released from ischemic cardiomyocytes now becomes protective. Adenosine receptors activate protective kinases, Akt and extracellular signal-regulated kinase, which, possibly through GSK-3$^{\beta}$, prevent formation of MPTP during reperfusion.

In the heart that is to be protected by postconditioning (Figure 7, lower panel), we suggest that the perfusion phases of postconditioning cycles deliver enough oxygen for mitochondria to produce ROS, but do not last long enough to allow pH to normalize. At end of index ischemia, the trigger pathway associated with preconditioning (eg, G$\text{\textsubscript{\alpha}}$-protein coupled receptor agonists, phosphatidylinositol 3-kinase, nitric oxide synthase, etc.) has already been activated to mitoK\textsubscript{ATP} opening, but signaling is stopped because ROS cannot be generated. Perpetuation of acidosis during postconditioning inhibits MPTP formation, whereas reoxygenation fuels redox signaling, which then proceeds to activate PKC. PKC activation initiates the signaling cascade of preconditioning, which permanently blocks MPTP opening. In the naïve heart (Figure 7, top panel), ROS will also be produced on reperfusion, but pH normalizes and MPTP opens before critical downstream signaling can be accomplished. Hence, protection is dependent on both signaling and perpetuation of acidic pH. If either is absent, protection is aborted. Diverse inhibitors of signaling such as adenosine receptor blockers, N\textsuperscript{\text{O}}-nitro-L-arginine methyl ester, 5-HD, and chelerythrine will block protection even if acidosis is maintained (Figure 6), whereas intact signaling in postconditioning is ineffective if myocardium is alkalotic (Figure 5). This acidosis hypothesis explains earlier observations. Postconditioning occlusion and reperfusion periods needed to be shorter than in in situ hearts to protect isolated hearts. This is probably related to much higher coronary flows in buffer-perfused hearts, which resulted in faster washout of H$^+$. Figure 3 suggests that tissue pH normalizes at reperfusion with an approximately 40-second time constant in the isolated heart, which implies that little acidosis would remain after 30 seconds of reperfusion.

A critical test of our proposed mechanism is whether ROS formation after pH normalizes. PKC is thought to be the target of ROS. Protection from postconditioning can be blocked by a PKC antagonist, and activation of PKC by phorbol ester infused at the end of a coronary occlusion causes protection similar to that seen with postconditioning. Furthermore, we have shown that protection is dependent on activation of an adenosine receptor by PKC. Although we proposed the A$\text{\textsubscript{3b}}$ receptor, others champion A$\text{\textsubscript{2a}}$ and A$\text{\textsubscript{2b}}$ receptors. Hearts can be preconditioned by inclusion of ROS in the perfusate, and that protection is PKC-dependent. PKC activation is also required to produce protection from acidic reperfusion. Additionally, opening mitoK\textsubscript{ATP} is required for protection from acidic reperfusion, and this same requirement has been reported in postconditioning. This is strong evidence that the signal transduction cascade in acidic reperfusion is identical to that seen in postconditioning, which largely recapitulates what is known to occur in preconditioning.

If our hypothesis is correct, then we can design an optimal postconditioning protocol. The shorter the cycles, the less likely pH will normalize during the reperfusion phase. Thus the cycles should be as short as is practical. Second, because we know that even 60 minutes of acidic reperfusion is protective, we speculate that postconditioning cannot last too long, although it can be too short. This obviously should be tested. It is possible that postreperfusion acidosis, possibly achieved by breathing CO\textsubscript{2}-enriched air for several minutes, may be a simple alternative cardioprotective intervention. Whereas postconditioning is only available for individuals who undergo angioplasty/stenting, high CO\textsubscript{2} could be used in patients who undergo reperfusion with noninvasive thrombolytic agents.

One obvious limitation of our present study is the absence of pH measurements. We measured pH only of coronary effluent. This allowed us to monitor washout of acidic substances from ischemic myocardium, however, and thus we could confirm that tissue was acidic and could estimate the time constant at which tissue pH normalized (Figures 2 and 3). Hypercapnia lowers intact heart pH, as well as cardiomyocyte pH, measured with pH-sensitive fluorochromes. Spitzer et al noted that an increase of CO\textsubscript{2} from 5% to 20% in the bath lowered the pH of cardiomyocytes by 0.7 of a pH unit from a control level of approximately pH 7.3. Nomura et al exposed contracting cardiomyocytes to 30% CO\textsubscript{2} and saw pH quickly fall by 0.32 of a pH unit from a stable pH of 7.1. We would expect perfusate saturated with 80% O\textsubscript{2}/20% CO\textsubscript{2} to have maintained acidic pH in recently ischemic tissue. Measurement of pH is needed to confirm this assumption.

In the present study, both previously ischemic and normally perfused myocardia were exposed to acidic perfusate after release of the coronary occlusion. It is unlikely that brief acidic perfusion of normal tissue was responsible for cardioprotection, although we cannot entirely exclude this. Nor is it possible to completely exclude some pH-independent effect of hypercapnia.

In summary, acidic reperfusion exactly mimics the protection of postconditioning both in time course and signal transduction pathway. We conclude that postconditioning...
protests by reoxygenating the heart while keeping it acidic. Reintroduced oxygen initiates preconditioning-like redox signaling, whereas acidosis inhibits MPTP formation.

Sources of Funding

The present study was supported in part by grants HL-20468 and HL-50688 from the Heart, Lung, and Blood Institute of the National Institutes of Health to Drs Cohen and Downey.

Disclosures

None.

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**CLINICAL PERSPECTIVE**

Pharmacological or ischemic preconditioning strongly limits infarct size, but this approach is not possible in patients who present after onset of ischemia caused by coronary occlusion. Postconditioning of the heart with several cycles of brief coronary reperfusion/reocclusion immediately after coronary recanalization has recently been found to be as cardioprotective as preconditioning. However, postconditioning with occlusion cycles is not possible in patients treated with intravenous thrombolytic agents. Thus we investigated why postconditioning is cardioprotective in hopes to develop a more generic approach to cardioprotection at the time of reperfusion. The low pH in ischemic myocardium inhibits ischemia-induced formation of mitochondrial permeability transition pores, but that inhibition is lost as pH rapidly normalizes during reflow. Mitochondrial permeability transition pore formation blocks ATP generation by mitochondria and thus leads to necrosis in reperfused myocardium. Our experiments reveal that the brief periods of reflow reintroduce oxygen that fuels redox signaling, which in turn triggers preconditioning-like cardioprotection. The occlusion cycles are also critical to keep myocardial pH low enough to inhibit mitochondrial permeability transition pore formation until the protective signaling can be established. In the present study, we mimicked the cardioprotective effect of postconditioning by reperfusing the heart for 2 minutes with oxygenated buffer that was made acidic by equilibration with 20% CO₂. Lowering the pH of the coronary perfusate by having the patient briefly inhale CO₂ prior to coronary reperfusion may be sufficient to confer this protection.
The pH Hypothesis of Postconditioning: Staccato Reperfusion Reintroduces Oxygen and Perpetuates Myocardial Acidosis
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Circulation. 2007;115:1895-1903; originally published online March 26, 2007; doi: 10.1161/CIRCULATIONAHA.106.675710

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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