Intravenous Pretreatment With A₁-Selective Adenosine Analogues Protects the Heart Against Infarction

Jon D. Thornton, BS; Guang S. Liu, PhD; Ray A. Olsson, MD; and James M. Downey, PhD

Background. Recent data from this laboratory indicate that pretreatment with adenosine can protect the heart against infarction via A₁-receptors, but because of systemic hypotension, adenosine had to be given into the coronary circulation.

Methods and Results. In this study, we tested whether that protection could be achieved by intravenous administration of the A₁-selective adenosine agonists N⁶-(phenyl-2R-isopropyl)-adenosine (PIA) and 2-chloro-N⁶-cyclopentyladenosine (CCPA). Nine groups of open-chest anesthetized rabbits were subjected to 30 minutes of regional coronary ischemia and 3 hours of reperfusion. Infarct size was determined by tetrazolium staining. Control hearts receiving no treatment had 38±4% of the risk zone infarcted. Preconditioning with 5 minutes of ischemia and 10 minutes of reperfusion before ischemia limited the infarct to 8±4%. Intravenous PIA 15 minutes before 30-minute ischemia also limited infarct size to 6±2% at the highest dose. CCPA offered similar protection. When the PIA was given at reperfusion, infarct size was 46±6%, indicating that receptor activation must precede ischemia to protect. Pretreatment with CGS 21680, a selective A₁-receptor agonist, caused identical hypotension but failed to limit infarct size (43±3%), indicating again that the A₁-receptor is involved. When rabbits pretreated with PIA were paced at 220 beats per minute, PIA still limited infarct size (16±4%), indicating that protection was not the result of bradycardia.

Conclusions. These results indicate that stimulation of adenosine A₁-receptors causes the heart to become resistant to ischemia and that this protection can be achieved with intravenous administration of A₁-selective agents. (Circulation 1992;85:659–665)

Preconditioning refers to a phenomenon whereby a brief period of ischemia renders the myocardium resistant to infarction from a subsequent ischemic insult. The phenomenon has been demonstrated by various investigators in dogs,¹⁻³ in pigs,⁴ and in rabbits.⁵⁻⁷ Preconditioning does not appear to involve opening of collateral vessels¹ or the synthesis of a protective protein.⁶ Recent work in our laboratory indicates that the buildup of adenosine during the first ischemic period may trigger protection by preconditioning. Adenosine-receptor–blocking agents eliminated the protective effect of preconditioning, and substituting a transient infusion of intracoronary adenosine for the brief ischemia conferred equal protection to the heart.⁸

The cardiac effects of adenosine are divided according to the two receptor types, A₁ and A₂. Stimulation of adenosine A₁-receptors results in a decrease in cyclic AMP⁹ with a negative chronotropic and inotropic effect.¹⁰ Adenosine A₂-stimulation has been shown to inhibit neutrophil function¹¹,¹² and to dilate vascular smooth muscle.¹³ Because we found that intracoronary infusion of the A₁-selective adenosine analogue N⁶-(phenyl-2R-isopropyl)-adenosine (PIA) was as protective as adenosine, we concluded that the protection was probably through the A₁-receptor.⁸ When adenosine was infused intravenously in that study,⁸ the A₂ effects predominated, causing widespread peripheral dilation. We found that A₂-mediated dilation and its subsequent hypotension pre-
vented us from giving sufficient intravenous adenosine to achieve a protective concentration in the coronary perfusate.

In the present study, we test whether an A₁-selective adenosine agonist could be tolerated at a dose sufficient to confer protection against infarction when given systemically. We also compare the effects of two different A₁-selective agonists with that of an A₂-selective adenosine agonist. Because A₁-selective agonists decrease heart rate, this study also tests whether A₁-induced protection is related to the resulting bradycardia. Finally, we test whether PIA can protect if administered at reperfusion rather than before ischemia.

Methods

Surgical Preparation of Animals

New Zealand White rabbits of either sex, weighing between 1.5 and 2.8 kg, were anesthetized with intravenous sodium pentobarbital (30 mg/kg) administered via a marginal ear vein. The neck was opened with a ventral midline incision, and a tracheotomy was performed; the rabbits were ventilated with 100% oxygen via a positive pressure respirator (MD Industries, Mobile, Ala.). Ventilation rate was 30–35 breaths per minute, and tidal volume was approximately 15 ml. The respiratory rate was adjusted to keep the blood pH in the physiological range. Catheters filled with heparinized saline (10 units/ml) were placed in the left carotid artery and jugular vein to monitor blood pressure and to inject drugs, respectively. Additional anesthesia was also administered through the jugular vein as needed. A left thoracotomy was performed in the fourth intercostal space, and the pericardium was opened to expose the heart. A 2-0 silk suture with an RB taper needle was passed around a branch of the left coronary artery, and the ends of the silk were threaded through a small vinyl tube to form a 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 effected by releasing the snare and was confirmed by visible hyperemia over the surface.

