Adenosine Receptor Involvement in a Delayed Phase of Myocardial Protection 24 Hours After Ischemic Preconditioning

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Background We previously reported a delayed phase of protection against infarction 24 hours after ischemic preconditioning in the rabbit. In the present study, we investigated the possibility that this "second window of protection," like the well-described early phase of protection in the rabbit, might be associated with adenosine receptor activation.

Methods and Results In the first series of experiments, we examined whether adenosine receptor blockade with 8-[(p-sulfophenyl)]-theophylline (SPT) during preconditioning could abolish the delayed protection against infarction 24 hours later. Open-chest rabbits were subjected to myocardial preconditioning (PC) with the four 5-minute coronary occlusions or they were sham operated on (SHAM). During these procedures, animals received either SPT (PC+SPT, n=6; and SHAM+SPT, n=6) or vehicle (PC+VEH, n=12; and SHAM+VEH, n=11). Twenty-four hours later, infarct development after a 30-minute coronary occlusion/120-minute reperfusion insult was assessed with triphenyltetrazolium staining. In vehicle-treated rabbits, the infarct-to-risk ratio (I/R) was reduced from 53.6±5.7% (SHAM+VEH) to 32.9±4.6% (PC+VEH) (P<.05), clearly indicating a delayed phase of protection. Although I/R was not significantly different between SHAM+VEH (53.6±5.7%) and SHAM+SPT (61.7±5.4%), in PC+SPT the delayed protection was abolished (I/R=56.8±3.8%). In the second series of experiments, we examined if pharmacological adenosine A1 receptor stimulation could evoke a delayed phase of protection. Conscious rabbits were pretreated with intravenous boluses of saline or the A1 receptor–selective agonist 2-chloro-N6-cyclopentyladenosine (CCPA), and infarct size in response to 30-minute ischemia/120-minute reperfusion was assessed 24 hours later. I/R was 54.5±2.7% in saline-pretreated controls (n=12). Pretreatment with 25 μg/kg CCPA (n=6), 50 μg/kg CCPA (n=6), or 100 μg/kg CCPA (n=6) resulted in I/R ratios of 37.1±4.2% (P<.01), 37.7±2.2% (P<.01), and 26.3±5.7% (P<.01), respectively. In both series of experiments, there were no differences in systemic hemodynamics during the infarct protocol, assessed as rate-pressure product, between the different experimental groups.

Conclusions Twenty-four hours after repetitive brief coronary occlusions, susceptibility to infarction in rabbit myocardium is reduced, an effect that may have clinical relevance. Results of the present study suggest that this second window of protection following preconditioning may, like the early phase of protection, be initiated by an adenosine-related mechanism. (Circulation. 1994;90:2993-3000.)

Key Words • adenosine • myocardium • infarction

The marked myocardial protection associated with ischemic preconditioning is known to wane as the interval between the preconditioning coronary occlusions and the sustained occlusion is extended. For example, Murry et al.1 showed that the infarct-limiting effect of preconditioning in canine myocardium was considerably reduced, although still marked, when the interval between the preconditioning stimulus and the prolonged ischemic insult was extended from 5 to 120 minutes. In rabbit myocardium, the waning of the protective effect may be even more rapid, such that the infarct-limiting effect is lost 120 minutes after preconditioning.2 This acutely manifested protection conferred by ischemic preconditioning is now widely recognized in the experimental literature. However, recent reports support the notion that sublethal coronary occlusion elicits a biphasic pattern of protection, ie, an acute phase of protection (the "classic" preconditioning effect) and a second, delayed phase of protection possibly arising as a consequence of a molecular adaptation to sublethal myocardial ischemia. Evidence for this "second window of protection" comes principally from two groups. We have observed a limitation of infarct size in rabbit myocardium 24 hours after a preconditioning protocol consisting of four 5-minute coronary artery occlusions,3 and Kuzuya et al.4 also reported similar myocardial protection in the dog heart 24 hours after an identical preconditioning stimulus. It may also be relevant that Szekeres et al.5 reported that a brief period of rapid atrial pacing in the rabbit attenuated ECG ST-segment deviation during further periods of rapid pacing 24 and 48 hours subsequently.

