Ischemic preconditioning and myocardial hypothermia in rabbits with prolonged coronary artery occlusion

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The present study was designed to answer two questions: first, whether combining hypothermia with IP would provide greater protection than either intervention alone, and second, whether a longer duration of IP (7 min/episode) could extend the window of protection to include a prolonged (2 h) coronary occlusion, and whether a longer duration of IP is superior to the standard 5-min preconditioning protocol in this setting.

**METHODS**

The rabbits used in this study were maintained in accordance with the policies and guidelines of the "Position of the American Heart Association on research animal use" (1) and the Guide for the Care and Use of Laboratory Animals [DHEW Publication No. (NIH) 85–23, Revised 1985, Office of Science and Health Reports, Bethesda, MD 20892]. The Good Samaritan Hospital is accredited by the American Association for Accreditation of Laboratory Animal Care.

**Preparation**

Male New Zealand White rabbits weighing 2.4-3.0 kg were anesthetized with an intramuscular injection of ketamine (200 mg) and xylazine (100 mg) given twice before the start of surgery. Additional pentobarbital sodium was given as needed throughout the protocol to maintain a deep level of anesthesia. The rabbits were shaved, intubated, and respirated with room air supplemented with 100% oxygen. Fluid-filled catheters were inserted into the jugular vein to give fluids and into the carotid artery to monitor arterial blood pressure and to withdraw blood samples during regional myocardial blood flow (RMBF) measurements. The chest was opened at the fourth intercostal space, and the pericardium was cut. A large anterolateral branch of the circumflex artery was encircled with a smallatraumatic needle attached to a 4-0 silk suture. The needle was removed, and the ends of the suture were threaded through a piece of tubing to form a snare, which could be used to occlude and reperfuse the artery. The needle of a 23-gauge butterfly catheter with the flanges removed was inserted into the left atrial appendage and held in place with a Schwartz clamp. This catheter was used for the injection of radioactive microspheres and blue dye at the conclusion of the protocol. A hypodermic (30 gauge) thermocouple probe (Omega, Stamford, CT) was inserted transversely into the free wall of the left ventricle to measure myocardial temperature. To ensure that the tip of the probe was located within the ventricular wall and not in the left ventricular cavity, cool saline was injected on the surface of the heart overlaying the probe. An immediate reduction in temperature indicated proper placement. A digital thermometer was placed in the rectum of the rabbit to measure body temperature. Heart rate, blood pressure, and myocardial and core body temperature were recorded throughout the study protocol. Body temperature was maintained using a heating pad.

**Experimental Protocols**

After all surgical preparations were completed, the rabbits were randomly assigned to one of the four experimental groups.
groups (n = 8 rabbits/group) (Fig. 1). The control (C) group received no preconditioning ischemia or hypothermia, the H group received hypothermia alone, the IP7 group received ischemic preconditioning for two 7-min episodes alone, and the H + IP7 group received preconditioning plus hypothermia. All rabbits received 120 min of occlusion and 3 h of reperfusion. Preconditioning consisted of two 7-min periods of ischemia followed by 5 min of reperfusion. Regional hypothermia was created by placing a bag filled with ice and water directly on the anterior surface of the heart (risk region). Hypothermia was initiated after 30 min of coronary artery occlusion and maintained until 15 min of reperfusion. The bag was then removed, and the hearts were allowed to warm.

To compare the effects of 7-min versus 5-min periods of ischemic preconditioning, an additional group (IP5, n = 6 rabbits) was added halfway through the initial protocol. Rabbids in this group received a 5-min period of preconditioning ischemia, 120 min of occlusion, and 3 h of reperfusion. This group was randomly assigned with the others. Our a priori intention was to compare this group only with the control and the 7-min preconditioned groups.

RMBF was determined at 90 min of coronary occlusion to confirm that the risk zone was ischemic and at 30 min of reperfusion to confirm reflow in the same zone. Measurement of RMBF was performed as described previously (7). At the end of 3 h of reperfusion, the coronary artery was reoccluded. Four milliliters of 50% Unisperse blue (Ciba-Geigy, Hawthorne, NY) were injected into the left atrial catheter and allowed to circulate throughout the vasculature. The rabbit was then euthanized by an overdose of xylazine (300 mg iv) followed by 12 meq of potassium chloride given into the left atrium. The ischemic risk region and the region of necrosis were delineated with triphenyltetrazolium chloride staining and measured as previously described (7).