Measurement of Infarct and Risk Area

At the end of each experiment, the heart was quickly removed, mounted on a Langendorff apparatus, and flushed with room-temperature saline for 60 seconds. The silk suture under the coronary branch was then tightly tied to occlude the artery, and a 0.5% suspension of fluorescent particles (1–10 µm diameter from Duke Scientific Corp., Palo Alto, Calif.) was infused into the perfusate to differentiate the risk zone as the tissue with no fluorescence. The heart was removed from the Langendorff apparatus, weighed, and then frozen. When frozen, the heart was cut into transverse slices 2 mm thick. The slices were thawed and stained by incubation for 20 minutes in 1% triphenyl tetrazolium chloride (TTC) in isotonic pH 7.4 phosphate buffer. TTC reacts with nicotinamide adenine dinucleotide and dehydrogenase enzymes and causes all tissue still having them to stain a deep red color. Because the infarcted area of the heart quickly loses either enzyme and/or cofactor, it does not stain. After staining, the slices were put between two glass plates 2 mm apart, and the region of infarcted tissue and the risk zone were traced. The area of infarct and the risk zone were determined by planimetry of the tracings. The volumes of infarcted myocardium and myocardium at risk were calculated by multiplying the planimeted areas by the slice thickness.

Chemicals

PIA and 2-chloro-N⁶-cyclopentyladenosine (CCPA) were obtained from Research Biochemicals Inc., Natick, Mass. 2-[4-(2- Carboxyethyl)phenethylamino]-5’-N-ethylcarboxamido adenosine (CGS 21680) was synthesized as described by Hutchison et al. All chemicals are water soluble and were prepared for injection by mixing with appropriate amounts of 0.9% saline.

Experiment Protocols

This experiment involved 85 animals divided into nine groups. The groups were treated serially, with control animals interspersed all through the study. Although no attempt was made to randomize systematically between control and treatment studies, the decision as to the nature of the day’s experiment was always made before the animal was seen by the investigator. Furthermore, the control group consisted of 14 historical controls from previous studies by the investigators and 11 contemporary controls used during the course of this study. There was no difference in the mean infarct size between historical and contemporary controls (37±5% and 38±5%, respectively, of the risk zone). All animals were subjected to a 30-minute coronary occlusion followed by 180 minutes of reperfusion. Group 1 was the control group and underwent only the above-mentioned procedure. Group 2, the preconditioned group, received 5 minutes of occlusion followed by 10 minutes of reperfusion before the 30-minute occlusion. Groups 3, 4, and 5 were treated with a 5-minute infusion of PIA, a moderately A₁-selective adenosine receptor agonist, followed by 10 minutes of recovery before the 30-minute occlusion; thus, PIA infusion was substituted for preconditioning. The dosages of the groups were 250, 500, and 915 µg/kg of PIA, respectively. Group 6 received a 5-minute infusion of 915 µg/kg of PIA starting 2 minutes before reperfusion after the single 30-minute occlusion. Because of the decrease in heart rate that is associated with PIA, group 7 received an infusion of 915 µg/kg PIA before ischemia and was paced at 220 beats per minute with a Grass SD9 stimulator (pulses of 5 V and 4-msec duration) beginning at the onset of drug infusion. Group 8 received a 5-minute infusion of 80 µg/kg CGS 21680, an A₂-selective adenosine receptor agonist, 15 minutes before the 30-minute occlusion. Group 9 received 0.25
mg/kg of CCPA, a highly \(A_2\)-selective adenosine receptor agonist,\(^{16}\) 15 minutes before the 30-minute ischemic period. A one-way analysis of variance was used to analyze both the hemodynamic and the infarct size data. Significance was assumed to be indicated at a value of \(p<0.05\).

**Statistics**

We tested for differences among the nine groups with a one-way analysis of variance using a Newman-Keuls post hoc test. A value of \(p<0.05\) was considered to be significant. Confidence limits in the text are SEM unless otherwise indicated.