The cellular mechanisms underlying the acute phase of protection are not clearly understood at present but, in the rabbit at least, considerable evidence points to involvement of the adenosine A1 receptor as both a trigger and a mediator of the early protection. Thus, Liu et al.6 showed that the acute protection could be abolished by pretreatment with adenosine receptor antagonists and, conversely, intracoronary administration of
Adenosine and R-phenyl-isopropyladenosine, an adenosine A₁ receptor agonist, limited infarct size to a degree similar to that seen with preconditioning. However, it is not known if the delayed protection in the rabbit is dependent on a similar mechanism. The aim of this study was to determine in our rabbit model of infarction (1) if the delayed protection could be inhibited by treatment during preconditioning with 8-(p-sulfophenyl)-theophylline (SPT), a nonselective adenosine receptor antagonist, and (2) if the delayed protection could be elicited by pretreatment with the adenosine A₁ receptor agonist 2-chloro-N⁶-cyclopentyladenosine (CCPA), administered in place of preconditioning coronary occlusion.

Preliminary accounts of some of these results were presented at the 66th Scientific Sessions of the American Heart Association in Atlanta, Ga, November 1993, and the Winter Meeting of the British Pharmacological Society in London, UK, January 1994.

Methods

Materials

We obtained 4% glucose/0.18% sodium chloride intravenous injection BP (dextrose-saline) from Baxter, SPT and CCPA from Research Biochemicals Inc through Semat, zinc-cadmium sulfide fluorescent microspheres (1 to 10 μm) from Duke Scientific, and triphenyltetrazolium chloride (TTC), from Sigma Chemical. All other chemicals were of analytical reagent quality.

Male New Zealand White rabbits (2.0 to 3.5 kg) were used for these studies. The care and use of animals in this work were in accordance with UK Home Office guidelines of the Animals (Scientific Procedures) Act 1986. Two studies were conducted: an adenosine antagonist study and an A₁ receptor agonist study. Each experiment had two parts. Part 1 involved preparation of the animal (either ischemic preconditioning with and without 8-SPT or pretreatment with CCPA). Part 2 was the infarction procedure. These protocols are summarized in Fig 1.

Adenosine Antagonist Study: Preparation

Animals were prepared surgically (as described below). There were four experimental groups. The vehicle-treated groups received a 2 mL/kg dextrose-saline bolus before the first preconditioning coronary occlusion followed by infusion of 6 mL/kg over 1 to 2 hours. Group 1 was preconditioned and received dextrose-saline only (PC+VEH), and group 2 was sham-operated and received dextrose-saline only (SHAM+VEH).

The SPT groups received SPT dissolved in dextrose-saline (3.5 mg/mL). A loading dose of 7.5 mg/kg was given 5 to 6 minutes before the first coronary occlusion followed by a slow infusion of 20 mg/kg over 1 hour. Group 3 was preconditioned and received SPT (PC+SPT), and group 4 was sham-operated and received SPT (SHAM+SPT).