Prospective exclusion criteria included an ischemic risk zone of <10% of the left ventricular weight, a regional blood flow of >0.2 ml·min⁻¹·g⁻¹ in the risk zone during coronary artery occlusion (lack of ischemia), or a regional blood flow of <0.2 ml·min⁻¹·g⁻¹ in the risk zone at 30 min of reperfusion (failure to reperfuse).

Controls and Data Analyses

The following end points were measured: core body temperature, myocardial risk zone temperature, mean arterial blood pressure, heart rate, RMBF, area at risk, and infarct size. As determined prospectively, the initial four groups (C, H, IP7, and H + IP7) were analyzed together. In addition, as determined prospectively, the ischemic preconditioning protocols and control protocols were analyzed together. All data summary and statistical analyses were performed using SAS (version 6.04, Cary, NC). Left ventricular weight, infarct size, and area at risk were compared using one-way ANOVA. Regional myocardial blood flow data were analyzed by ANOVA. In cases of P < 0.05, differences among means were assessed using Duncan’s multiple range test. Rectal temperature, heart rate, and blood pressure were analyzed by repeated-measures ANOVA. Data are means ± SE.

RESULTS

Animal Population

Forty-six rabbits entered the study, but eight were excluded. Two IP7 rabbits and one H + IP7 rabbit died of hypotension before completing the protocol; one IP7 rabbit was excluded because the risk zone was <10% of the left ventricle; and one control, one IP7, and two H + IP7 rabbits were excluded because RMBF in the risk zone at 110 min of occlusion exceeded 0.2 ml·min⁻¹·g⁻¹. Data presented are for eight rabbits in the control group, the IP7 group, the H group, and the H + IP7 group and for six rabbits in the IP5 group.

Effects of Cooling, Preconditioning, and Combined Therapy

Temperature. Myocardial risk zone temperatures were similar in all four groups at baseline (range 38.4–38.7°C), before occlusion (range 38.4–39.0°C), and at 30 min of coronary occlusion (range 38.4–39.0°C).
Myocardial cooling was initiated in the H and H + IP7 groups after 30 min of occlusion. Average risk zone temperatures during the remaining 90 min of occlusion were 32.8 ± 0.6°C in the H group receiving topical cooling and 32.3 ± 0.4°C in the H + IP7 group receiving topical cooling. In the two groups not receiving topical cooling, average risk zone temperatures during the remaining 90 min of occlusion were 39.0 ± 0.2°C in the control group and 38.4 ± 0.2°C in the IP7 group. By 60 min of reperfusion, temperature had returned to preocclusion values in all groups (range 38.3–38.7°C).

Infarct size data. There were no significant differences among the groups in body weight, left ventricle weight (data not shown), or the size of the ischemic risk region, which was expressed as a percentage of the left ventricle (Fig. 2). Infarct size, expressed as a percentage of the risk zone in H hearts, was 42 ± 6% in control hearts. Cooling of the heart (H group), preconditioning for 2×7-min periods (IP7 group), and combined therapy (H + IP7 group) all significantly reduced infarct size compared with the control group. Infarct size was 50 ± 7% of the risk zone in H hearts (P < 0.05 vs. control) and 49 ± 5% in IP7 hearts (P < 0.05 vs. control). Infarct size was 42 ± 6% significantly lower than the control value in H + IP7 hearts (P < 0.05 vs. control) but not significantly different from individual treatments. With the use of analysis of covariance in testing for a group effect on the relationship between the risk region and the necrotic regions (both expressed in grams), there was a significant group effect (P < 0.026). The scattergram representing this relationship is shown in Fig. 3.

Rate-pressure product. Values of the rate-pressure product (RPP) (heart rate × systolic blood pressure) throughout the protocol are given in Table 1. As observed in previous studies, RPP decreased from baseline to the end of reperfusion (P < 0.0001 for time effect by repeated-measures ANOVA); however, there was no significant group effect.

Regional myocardial blood flow. There were no significant differences among groups in RMBF at 110 min of coronary artery occlusion in either the ischemic risk zone or in the adjacent nonischemic areas (Table 2). However, at 30 min of reperfusion, hearts that received combined therapy (H + IP7 group) had significantly lower RMBF than the control group (P = 0.0001 for group effect). There were no significant group effects. Rate-pressure product calculated by heart rate (beats/min) times systolic blood pressure (mmHg) (×10) was 198 ± 22 in control hearts, 178 ± 18 in IP5 and 163 ± 15 in IP7 hearts. Values are means ± SE measured by repeated-measures ANOVA, and values in all groups decreased over time (P < 0.001 for group effect); there were no significant group effects.