**Results**

**Hemodynamic Effects of \(A_1\) and \(A_2\)-Agonists**

Table 1 shows that PIA reduced heart rate by about 30% at all concentrations of the drug tested (\(p<0.02\) versus control). There was also a significant decrease in mean arterial blood pressure (MAP) with the 500-\(\mu\)g/kg and the 915-\(\mu\)g/kg doses. MAP was 27±3 and 27±2 mm Hg for the two doses, respectively (\(p<0.02\) versus control value of 71±3 mm Hg). Pacing the heart at the onset of PIA infusion eliminated the decrease in heart rate (222±6 beats per minute) but did not eliminate the fall in blood pressure (MAP, 33±3 mm Hg, \(p<0.02\) versus control). Figure 1 demonstrates the effects of PIA (high dose), CCPA, and CGS 21680 on blood pressure and heart rate. CCPA caused significant bradycardia (163±8 beats per minute, \(p<0.02\) but less hypotension than that seen with PIA (MAP, 39±7 mm Hg, \(p<0.02\)). The \(A_2\)-agonist CGS 21680 had no effect on heart rate (239±9 beats per minute), but MAP fell to a level comparable to that seen with PIA (41±3 mm Hg, \(p<0.02\)). CCPA is highly \(A_2\)-selective, and it is assumed that hypotension from CCPA was primarily the result of cardiac depression. Similarly, the hypotension accompanying CGS 21680 was assumed to be primarily the result of decreased peripheral resistance, as it is highly \(A_2\)-selective. We also assume that the difference in hypotension between the less

**FIGURE 1.** Bar charts demonstrate effects of PIA, CCPA, and CGS on blood pressure and heart rate. Top panel: heart rate. Pressure and rates were measured just before 30-minute occlusion and compared with values taken 15 minutes earlier (before drug infusion or preconditioning). PC, preconditioned; PIA, N\(^6\)-(phenyl-2-R-isopropyl) adenosine; CCPA, 2-chloro-N\(^6\)-cyclopentyladenosine; CGS, CGS 21680 (2-[4-(2-carboxyethyl) phenethylamino]-5'-N-ethylcarboxamido adenosine). Bottom panel: change in blood pressure induced by preconditioning or the three adenosine analogues.
selective PIA and CCPA is a result of more $A_2$-mediated peripheral dilation with PIA, because the $A_1$ effects on heart rate were similar between the two agents. Note also from Table 1 that all three agents caused a prolonged hypotension, because none of the analogues is as rapidly metabolized as adenosine.

Infarct Size Data

There were four exclusions in the studies. One animal died prematurely in the paced group. Technical problems associated with staining occurred in three other animals—one in the CGS 21680 group, one in the preconditioned group, and one in the contemporary control group. These four animals do not appear in the group sizes in Table 2. Table 2 indicates that there were no significant differences among the risk areas of the various groups. Infarct size in the control hearts averaged 37.9±3.6% of the risk zone. It can be seen from the infarct size data in Table 2 that 915 $\mu$g/kg PIA protected the heart from infarction to the same degree as preconditioning (6.4±2.0% and 8.8±2.1%, respectively; $p<0.001$ versus control). PIA appeared to offer some protection at the lower doses as well but was inconsistent (percent infarction averaged 21.3±6.9% and 21.5±11.1% for doses of 250 and 500 $\mu$g/kg, respectively). The latter dose barely achieved significance against the control group, but the former did not. Figure 2 displays these results in graph form. The first two panels show the control and the preconditioned groups. The next three panels show the PIA groups. There was no significant difference among the infarct sizes for the three doses, but the data suggest that only the high dose of drug resulted in consistent protection.

Figure 3 shows the infarct sizes from the control group and groups 5–9. PIA NO PACE is the same high-dose PIA pretreatment group shown in Figure 2 and is included as a reference. When the high dose (915 $\mu$g/kg) of PIA was administered at reperfusion in group 6, infarct size was 45.6±6.6%, which was not different from that seen in controls. Clearly, PIA had to be administered before ischemia to be protective in this model. In addition to the PIA data, Figure 3 also includes data from the CCPA group. Note that pretreatment with the highly selective $A_2$-agonist CCPA was also protective, resulting in only 10.9±3% infarction ($p<0.02$ versus control).

Two groups were included to see whether either the hypotension or the bradycardia was directly responsible for the protection seen with the $A_2$-selective adenosine agonists. The data from group 7 indicate that pacing the heart to prevent the bradycardia did not block the protective effect of PIA: infarction/risk area, I/R=16.25±4.31, $p<0.05$ versus control. The PIA PACE group in Figure 3 shows those data in graph form.