Animals in the antagonist study were anesthetized with a combination of 2 mg/kg diazepam IP and 0.3 mL/kg fentanyl citrate and fluanisone mixture IM (Hypnorm, Janssen Pharmaceuticals). They were intubated with an orotracheal tube using a pediatric laryngoscope and ventilated with 100% O₂ at a tidal volume of 5 mL/kg and a rate of 58 cycles/min (small animal ventilator, Harvard Apparatus). Electrodes were attached to shaved areas on each limb for recording of the surface ECG. A marginal ear vein was cannulated to administer drugs and fluids. The standard infusion fluid used during the surgical preparation of all rabbits was dextrose-saline. SPT was dissolved in this solution as detailed above. A median sternotomy and pericardiotomy were performed. An anterolateral branch of the circumflex coronary artery was identified, and a 3-0 silk suture (Mersilk type 504, Ethicon) was passed underneath the vessel at a point approximately halfway between the left atrioventricular groove and the apex. The ends of the suture were threaded through a 1.5-cm polypropylene tube to form a snare. Heparin sodium 500 IU was administered before the first coronary artery occlusion. The artery was occluded by pulling the ends of the suture taut and clamping the snare onto the epicardial surface. Snaring of the artery caused epicardial cyanosis and regional hypokinesis within 20 to 30 seconds and was usually accompanied by ST-segment elevation or depression in the ECG within 2 minutes. After 5 minutes, reperfusion was instituted by releasing the snare. Successful reperfusion was confirmed by conspicuous blushing of the previously ischemic myocardium and gradual resolution.

Fig 1. Diagram of protocol and time course of experiments in the two studies. The two studies were performed sequentially, but within each study animals were assigned randomly to the various treatment groups. SPT indicates 8-(p-sulfophenyl)-theophylline; CCPA, 2-chloro-N⁶-cyclopentyladenosine; Rep, reperfusion; and Isch, ischemia.
of the ECG changes. The preconditioning protocol consisted of four 5-minute occlusions, each separated by 10-minute reperfusion. After preconditioning, the loose suture was left in situ, the thorax was evacuated, and the sternotomy was closed. Animals were extubated and allowed to recover from anesthesia with antibiotic cover (5 mg/kg enrofloxacin) and postoperative analgesia (30 μg/kg buprenorphine HCl).

Sham-operated animals underwent the same surgical procedure, including pericardiotomy, but as in our previous study, no coronary artery suture was positioned.

**A1 Receptor Agonist Study: Preparation**

Conscious rabbits did not undergo surgery, but 24 hours before the infarction protocol they received intravenous bolus injections of saline solution (0.9% NaCl) or CCPA (25, 50, or 100 μg/kg administered in −1.5 mL saline) via a marginal ear vein. Animals were immediately returned to their pens with no further manipulation.

**Infarction Procedure and Infarct Size Assessment**

Approximately 24 hours after preparation (preconditioning or sham operation, CCPA or saline bolus), rabbits were anesthetized with a combination of 40 mg/kg pentobarbital sodium IV and 0.15 mL/kg Hypnorn IM. They were ventilated with 100% O2, and the right common carotid artery was cannulated for periodic hemodynamic and arterial blood gas measurements. Tidal volume was adjusted as necessary throughout the procedure to maintain arterial pH between 7.3 and 7.5 and PCO2 at <5.0 kPa. The thorax was reopened, and the coronary artery branch was identified. In the preconditioned groups, the in situ coronary ligature was used again. In all other groups, a ligature was positioned as described above. After stabilization, 1000 IU heparin sodium was given intravenously before coronary occlusion. The artery was occluded for 30 minutes, followed by 120-minute reperfusion by manipulating the ligature and snare as described above.

At the end of 120-minute reperfusion, 1000 IU heparin sodium was administered before the heart was excised and Langendorff-perfused with saline solution to remove blood. The ligature was tightened again, and zinc–cadmium sulfide microspheres were infused through the aorta to delineate the myocardium at risk under ultraviolet light. After freezing at −18°C, the heart was sliced transversely from apex to base in 2-mm sections. The slices were defrosted, blotted, and incubated at 37°C with 1% w/v TTC in phosphate buffer (pH 7.4) for 10 to 20 minutes and fixed in 4% v/v formaldehyde solution to clearly distinguish between stained viable tissue and unstained necrotic tissue. The volumes of the infarcted tissue (I) and the tissue at risk (R) were determined by a computerized planimetric technique (Summa Sketch II, Summa Graphics).