### Table 1. Rate-pressure product values

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>H</th>
<th>IP7</th>
<th>H + IP7</th>
<th>IP5</th>
</tr>
</thead>
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<tr>
<td>Baseline</td>
<td>198 ± 22</td>
<td>210 ± 6</td>
<td>172 ± 17</td>
<td>180 ± 9</td>
<td>206 ± 23</td>
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<td>Preocclusion</td>
<td>178 ± 18</td>
<td>189 ± 8</td>
<td>163 ± 15</td>
<td>157 ± 8</td>
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<td>Occlusion, min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>157 ± 16</td>
<td>160 ± 9</td>
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<td>60</td>
<td>143 ± 7</td>
<td>138 ± 5</td>
<td>131 ± 8</td>
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<td>90</td>
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<td>148 ± 5</td>
<td>134 ± 10</td>
<td>117 ± 7</td>
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<tr>
<td>120</td>
<td>120 ± 7</td>
<td>140 ± 11</td>
<td>129 ± 10</td>
<td>114 ± 8</td>
<td>142 ± 3</td>
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<tr>
<td>Reperfusion, min</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>127 ± 4</td>
<td>113 ± 7</td>
<td>104 ± 8</td>
<td>107 ± 6</td>
<td>133 ± 5</td>
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<tr>
<td>30</td>
<td>123 ± 4</td>
<td>116 ± 9</td>
<td>106 ± 6</td>
<td>119 ± 3</td>
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<td>60</td>
<td>114 ± 6</td>
<td>114 ± 7</td>
<td>106 ± 6</td>
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<td>180</td>
<td>107 ± 12</td>
<td>106 ± 9</td>
<td>104 ± 8</td>
<td>106 ± 5</td>
<td>122 ± 5</td>
</tr>
</tbody>
</table>

Values are means ± SE measured by repeated-measures ANOVA, and values in all groups decreased over time (P < 0.001 for group effect); there were no significant group effects. Rate-pressure product calculated by heart rate (beats/min) times systolic blood pressure (mmHg) (×10). C, control; H, hypothermia alone; IP7, ischemic preconditioning alone for two 7-min periods; H + IP7, hypothermia plus ischemic preconditioning for two 7-min periods; IP5, ischemic preconditioning for one 5-min period.
greater flow in the previously ischemic region than control hearts or hearts that had received either treatment alone. Blood flows in nonrisk areas at this time were statistically similar in all groups.

Comparison of the Effects of the Two Preconditioning Protocols on Infarct Size

The ischemic risk zone was similar in both the 5- and 7-min preconditioned groups (31 ± 2 and 28 ± 3% of the left ventricle, respectively) as well as in the control group (34 ± 3%). One 5-min preconditioning episode of ischemia failed to confer protection after the long duration of ischemia. Infarct size averaged 67 ± 5% of the risk zone in this group compared with the average infarct size in controls (72 ± 4%, P = not significant, Fig. 2). In contrast, infarct size in hearts that had received two 7-min preconditioning episodes had a marked reduction in infarct size compared with the other two groups (49 ± 5%, P < 0.05 compared with the two other groups). With the use of analysis of covariance, we tested for a group effect on the relationship between the risk region and the necrotic regions (both expressed in grams), and we found that there was a significant group effect (P < 0.03). Other parameters such as the RPP throughout the protocol (Table 1) and RMBF during ischemia and reperfusion were similar in all groups. In the IP5 group, blood flow to the ischemic zone during occlusion averaged 0.04 ± 0.02 ml·min⁻¹·g⁻¹, and at 30 min of reperfusion, flow to this region was 0.86 ± 0.28 ml·min⁻¹·g⁻¹.

DISCUSSION

The most important new observation in this study is that the protection provided by ischemic preconditioning can encompass long-term ischemia (120 min in the rabbit) if the duration of the ischemic preconditioning episodes is extended from 5 to 7 min. In control hearts subjected to this extended period of ischemia, necrosis comprises almost 75% of the nonperfused myocardium. Preconditioning before ischemia resulted in salvage of over 30% of the nonperfused tissue.

