Protection Against Infarction Afforded by Preconditioning is Mediated by A₁ Adenosine Receptors in Rabbit Heart

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**Background.** Preconditioning (5 minutes of ischemia followed by 10 minutes of recovery) renders the heart very resistant to infarction from subsequent ischemia. This study tests whether adenosine receptors might mediate preconditioning protection.

**Methods and Results.** We examined the effect on infarct size of pretreatment with either of two adenosine receptor antagonists in both control and preconditioned in situ rabbit hearts. Hearts underwent 30 minutes of regional ischemia plus 3 hours of reperfusion, and infarct size was measured with tetrazolium. Infarct size averaged 39% of the zone at risk in controls but only 8% in preconditioned hearts. Preconditioned and nonpreconditioned hearts receiving either blocker had infarcts not different in size from the controls. A 5-minute intracoronary infusion of adenosine was as effective as 5 minutes of ischemia in protecting parabiotically perfused isolated hearts against infarction from a 45-minute ischemic insult. Similarly, intracoronary infusion of N₆,1-(phenyl-2R-isopropyl)adenosine, an A₁-selective adenosine receptor agonist, at a dose that delayed conduction but did not dilate the coronary vessels, also limited infarct size. The protection disappeared when we reduced the coronary concentration of drug by intravenous infusion of adenosine, indicating that cardiac rather than peripheral receptors were involved in the protection.

**Conclusions.** We conclude that adenosine released during the preconditioning occlusion stimulates cardiac A₁ receptors, which leaves the heart protected against infarction even after the adenosine has been withdrawn. (Circulation 1991;84:350–356)

A brief period of ischemia followed by reperfusion renders the heart very resistant to infarction from a subsequent ischemic insult. This phenomenon, termed preconditioning, has been described in dogs,¹⁻³ in pigs,⁴ and in rabbits.⁵⁻⁶ The mechanism for preconditioning is unknown, but previous work has shown that it does not involve opening of collateral vessels,¹ nor does it involve synthesis of a protective protein.⁶ The present study tests the hypothesis that adenosine released during the preconditioning occlusion might be the mediator of this response. Adenosine is a likely candidate since it is released by ischemic myocytes⁷ and because it has been reported to have cardioprotective properties. Pretreatment with adenosine has been reported to both stimulate glycolysis in hypoxic hearts⁸ and delay the onset of ischemic contracture.⁹ Both effects were mediated by A₁ receptors. Adenosine is also reported to have an antineutrophil effect¹⁰ that is mediated by an A₂ receptor.¹¹ Adenosine infused during reperfusion has also been reported to limit infarct size.¹²

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**Methods**

**Surgical Preparation of Animals**

The **in situ model.** New Zealand White rabbits of either sex, weighing between 1.3 and 2.5 kg, were intravenously anesthetized with sodium pentobarbital (30 mg/kg). Additional anesthesia was administered during the experiment as needed. The neck was opened with a ventral midline incision, and a tracheotomy was performed. The rabbits were orally intubated and mechanically ventilated on a positive pressure respirator (MD Industries, Mobile, Ala.)
with 100% oxygen. Ventilation rate was 30–35
breaths per minute, and tidal volume was approxi-
ately 15 ml. The respiration rate was adjusted to
keep the blood pH in the physiological range. Cat-
heters were placed in the left carotid artery and
jugular vein for blood pressure monitoring and drug
injection, respectively. A left thoracotomy was per-
formed in the fourth intercostal space, and the
pericardium was opened to expose the heart. A 2-0
silk thread was passed around a branch of the left
coronary artery with a taper needle, and the ends of
the tie were threaded through a small vinyl tube to
form a snare. The rabbits were given 500 units/kg
sodium heparin to prevent clot formation around
the snare. The coronary branch was occluded by pulling
the snare, which was then fixed by clamping the tube
with a mosquito hemostat. Myocardial ischemia was
confirmed by regional cyanosis. Reperfusion was
confirmed by hyperemia over the surface after releas-
ing the snare.