**Acute Hemodynamic Effects of CCPA**

In a separate series of experiments, the short-term effects of 100 μg/kg CCPA on arterial blood pressure and heart rate were assessed in anesthetized rabbits. Rabbits were anesthetized with 40 mg/kg pentobarbital sodium IV and 0.15 mL/kg Hypnorn IM. They were ventilated, and the right carotid artery was cannulated for blood pressure and heart rate determination, as described above. CCPA (100 μg/kg) or 0.9% saline was administered by intravenous bolus, as described above, and the hemodynamic effects were monitored continuously for 120 minutes.

**Statistical Analysis**

The data are presented throughout as mean±SEM values. The significance of differences in mean values was evaluated by a one-way ANOVA. When treatment constituted a significant source of variance, Fisher’s least significant difference test or Dunnnett’s test, as appropriate, was used post hoc for predetermined individual group comparisons. The null hypothesis was rejected when P<0.05.

**Results**

**Exclusions**

A total of 89 rabbits were used for these studies. Eighty-three were used for the adenosine antagonist study and the A1 receptor agonist study, and 6 were used for hemodynamic assessments after CCPA or saline administration. In the antagonist study, 7 rabbits were lost due to hemorrhage or respiratory failure during the preconditioning or sham operation protocol. In the A1 agonist study, no animals were lost after preliminary treatment with CCPA or saline. Thus, 76 animals survived the preparation protocols. During part 2, 3 animals were excluded due to severe VF during the infarct protocol (1 in PC+VEH, 1 in SHAM+VEH, 1 in saline-pretreated group); 3 were excluded due to hemorrhage or persistent tachycardia throughout the infarct protocol (2 in PC+VEH, 1 in SHAM+SPT); 4 were excluded due to absence of a risk zone or a risk zone less than 0.4 cm² (1 in PC+VEH, 2 in SHAM+VEH, 1 in SHAM+SPT); and one heart was excluded due to failure of the TTC stain (PC+SPT). The final numbers of animals in each group were 11 in SHAM+VEH; 12, PC+VEH; 6, SHAM+SPT; 6, PC+SPT; 12, saline pretreatment; 6, CCPA 25 μg/kg; 6, CCPA 50 μg/kg; and 6, CCPA 100 μg/kg.

**Adenosine Antagonist Study**

**Systemic Hemodynamic Changes During Infarct Protocol**

Table 1 describes alterations in rate-pressure product (mean arterial pressure×heart rate [RPP]) during the infarct protocol in this first series of experiments. Twenty-four hours after surgical preparation, there were no differences in RPP between the experimental groups. Although there was a decrease in blood pressure during the first few minutes after coronary occlusion, with no tendency to recovery of RPP during reperfusion, this decline was similar in all groups. The similarity of hemodynamic parameters across the experimental groups during the infarct protocol makes it unlikely that these contributed to the differences in infarct size. We did not measure blood pressure during the preconditioning or sham operation protocol or during the subsequent 24-hour interval. We cannot exclude the possibility, albeit unlikely, that differences in hemodynamic parameters at this stage contributed to subsequent limitation of infarct size.