In the rabbit model, most investigators have used an ischemia duration of 30–40 min. In this situation, infarct size is typically 30–40% of the risk zone. Therapies, such as preconditioning and hypothermia, have reduced infarct size by 50% or more in some studies. There are few data available on the effects of infarct-limiting maneuvers in the rabbit model with a longer duration of ischemia. Yet in many patients reperfusion therapy is delayed for several hours. Therefore, it is important to determine whether infarct size can be reduced in this setting of prolonged ischemia. The present study is one of the first that addresses this issue in the commonly used rabbit model. Although the protection provided by preconditioning and hypothermia was not as great as that provided by a shorter ischemic insult, such as 30 min, these maneuvers still salvaged myocardium compared with control, suggesting that even 2-h occlusions are amenable to infarct-limiting therapy in the rabbit.

The duration and number of preconditioning cycles used in the rabbit model is variable, depending on the investigator and study. In the rabbit if the duration of preconditioning ischemia is 2 min, the intervention appears to be ineffective (12, 21), whereas two 5-min episodes were no better than one. Typically, one to four episodes of 5-min ischemia have been used in the rabbit model. Yamasaki et al. (22) tested the maximum ischemic period to induce a beneficial preconditioning effect, comparing the protection offered by one ischemic episode of 5, 10, or 15 min. They found no difference in protection between the 5- and 10-min treatments: both durations significantly reduced infarct size. However, when the duration of the preconditioning episode was extended to 15 min, eventual infarct size was greater than in controls. It should be noted that in all the above studies the duration of the s"sustained" ischemia after preconditioning was 30 min. In our study of 120 min of prolonged ischemia, the 5-min preconditioning interval did not protect the heart but the 7-min interval did.

As we have shown previously, cooling of ischemic myocardium is also protective in long-term ischemia, even when it is initiated 30 min after coronary artery occlusion (6). Data from the present study indicate that the combination of cooling and ischemic preconditioning was not significantly better than either therapy alone. There was no clear-cut additive effect of ischemic preconditioning (even using the 7-min preconditioning ischemia) with hypothermia. However, we cannot exclude the possibility that in a large population the combined therapy might show benefit in some.

Our results do suggest that the combined therapy afforded protection to the coronary vasculature. At 30 min of reperfusion, RMBF to the previously ischemic risk region was significantly higher in hearts receiving combined therapy than it was in either control hearts or in hearts that received individual therapy.

Van den Doel and co-workers (20) performed an extensive study in rat hearts testing the effects of preconditioning combined with hypothermia. The dura-

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Table 2. Regional myocardial blood flow

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>H</th>
<th>IP7</th>
<th>H + IP7</th>
<th>IP5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occlusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Risk zone</td>
<td>0.03 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>Normal zone</td>
<td>1.47 ± 0.19</td>
<td>1.34 ± 0.13</td>
<td>1.21 ± 0.13</td>
<td>1.20 ± 0.11</td>
<td>1.39 ± 0.09</td>
</tr>
<tr>
<td>Reperfusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Risk zone</td>
<td>0.65 ± 0.10</td>
<td>0.91 ± 0.13</td>
<td>0.69 ± 0.14</td>
<td>2.23 ± 0.41*</td>
<td>0.86 ± 0.28</td>
</tr>
<tr>
<td>Normal zone</td>
<td>1.43 ± 0.18</td>
<td>1.09 ± 0.12</td>
<td>1.07 ± 0.13</td>
<td>1.36 ± 0.15</td>
<td>1.63 ± 0.16</td>
</tr>
</tbody>
</table>

Values are means ± SE (in ml·min⁻¹·g⁻¹). *P < 0.01 vs. other groups.
tion of preconditioning ischemia used in their study was 15 min. Infarct size was reduced for up to 60 min of coronary artery occlusion using this regimen, but infarct size was not reduced after 120 min of occlusion. Hypothermia, when used alone (30–31°C), protected myocardium during a 30-min coronary artery occlusion, but protection was lost by 60 min. When the two interventions were combined, significant protection was seen up to 120 min of ischemia. These results are somewhat different from our study in the rabbit, in which preconditioning still reduced infarct size after 120 min of coronary occlusion. One possible explanation for this difference is that preconditioning in our study was performed under normothermic conditions, and hypothermia was instituted later. In the above study, preconditioning was performed under hypothermic conditions, which may have altered the outcome. This prospect is supported by the recent work of Dote et al. (5), which shows that when ischemic preconditioning is performed under hypothermic conditions (25°C) in isolated rabbit hearts, one episode of 5 min was not sufficient to precondition the hearts. Instead, hypothermia appeared to raise the threshold for obtaining infarct limitation such that four 5-min episodes were required to obtain a reduction in infarct size.