The isolated heart model: Donor rabbits. New
Zealand White rabbits (1.7–2.5 kg) of either sex were
anesthetized with 30 mg/kg sodium pentobarbital,
administered via a marginal ear vein. A tracheotomy
was performed, and animals were ventilated with
100% oxygen as described above. As above, a left
thoracotomy was performed to expose the heart, and
a silk ligature on a curved taper needle was passed
underneath a prominent branch of the left coronary
artery. The rabbits were given 500 units/kg sodium
heparin, then the heart was rapidly removed by cutting
the great vessels, was put in room tempera-
ture saline, and was mounted on the Langendorff
apparatus within 1 minute.

The isolated heart model: Support rabbits. New
Zealand White rabbits (2.0–3.9 kg) of either sex were
anesthetized and ventilated with 100% oxygen as
described above. The right carotid artery was cannu-
lated with a 13-gauge needle adapter to supply
arterial blood to the isolated heart. A large-bore
Tygon catheter was placed in the left jugular vein for
return of venous blood from the isolated heart cham-
ber. The support rabbit’s aortic blood pressure was
measured via a catheter that was inserted in the right
femoral artery and advanced into the thoracic aorta.
To anticoagulate the circulating blood, animals were
given a 1,000 units/kg bolus of sodium heparin plus a
500 units/kg supplement every 60 minutes.

The perfusion circuit consisted of silicon tubing
running from the support animal’s carotid artery
through a roller pump (model 1215, Harvard Appar-
atus, South Natick, Mass.) to a Teflon cannula
encased in a water jacket heated to 37°C. The
isolated heart was suspended via the aorta from the
cannula and placed in a glass water-jacketed cham-
ber covered with plastic film. The chamber was
siliconized with Sigmacote (Sigma Chemical Co., St.
Louis, Mo.). The temperature of the chamber was set
at 37°C. The pulmonary artery was cut, allowing
blood to drip into the chamber from which the blood
was returned to the support rabbit’s jugular vein by
gravity. Perfusion pressure to the isolated heart was
measured via a side arm in the heart cannula. The
hearts were perfused directly with the support rab-
bit’s arterial pressure if a perfusion pressure at or
above 50 mm Hg could be achieved. If the perfusion
pressure was below 50 mm Hg, a roller pump was
used to maintain perfusion pressure at 60 mm Hg.
Pacing electrodes were placed in the right atrium,
and the isolated heart was paced at 180 beats/min
with pulses of 5 V of 4-msec duration. A bipolar
electrode was allowed to rest lightly on the ventricu-
lar surface of some of the hearts in order to measure
the electrocardiogram. A fluid-filled latex balloon,
which was connected to a transducer by PE240
tubing, was inserted into the left ventricle. Balloon
volume was adjusted to maintain the left ventricular
systolic pressure near 90 mm Hg. Total coronary
artery flow was measured by timed collection of
blood from the chamber into a graduated cylinder.
The heart was allowed to stabilize for at least 10
minutes before the experiment was begun.

Measurement of Infarct and Risk Area

At the end of each in situ study the heart was
quickly removed and mounted on the Langendorff
apparatus (in the isolated heart studies, the heart was
already mounted on a Langendorff apparatus). All
hearts were flushed with room temperature saline for
60 seconds. The coronary branch was then reoc-
cluded and fluorescent particles (1–10 μm in diam-
ter from Duke Scientific Corp., Palo Alto, Calif.)
were infused into the perfusate to mark the risk zone.
The heart was removed from the Langendorff appa-
ratus, weighed, and frozen. While frozen, the heart
was cut into 2-mm transverse slices. The slices were
incubated in 1% triphenyl tetrazolium chloride
(TTC) in pH 7.4 buffer for 20 minutes. TTC caused
living tissue to stain a deep red color. After staining,
the area of infarcted tissue (TTC-negative tissue)
and the risk zone (area lacking fluorescence under
ultraviolet light) were traced. The area of infarct and
risk zone was determined by planimetry of the trac-
ings. The volume of infarcted myocardium and myo-
cardium at risk was calculated by multiplying the
planimetered areas by the slice thickness.

Chemicals

Adenosine was obtained from Sigma. N\(^\text{6}-1\)-(phe-
nyl-2R-isopropyl)adenosine (R-PIA) and 8-p-
sulfophenyl theophylline (SPT) were obtained from
Research Biochemicals Inc., Natick, Mass. PD
115,199, N-[2-(dimethylamino) ethyl] N-methyl-4-
(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-
8-yl) benzosulfonamide, was provided as a gift from
Parke-Davis, Ann Arbor, Mich.