**Infarct Limitation**

Table 2 summarizes the infarct size and risk volume data. Fig 2 is a graphic representation of the I/R ratios. In the vehicle-treated controls, absolute infarct volume (I) was reduced from 0.60±0.08 cm³ in SHAM+VEH to 0.36±0.06 cm³ in PC+VEH (P<0.05), and the percentage of infarcted tissue within the risk zone (I/R) was reduced from 53.6±5.7% to 32.9±4.6%, respectively (P<0.05). These reductions in absolute and relative infarct sizes clearly indicate the presence of a delayed phase of protection 24 hours after preconditioning. The administration of the adenosine receptor antagonist SPT during the sham operation had no significant effect on I/R ratio in rabbits prepared by sham operation (61.7±5.4% in SHAM+SPT versus 53.6±5.7% in SHAM+VEH). However, when SPT was given during
Figure 4 depicts the effects of 100 μg/kg CCPA or saline on mean arterial pressure and heart rate in anesthetized rabbits. Baseline parameters were similar in both groups (CCPA-treated group: heart rate, 264±9 beats per minute; mean arterial pressure, 76±7 mm Hg; saline-treated control group: heart rate, 249±11 beats per minute; mean arterial pressure, 75±10 mm Hg). This dose of CCPA, the highest used in the A₃ receptor agonist study, caused a rapid reduction in heart rate. Maximal bradycardia (a reduction of 33.1±4.0% of baseline value) was observed 20 minutes after CCPA administration. By 90 minutes after the injection of CCPA, the magnitude of the bradycardia was not different from that seen in saline-treated controls, in which some bradycardia due to anesthesia was observed. The effect of 100 μg/kg CCPA on arterial pressure was more modest, with the peak hypotensive effect (a reduction in mean arterial pressure of 18.5±7.7% of baseline value) observed 10 minutes after administration. Thirty minutes after the injection, arterial pressure was similar to that seen in the saline-treated controls.

### Table 2. Adenosine Antagonist Study Values for Absolute Infarct Volume, Ischemic Risk Zone Volume, and Infarct-to-Risk Ratios

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Animals</th>
<th>Infarct Volume, cm³</th>
<th>Ischemic Risk Zone Volume, cm³</th>
<th>Infarct-to-Risk Ratio, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM+VEH</td>
<td>11</td>
<td>0.60±0.08</td>
<td>1.1±0.1</td>
<td>53.6±5.7</td>
</tr>
<tr>
<td>PC+VEH</td>
<td>12</td>
<td>0.36±0.06†</td>
<td>1.2±0.2</td>
<td>32.9±4.6*</td>
</tr>
<tr>
<td>SHAM+SPT</td>
<td>6</td>
<td>0.58±0.12</td>
<td>0.9±0.1</td>
<td>61.7±5.4</td>
</tr>
<tr>
<td>PC+SPT</td>
<td>6</td>
<td>0.58±0.04</td>
<td>1.0±0.1</td>
<td>56.8±3.8</td>
</tr>
</tbody>
</table>

SHAM indicates sham-operated; VEH, vehicle; PC, preconditioned; and SPT, 8-(p-sulfophenyl)-theophylline. Values are mean±SEM.

*P<.05 compared with all other groups; †1.1>P>.05 compared with sham control (ANOVA).
Discussion

Adenosine and the Second Window of Protection

These results provide further evidence of a delayed phase of myocardial protection after ischemic preconditioning and support previous observations from our laboratory and another. Furthermore, we have begun to investigate the mechanism underlying this delayed protection and present two lines of evidence in support of a role of the adenosine A_1 receptor in triggering this protection. First, we have demonstrated clearly that the second window of protection associated with preconditioning could be abrogated by adenosine receptor blockade with SPT during preconditioning. In interpreting the results of this antagonist study, we could not preclude the possibility of some nonspecific, i.e., not receptor related, effect of SPT on the myocardium that abolished or masked protection 24 hours later. Due to the sulphonphenyl moiety, SPT does not cross the myocyte plasma membrane like some other xanthine derivatives, and therefore inhibition of cyclic AMP phosphodiesterases is not likely to account for the loss of protection.

In our second series of experiments, we were able to elicit myocardial protection against a lethal coronary occlusive insult by pretreatment 24 hours beforehand with three doses of the highly selective adenosine A_1 receptor agonist CCPA. It is clear that all the animals in the infarct study were hemodynamically similar 24 hours after CCPA or saline treatment, and subtle differences in systemic hemodynamics during the infarct procedure are unlikely to account for the marked reductions in

Table 3. Systemic Hemodynamic Data (Summarized as Rate-Pressure Product) for the Infarction Protocol in the A_1 Agonist Study