In the rabbit and rat, 7–10 min of preconditioning ischemia seems to be the ideal length of time, because both Yamasaki et al. (22) and van den Doel et al. (20) reported that 15 min of preconditioning ischemia caused some degree of necrosis by itself. Our results differ from those of Miki et al. (11), who found that hypothermia enhances the protection provided by ischemic preconditioning. Rabbit hearts received ischemic preconditioning (5 min of ischemia plus 10 min of reperfusion). Whole body cooling was initiated 20 min after the onset of a 45-min coronary artery occlusion. Ischemic preconditioning alone reduced infarct size by ~46%. However, when the two interventions were combined, infarct size was reduced by 88% compared with control hearts. The different outcome in the work of Miki et al. compared with our outcome may be a result of the duration of ischemia: 45 min versus 120 min.

Valen and co-workers (19) found that if ischemic preconditioning was performed before warm global ischemia in isolated rat hearts, then ischemia-induced increases in LV end-diastolic pressure and decreases in coronary flow were attenuated. However, preconditioning before cold cardioplegia (6–8°C) of 3.5–5 h was ineffective.

Data from our in vitro study are in general agreement with an in vitro study of human isolated right atrial trabeculae (3). Using function as an end point, this study found ischemic preconditioning and hypothermia to protect equally against ischemia; however, the two treatments were not additive. Using developed force in human isolated right atrial trabeculae as an end point, Cleveland and co-workers (3) tested the effect of 5 min of simulated ischemia followed by 10 min of perfusion (preconditioning), hypothermic perfusion at 4°C, and a combination of the two. Control trabeculae recovered 24% of baseline developed force after 45 min of ischemia and 120 min of reperfusion. Preconditioned trabeculae recovered 51% of developed force, hypothermic trabeculae recovered 47%, and trabeculae that received combined treatment recovered 56%.

Despite extensive research, the exact mechanisms for the protective effect of ischemic preconditioning, as well as for the protective effect of hypothermia, remain unknown. One possible protective effect of hypothermia may be a reduction in myocardial high-energy phosphate utilization. Jones and co-workers (9) have shown that lowering the temperature from 40 to 28°C slows the rate of high-energy phosphate utilization in dog hearts. Topical hypothermia of 30°C has been shown to significantly protect ATP levels in 15 and 30 min of ischemia in the liver of rabbits (5a).

Ning and co-workers (16) studied isolated, perfused rabbit hearts that were given either normothermic (37°C) or hypothermic (31°C) perfusion for 20 min before 120 min of ischemia followed by reperfusion. Results of their study showed that, compared with normothermia, hypothermic perfusion resulted in increased myocardial ATP preservation during both ischemia and reperfusion.

In humans undergoing valve replacement, ischemic preconditioning (2 cycles of 3 min by occlusion of the vena cava with aortic cross-clamping) combined with cold cardioplegia resulted in improved indexes of postoperative recovery such as decreased S-T segment elevation and MB isozyme of creatine kinase (CK-MB) release. In addition, preconditioning also improved myocardial contractility after reperfusion (10).

The use of topical hypothermia in humans has recently been associated with increased postsurgical diaphragmatic paralysis and pericardial effusions possibly caused by phrenic nerve paralysis (15). However, it should be pointed out that, in these patients, myocardial temperatures were much lower (~15°C) than those in our study (~32°C). In our acute studies phrenic nerve function was not tested; however, a more moderate decrease in myocardial temperature may be less harmful to the phrenic nerve.

In summary, data from this study indicate that increasing the duration of the preconditioning ischemia to two episodes of 7 min extends the window of protection to 120 min of coronary artery occlusion in the rabbit model. The data confirm our previous research that hypothermia is protective and reduces infarct size, even if begun 30 min after the start of a 2-h coronary artery occlusion (6). The combination of hypothermia plus ischemic preconditioning was not significantly better in protecting ischemic myocardium than either intervention alone in this study.

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