Experiment Protocols

In situ experiments. The animals were randomly
divided into seven groups. Group 1, the control
group, was subjected to 30 minutes of coronary
branch occlusion followed by 180 minutes of reper-
fusion. Group 2, the preconditioned group, received 5 minutes of coronary branch occlusion followed by 10 minutes of reperfusion prior to the 30-minute occlusion. Groups 3 and 4 received the adenosine receptor blocker SPT (7.5 mg/kg) or PD 115,199 (3 mg/kg), respectively. The blocker was given as an intravenous bolus 20 minutes before a single 30-minute occlusion and reperfusion. Groups 5 and 6 received SPT or PD 115,199, respectively (same dose as groups 3 and 4), 5 minutes before preconditioning the animal. After preconditioning, the animals experienced a 30-minute reocclusion and reperfusion. Group 7 received an intravenous infusion of 5 mg adenosine for 5 minutes. After a 10-minute recovery period, the hearts underwent a 30-minute coronary branch occlusion, followed by reperfusion. In all groups, infarct size was measured at the end of the study by TTC staining. The dose of SPT was chosen as one that attenuated the hypotension to a 200-µg i.v. bolus by 70%. A doubling of that dose caused very little additional block against that test. The dose of PD 115,199 was recommended by the manufacturer and completely blocked the hypotensive response to intravenous adenosine in our model.

Isolated heart experiments. Four groups were studied. Group 1, the control group, underwent 45 minutes of coronary branch occlusion followed by 120 minutes of reperfusion. Group 2, the preconditioned group, received a 5-minute coronary branch occlusion followed by 10 minutes of reperfusion before experiencing a 45-minute reocclusion and 120 minutes of reperfusion. Groups 3 and 4 received a 5-minute infusion of either adenosine (1.4 mg) or the A1-selective agonist R-PIA (26.6 µg), respectively, in place of the 5 minutes of coronary branch occlusion. After 10 minutes of recovery, a coronary branch was occluded for 45 minutes and then reperfused. Before treatment, control values for heart rate, mean perfusion pressure, coronary flow, left ventricular systolic pressure, and left ventricular diastolic pressure were recorded. Before infusion of either adenosine or R-PIA the roller pump was engaged to maintain a perfusion pressure of 60 mm Hg. The flow rate was not changed during the infusion so that any coronary dilation would be reflected as a fall in perfusion pressure.

**Data Analysis**

All results are expressed as group mean±SEM. The significance (considered as p<0.05) of differences between groups was determined by a one-way analysis of variance with a Newman-Keuls post hoc test. Groupings for the analysis of variance were between the seven in situ groups for the first set and the four isolated hearts groups for the second set.

**Results**

**In situ heart experiments.** Sixty-three rabbits were used in the study: 20 in the control group, nine in the preconditioned group, eight and four in the SPT and PD 115,199 groups without preconditioning, respectively; 14 and five in the SPT and PD 115,199 groups with preconditioning, respectively; and three in the intravenous adenosine infusion group.

Hemodynamic and infarct size data for the in situ groups are summarized in Table 1. There are no significant differences in heart rate or mean aortic pressure (measured at the onset of the 30-minute coronary occlusion) among any of the seven groups. Mean aortic pressure fell from 94±6.9 to 51±6.8 mm Hg during the 5-minute intravenous adenosine infusion (p<0.02) but quickly recovered when the infusion was stopped. Table 1 also reveals the size of the risk zone and the infarct for the seven in situ groups. The size of the area at risk was not different among the seven groups. The myocardial infarct size, normalized as a percentage of the area at risk, averaged 38.9±4.2% in the control group (n=20), whereas preconditioning caused infarcts to be much smaller from the same ischemic insult (7.8±1.8%, n=9). These were significantly different (p<0.05). The protection afforded by preconditioning disappeared when either adenosine receptor blocking agent (SPT or PD 115,199) was given before preconditioning. Infarct size averaged 38.9±7.0% (n=14) for the SPT group and 51.1±5.9% (n=5) for the PD 115,199 group. Neither of the blocked or preconditioned groups had infarct sizes different from those in the control group. Similarly, both blocked and preconditioned groups had significantly larger infarcts than those in the preconditioned-only group (p<0.05).