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Animals</th>
<th>Baseline</th>
<th>5</th>
<th>29</th>
<th>30</th>
<th>60</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>12</td>
<td>19.3±1.2</td>
<td>12.3±1.5</td>
<td>14.0±1.1</td>
<td>12.0±0.7</td>
<td>12.5±0.7</td>
<td>11.8±0.8</td>
</tr>
<tr>
<td>CCPA 25 μg/kg</td>
<td>6</td>
<td>19.9±1.7</td>
<td>13.5±2.7</td>
<td>14.6±1.3</td>
<td>13.6±1.2</td>
<td>12.6±1.2</td>
<td>12.2±1.3</td>
</tr>
<tr>
<td>CCPA 50 μg/kg</td>
<td>6</td>
<td>21.8±1.7</td>
<td>15.5±2.7</td>
<td>17.5±2.1</td>
<td>13.5±1.4</td>
<td>12.3±1.0</td>
<td>12.6±0.9</td>
</tr>
<tr>
<td>CCPA 100 μg/kg</td>
<td>6</td>
<td>18.3±1.3</td>
<td>12.7±2.2</td>
<td>14.5±0.7</td>
<td>12.6±0.9</td>
<td>12.9±1.1</td>
<td>11.8±0.8</td>
</tr>
</tbody>
</table>

CCPA indicates 2-chloro-N^6-cyclopentyladenosine. Values are mean±SEM.

Baseline readings of blood pressure and heart rate were made during the 5-minute period before the 30-minute coronary artery occlusion. Rate-pressure product declined immediately after coronary occlusion and showed little recovery during the remainder of the protocol. Blood pressure was the main determinant of change in the rate-pressure product. There were no significant differences in rate-pressure product between the experimental groups at any time point.
TABLE 4. A, Agonist Study Values for Absolute Infarct Volume, Ischemic Risk Zone Volume, and Infarct-to-Risk Ratios

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Animals</th>
<th>Infarct Volume, cm³</th>
<th>Ischemic Risk Zone Volume, cm³</th>
<th>Infarct-to-Risk Ratio, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>12</td>
<td>0.68±0.06</td>
<td>1.2±0.1</td>
<td>54.5±2.7</td>
</tr>
<tr>
<td>CCPA 25 µg/kg</td>
<td>6</td>
<td>0.40±0.08*</td>
<td>1.0±0.1</td>
<td>37.1±4.2†</td>
</tr>
<tr>
<td>CCPA 50 µg/kg</td>
<td>6</td>
<td>0.43±0.05*</td>
<td>1.1±0.1</td>
<td>37.7±2.2†</td>
</tr>
<tr>
<td>CCPA 100 µg/kg</td>
<td>6</td>
<td>0.27±0.09†</td>
<td>1.0±0.1</td>
<td>26.3±5.7†</td>
</tr>
</tbody>
</table>

CCPA indicates 2-chloro-N⁶-cyclopentyladenosine. Values are mean±SEM.

*P<.05 or †P<.01 compared with saline-treated control group (ANOVA with Dunnett’s test).

Infarct size observed in either the ischchemically preconditioned or CCPA-pretreated groups. Our hemodynamic observations with the highest dose of CCPA in anesthetized rabbits showed that a single bolus of 100 µg/kg CCPA caused bradycardia over a period of ~90 minutes. Because the drug was administered to uninstrumented conscious animals for the A₁ receptor agonist study, we have no data on the immediate hemodynamic effects of the CCPA bolus in these animals, although it is likely that in the conscious state the immediate hemodynamic effects are less pronounced than they are in the anesthetized state. We cannot exclude the possibility that CCPA could have evoked preconditioning of the heart indirectly through its hemodynamic effects. Although we believe it unlikely that the combined bradycardia and hypotension would be sufficient to diminish coronary perfusion so as to cause myocardial hypoxia, and therefore preconditioning, it is possible that a compensatory sympathetic activation occurred. Catecholamine release in response to the bradycardia might reasonably be expected, and this may explain the different time courses of the bradycardia and the hypotension after CCPA administration (see Fig 4). There is some evidence that norepinephrine through an a₁-adrenergoreceptor effect can mimic, and may be the trigger for, the early preconditioning response in the rat. Although in the rabbit tyramine-evoked catecholamine release induces protection against infarction, adrenergoreceptor activation does not appear to be the primary mechanism of classic preconditioning protection in either the rabbit or the dog. Furthermore, it has been reported recently that norepinephrine administration to rats evokes myocardial protection in a biphase manner, which may have some relevance to our present observations (see below). On the other hand, Thornton et al showed that adenosine A₁ receptor activation with R-phenylisopropyladenosine mimicked the early phase of preconditioning protection directly and not as a consequence of the bradycardia since cardiac pacing to overcome the bradycardia did not abolish the protection. Thus, although it is apparent in our study that adenosine receptor blockade during preconditioning abolished the delayed protection, at present we are not able to state conclusively that A₁ receptor activation with CCPA directly triggers the delayed protection. The protection we have observed appears to be dependent on adenosine receptor activation, but further work is required to establish the contribution of factors other than A₁ receptor activation to this delayed protection.

Characterization and Potential of the Second Window of Protection

Although the degree of infarct reduction in the second window of protection is not as marked as that seen during the immediate phase of protection after ischemic preconditioning, this effect appears to be consistent and reproducible in the two laboratories where the protection has been observed. Thus, in our previous report we noted a decrease in I/R from 52.0% in sham-operated rabbits to 28.8% in preconditioned rabbits, a 45% reduction. In the present study, the reduction in I/R with ischemic preconditioning was similar at 39%. Kuzuya et al showed a reduction in I/R of 46% 24 hours after preconditioning in canine myocardium. In the present study, CCPA pretreatment in place of coronary occlusion led to reductions in I/R of between 31% and 52%.
The present study represents the third report of a delayed anti-infarct effect of preconditioning, but there also has been a negative study. Tanaka et al\textsuperscript{16} were unable to demonstrate a delayed phase of protection in the rabbit 24 hours after repeated brief coronary occlusions. The reasons for the failure in that study are not immediately apparent, but it may be that the lateral thoracotomy used in the negative study was more stressful than the median sternotomy used in the present study. In addition, lateral thoracotomy necessitates displacement of a lung to approach the heart. It is also possible that the gas used to ventilate the animals during preconditioning had some effect. In this and our previous study\textsuperscript{3} and the positive study of Kuzuya et al\textsuperscript{4}, 100% O\textsubscript{2} was administered. In contrast, Tanaka et al\textsuperscript{16} used air to ventilate. However, the possibility that 100% O\textsubscript{2} was a major determinant of the subsequent myocardial protection seems remote, particularly in view of our present finding that CCPA administration to conscious, unventilated animals elicited a similar delayed protection.

So far, the duration of the second window of protection is unknown, but it is almost certainly longer than the 1 or 2 hours of opportunity that characterize the early period of protection after preconditioning. Because we hypothesize that the delayed protection is finally mediated by alterations in the gene expression of cytoprotective proteins (see below), we believe it likely that the time course of appearance and disappearance of these proteins will be gradual and slow. If this is the case, then the second window of protection offers the potential of valuable clinical exploitation. For example, coronary artery bypass graft surgery and percutaneous transluminal coronary angioplasty are two procedures that, of necessity, involve rendering the myocardium ischemic. If by some pharmacological or other pretreatment one could induce in patients being prepared for these procedures a period of enhanced resilience to ischemia, the period of ischemic manipulation available to the surgeon or physician might be extended with improved outcome for the patient.