**Table 1. Hemodynamics and Infarct Sizes in 63 Rabbits In Situ**

<table>
<thead>
<tr>
<th></th>
<th>Heart rate (beats/min)</th>
<th>Aortic pressure (mm Hg)</th>
<th>Risk zone size (cm³)</th>
<th>Infarct size (cm³)</th>
<th>Infarction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=20)</td>
<td>250±7</td>
<td>71±3</td>
<td>0.97±0.11</td>
<td>0.38±0.06</td>
<td>38.9±4.2</td>
</tr>
<tr>
<td>PC (n=9)</td>
<td>225±12</td>
<td>61±9</td>
<td>0.58±0.02</td>
<td>0.06±0.01*</td>
<td>7.8±1.8*</td>
</tr>
<tr>
<td>SPT (n=8)</td>
<td>275±9</td>
<td>76±5</td>
<td>0.89±0.08</td>
<td>0.47±0.09</td>
<td>53.2±8.6</td>
</tr>
<tr>
<td>SPT+PC (n=14)</td>
<td>260±8</td>
<td>77±3</td>
<td>0.84±0.08</td>
<td>0.38±0.09</td>
<td>38.9±7.0</td>
</tr>
<tr>
<td>PD (n=4)</td>
<td>263±12</td>
<td>74±15</td>
<td>0.85±0.14</td>
<td>0.41±0.16</td>
<td>41.9±11.4</td>
</tr>
<tr>
<td>PD+PC (n=5)</td>
<td>271±8</td>
<td>71±7</td>
<td>0.68±0.11</td>
<td>0.35±0.07</td>
<td>51.1±5.9</td>
</tr>
<tr>
<td>IV AD (n=3)</td>
<td>254±28</td>
<td>73±7</td>
<td>0.94±0.16</td>
<td>0.39±0.02</td>
<td>44.0±8.3</td>
</tr>
</tbody>
</table>

Values are mean±SEM. PC, preconditioned; SPT, 8-p-sulfophenyl theophylline; PD, PD 115,199; IV AD, intravenous adenosine.

*p<0.01 vs. control.
Neither SPT nor PD 115,199 had any effect on infarct size in nonpreconditioned animals when compared with controls. Five minutes of intravenous infusion of adenosine did not substitute for preconditioning as the infarcts in that group (44.0±8.3, n=3) were no different from those seen in the controls (see Figure 1).

Isolated heart experiments. Thirty-eight heart-donor rabbits comprised these four groups. Left ventricular systolic and diastolic pressure, coronary perfusion pressure, and coronary flow are summarized in Table 2. Risk zone and infarct size data for each group are also shown in Table 2. There were no differences in risk zone size among the groups. The infarct size in the control group (n=13) averaged 32.1±4.1%, whereas that in preconditioned hearts (n=10) was significantly smaller at 7.8±1.7% (p<0.001). A 5-minute infusion of adenosine or R-PIA directly into the perfusate to the isolated hearts was just as protective as a 5-minute occlusion. The infarct sizes averaged 7.4±1.4% and 7.6±2.7% for the adenosine and R-PIA groups, respectively, which were not different from those in the preconditioned group and significantly smaller than those in the control group (p<0.01). Although adenosine caused a marked coronary dilation as evidenced by a fall in the perfusion pressure to the heart, R-PIA was given at a dose that was not dilatory. Both adenosine and R-PIA lengthened the stimulus-QRS interval by more than twofold but because of the pacing, heart rate was unchanged. An even greater dose of adenosine given intravenously was not protective, presumably because it was too diluted when it reached the coronary circulation. Apparently the effect of adenosine must be directly on the heart (see Figure 2).