It is possible that the second window of protection after preconditioning in this experimental model has some parallels in the natural history of certain clinical ischemic syndromes. In particular, a recent clinical investigations have examined the potential for antecedent ischemia (unstable angina) 24 to 48 hours before myocardial infarction to influence infarct size and clinical outcome. Ottani et al\textsuperscript{17} studied 25 patients presenting with first myocardial infarction who received intravenous thrombolytic therapy. Angiography was performed 3 weeks after thrombolysis, revealing patency of the infarct-related artery and absence of collateral vessels in all patients. However, those with preceding angina 24 hours before infarction had an apparent reduction in infarct size, determined by peak creatine kinase–MB release and extent of segmental hypokinesis in the ventriculogram. Kloner et al\textsuperscript{18} recently reported the results of their analysis of the outcome of 350 patients enrolled in the TIMI-4 trial. Patients who had experienced angina in the 48 hours preceding infarction were compared with those who had not. In the group with antecedent angina, the incidences of cardiac failure/cardiogenic shock and in-hospital mortality were reduced. Again, collateralization was not detected as different in the two groups.

**Possible Mechanisms for the Delayed Protection**

The molecular basis of the protection we observed is unknown. It is well known that the rabbit is a species deficient in preformed collateral vessels,\textsuperscript{19} and it is most unlikely that angioneogenesis, providing collateral support to the ischemic zone, could occur within 24 hours in the preconditioned group. We believe it more probable that the myocardial protection is the direct result of enhanced cellular resistance to ischemia. Although the final mediators of this cellular resistance are unknown, we suggest that the long-term adaptation to the preconditioning stimulus may involve genetic modifications that lead to the appearance of cytoprotective substances in the myocardium. In this connection, we have been interested by recent work examining the role of protein kinase C (PKC) in the myocardial response to transient ischemia. Downey's group,\textsuperscript{20} Banerjee's group,\textsuperscript{21} and our group\textsuperscript{22} have provided evidence that activation of PKC may be a crucial event in the mediation of classic preconditioning protection. In addition to translocation of the calcium-dependent \( \alpha \) subtype and the calcium-independent \( \delta, \zeta, \) and \( \epsilon \) subtypes from the cytosol to the sarcolemma during brief periods of ischemia,\textsuperscript{23} it is possible that other subtypes may be translocated into the nucleus. This nuclear translocation may be a key event in the modification of gene-regulatory processes that could underlie the second window of protection. Because PKC translocation is proposed to occur after GTP regulatory protein (G protein) transduction, several receptor types, including the adenosine \( A_1 \) receptor, may influence PKC activity.

Many oncogenes and nuclear transcription factors may be activated by PKC (for review, see Reference 24). Interestingly, Meng et al\textsuperscript{14} described a biphasic time course of myocardial protection after norepinephrine administration with enhanced postischemic recovery observed at 1, 24, and 72 hours later but not at 4 hours. This effect of norepinephrine was evidently mediated via the \( \alpha_1 \)-adrenoreceptor, a receptor subtype linked to a G protein and therefore to the PKC signaling pathway. It is already known that the late myocardial response to sublethal ischemia involves the augmentation of various proteins, including stress proteins (heat shock proteins [HSPs]),\textsuperscript{3,25,26} and endogenous antioxidant proteins, especially catalase\textsuperscript{27} and the mitochondrial manganese superoxide dismutase.\textsuperscript{28,29} In the study of Meng et al,\textsuperscript{14} altered levels of c-fos, c-jun, and HSP70 mRNAs were found several hours after norepinephrine administration. However, it is not yet clear what roles these putative cytoprotective proteins play in the delayed myocardial protection, to what extent their effects are interdependent, or how their appearance and activity may be affected by adenosine receptor modulation.

In conclusion, we have provided evidence that a delayed myocardial protection after preconditioning in the rabbit may be linked to adenosine receptor activation. Further work is under way in our laboratory to explore the myocardial stress protein and antioxidant responses to sublethal ischemia, how these might be modified by adenosine receptor blockade and activation, and the position of PKC activation in this intrigu-
ing and potentially exploitable adaptive response of the myocardium to ischemic stress.

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