**Table 2. Hemodynamics and Infarct Sizes in 38 Isolated Rabbit Hearts**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=13)</th>
<th>PC (n=10)</th>
<th>Adenosine (n=8)</th>
<th>R-PIA (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary perfusion pressure (mm Hg)</td>
<td>64±3</td>
<td>62±4</td>
<td>66±4</td>
<td>66±5</td>
</tr>
<tr>
<td>Systolic pressure (mm Hg)</td>
<td>93±3</td>
<td>93±3</td>
<td>99±1</td>
<td>97±3</td>
</tr>
<tr>
<td>Diastolic pressure (mm Hg)</td>
<td>7±2</td>
<td>7±2</td>
<td>9±2</td>
<td>9±1</td>
</tr>
<tr>
<td>Coronary flow (ml/min/g)</td>
<td>0.90±0.10</td>
<td>0.88±0.08</td>
<td>0.84±0.14</td>
<td>1.04±0.13</td>
</tr>
<tr>
<td>Risk zone size (cm²)</td>
<td>0.64±0.06</td>
<td>0.60±0.18</td>
<td>0.63±0.08</td>
<td>0.56±0.06</td>
</tr>
<tr>
<td>Infarct size (cm²)</td>
<td>0.19±0.02</td>
<td>0.05±0.01*</td>
<td>0.05±0.01*</td>
<td>0.04±0.02*</td>
</tr>
<tr>
<td>Infarction (%)</td>
<td>32.1±4.1</td>
<td>7.8±1.7*</td>
<td>7.4±1.4*</td>
<td>7.6±2.7*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. PC, preconditioned; R-PIA, N6-1-(phenyl-2R-isopropyl)adenosine.

*p<0.01 vs. control.
A brief period of \( A_1 \) receptor stimulation apparently triggers a long-lasting change within the myocardium that causes it to be more resistant to the ischemic insult. At this point we do not know the nature of that change and can only speculate on what it might be. The protective change could involve improved glycolytic capacity during the ischemic phase. Wyatt et al.\(^1\) reported that hypoxic rat hearts had a fourfold increase in glycolysis when stimulated with either adenosine or \( R \)-PIA. Improved glycolysis could allow the heart to use its glycogen stores more efficiently during the ischemic period. That result is controversial, however, in that at least two groups have not been able to reproduce that result.\(^1,14\) In light of the present experiment, failure of the latter groups to see a stimulated glycolytic rate from exogenous adenosine could have been related to excessive ischemia during the mounting process, which may have preconditioned the hearts. If that were the case, no further increase in glycolytic capacity might have been possible. We found that a single 5-minute period of ischemia will fully precondition the rabbit heart, but 2 minutes of ischemia would not.\(^15\) In the present study, the normothermic transfer between the donor rabbit and the perfusion apparatus was always accomplished in less than 60 seconds, well below the threshold for preconditioning. Whether collecting the heart in iced saline, as is often done, raises or lowers the threshold for preconditioning is unknown. Another problem with the glycolysis theory is the observation of Murry et al.\(^16\) that glycolysis was actually slower in preconditioned dog hearts, as evidenced by reduced rates of glycogen breakdown and lactate production.

Adenosine has recently been reported to be protective to the ischemic heart when it was started just before reperfusion.\(^17,18\) Adenosine was thought to be beneficial in those studies through an inhibition of neutrophil function.\(^19\) That mechanism, however, seems less likely to explain preconditioning for several reasons. First, \( A_2 \) receptors are thought to mediate the inhibitory effect of adenosine on circulating neutrophils,\(^11\) but the present experiments implicate \( A_1 \) receptors in preconditioning. Second, adenosine was only protective when it reached the heart in high concentration by intracoronary administration. When an even larger dose of adenosine was given intravenously, causing it to be greatly diluted by the time it reached the heart, it failed to stimulate \( A_1 \) receptors (as evidenced by an unaltered heart rate) or to limit infarct size. Yet a higher adenosine concentration would have been seen by the circulating leukocytes in the intravenous protocol. Finally, adenosine has a very short half-life in the circulation\(^20\) so that virtually all of the infused adenosine was gone by the time the coronary artery was occluded, the time when neutrophils would begin to collect in the heart. It would appear that a brief period of \( A_1 \) stimulation triggered a biochemical change in the heart that persisted long after the adenosine was withdrawn. The nature of that change is currently unknown, but if it involves a reduced affinity for leukocytes, then the effect must be on the heart tissue rather than the leukocyte. Schrier et al.\(^21\) have reported that some aspects of inflammation in the lung are inhibited by \( A_1 \) receptors and suggest an altered blood vessel affinity as a possible mechanism.

Neither of the two inhibitors we employed are very specific for a given receptor type. Radioligand studies indicate that SPT is an unselective antagonist having a \( K_i \) of 4.5 and 6.3 \( \mu M \) for the \( A_1 \) and the \( A_2 \) receptors, respectively.\(^22\) As given here, SPT caused about a 75% inhibition of the hypotension resulting from an intravenous bolus of 200 \( \mu g \) adenosine. The main advantage of SPT is that the sulfophenyl group prevents the molecule from entering the cells. Thus, the extracellular adenosine receptors are blocked with no effect on intracellular phosphodiesterase. The extracellular compartmentalization apparently gives SPT a short plasma half-life because within 45 minutes of administration of SPT, the blood pressure response to intravenous adenosine had almost completely recovered. PD 115,199 is more potent than SPT, having a \( K_i \) around 10 \( \mu M \) for both receptor subtypes.\(^23\) PD 115,199 completely abolished hypotension from 200 \( \mu g \) of intravenous adenosine for the duration of the study. Both inhibitors completely abolished the protection afforded by preconditioning. The \( R \)-PIA results would support an \( A_1 \) receptor type for this protection. In another study we found that \( R \)-PIA could be given intravenously before ischemia and still protect.\(^24\) Intravenous \( R \)-PIA slowed the heart rate and caused only moderate hypotension. When the \( A_2 \)-selective agonist CGS-21680 was given to produce the same degree of hypotension, the heart rate was unaltered and the hearts were not protected. That again supports our hypothesis that the protection is \( A_1 \) mediated.

Infarct size as indicated by TTC staining after only several hours of reperfusion was the end point for these studies. The use of TTC has been controversial because positive staining with TTC simply means that the tissue contains both the dehydrogenase enzymes and cofactor needed to promote the reaction,\(^25\) not that it is alive. While most would agree that tissue that has lost these constituents would be unable to recover and that recoverable tissue will be tetrazolium positive, it does not necessarily follow that TTC-positive tissue must always be viable. While many investigators have seen a remarkably good correlation between ultimate infarct size and that revealed by TTC soon after reperfusion in untreated hearts,\(^26,27\) we found that at least one treatment, superoxide dismutase, delayed the washout of enzyme and cofactor so that some dead myocardium retained its ability to stain with TTC after a full 24 hours of reperfusion.\(^28\) How does this affect the present experiment? When rabbit infarcts were sized by histology after 3 days of reperfusion, preconditioning yielded protection that was almost identical to that seen in the present study,\(^8\) confirming the validity of TTC in the setting of preconditioning. Because adenosine blockers resulted in a greater amount of
tissue to be TTC negative (and thus unrecoverable) than was seen in the nonblocked but preconditioned hearts, we can conclude that the protection afforded by preconditioning really had been blocked. The only assumption that remains to be made is that concerning the intracoronary adenosine receptor agonists. We cannot exclude the possibility that a true reduction of infarct size had not occurred but some nonspecific effect delayed the egress of enzyme and cofactor from irreversibly injured tissue, as was the case with superoxide dismutase. The only way to absolutely exclude this possibility is with a long-term reperfusion and measurement of the infarcts by histology, a procedure not feasible with the isolated heart. However, if, as the data strongly indicate, adenosine-mediated protection and that from preconditioning are the same, then the protection seen here is real.

It may be possible to maintain the heart in a preconditioned state indefinitely with the administration of an A1 receptor agonist. Such treatment would be indicated for patients who are at high risk of myocardial infarction as it would allow them to better tolerate a coronary occlusive event. Adenosine would be inappropriate for systemic treatment because of its short half-life in the circulation and the severe hypotension that would result from A2-mediated vasodilation. However, those problems could be eliminated by designing an agent that is very A2 specific and nonmetabolizable. We have recently found that intravenous R-PIA is A2 selective enough to limit infarct size to a similar degree as preconditioning when a large dose (1 mg/kg) was given intravenously 15 minutes before the onset of ischemia. When the same dose of R-PIA was begun just before reperfusion, no protection was seen.24 Whether the heart can be maintained in a prolonged preconditioned state for extended periods and whether there are unanticipated side effects of prolonged A2 stimulation is currently unknown and must be the subject of future investigations.

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