Although we could not think of a way to selectively circumvent the hypotension caused by the $A_2$-selective agonists, we were able to produce comparable hypotension with an $A_2$-selective agonist, CGS 21680. We have included those data in Figure 3 as the leftmost panel (CGS). CGS 21680 produced a similar degree of hypotension but had no effect on infarct size (percent infarction, 42.5±3.4%). To investigate further whether hypotension could explain why salvage was seen in some groups and not in others, we plotted the infarct size against blood pressure for two time periods during the study. Those data are shown in Figure 4. Infarct size in the upper panel is plotted against aortic pressure taken 1 minute after the onset of reperfusion and in the lower panel against that taken 60 minutes after reperfusion. Note that in both graphs, the two non-protective interventions (solid symbols) experienced aortic pressures equivalent to the three protected groups (open symbols). In none of the protected groups did the salvage correlate with the blood pressure at either 1 or 60 minutes of reperfusion.

### Table 2. Infarction Data for Groups

<table>
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<th>Group</th>
<th>Identifier</th>
<th>n</th>
<th>Risk region</th>
<th>Infarct region</th>
<th>Infarction (%)</th>
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<td>23</td>
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<td>39±4</td>
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<td>0.06±0.02*</td>
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<tr>
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<td>0.16±0.09</td>
<td>21±11†</td>
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<tr>
<td>PIA 915</td>
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<td>0.06±0.02*</td>
<td>7±2*</td>
</tr>
<tr>
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<tr>
<td>PIA pace</td>
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<td>16±4*</td>
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<tr>
<td>CGS 21680</td>
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<td>0.34±0.06</td>
<td>42±3</td>
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<tr>
<td>CCPA</td>
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<td>0.74±0.07</td>
<td>0.08±0.02*</td>
<td>10±3*</td>
</tr>
</tbody>
</table>

Region sizes are in cubic centimeters.
Infarction, percentage of the risk zone infarcted; PC, preconditioned; PIA, N*(phenyl-2R-isopropyl)-adenosine; CGS 21680, 2-[4-(2-carboxyethyl)phenethylaminol]-5'-N-ethylcarboxamido adenosine; CCPA, 2-chloro-N*-cyclopentyladenosine.

*p<0.02 against the control group; †p<0.05 against the control group.
We also monitored rectal temperature in three of the PIA pretreatment animals. Rectal temperatures 60 minutes after administration of the drug were 37.2, 37, and 36.5°C. These temperatures were very similar to those seen in untreated open-chest rabbits undergoing the same procedure (36.8±0.21°C, n=5). Thus, altered temperature cannot explain the protective effect of PIA either.

**Discussion**

The present results further support the hypothesis that preconditioning is mediated by A<sub>1</sub>-adenosine receptors and that intravenous administration of A<sub>1</sub>-selective agonists can confer a similar protection. We previously found that a dose of adenosine that was protective when given into the coronary circulation failed to protect when given intravenously. Our explanation was that adenosine's effect was directly on the heart and that the adenosine had been too diluted to populate the A<sub>1</sub>-receptors adequately when it reached the coronary circulation. When we attempted to increase the intravenous dose of adenosine, hypotension became a limiting factor, because adenosine is a powerful vasodilator as a result of the action of A<sub>2</sub>-receptors on the peripheral vessels. The obvious way to eliminate the hypotensive side effects of adenosine would be to use an A<sub>1</sub>-selective analogue to confer the protection. In practical terms, the present data would indeed indicate that the protective effects of preconditioning can be conferred by parenteral administration of an A<sub>1</sub>-selective adenosine analogue.

It should be noted that both A<sub>1</sub>-selective analogues caused considerable hypotension. Some of the hypotension in the PIA animals may have been caused by some residual A<sub>2</sub>-mediated dilatation. The CCPA, however, is so selective that it is unlikely that it would have caused any peripheral dilation. Decreased heart rate and contractility are the more likely explanations for the hypotension. Even eliminating the bradycardia with pacing did not completely eliminate the hypotension caused by PIA. This undesirable side effect may be an insurmountable obstacle in achieving a practical therapy based on parenteral administration of A<sub>1</sub>-selective agonists.

Infarct size in this experiment was determined by staining with TTC after only 3 hours of reperfusion. Positive staining with TTC means only that the tissue contains the dehydrogenase enzymes and the cofactors needed to reduce tetrazolium to its formazan pigment, not that the tissue is viable. Most investigators have seen a good correlation between ultimate infarct size and that revealed by TTC soon after reperfusion in the untreated heart. Preconditioning, when analyzed by histology after 72 hours of reperfusion, yielded protection in both rabbits and dogs that was almost identical to that seen in the
present study. If an intervention delays the washout of enzymes from irreversibly injured tissue, it can give the appearance of protection where none actually exists. Because our previous results would suggest that adenosine is the mediator for preconditioning and because preconditioning has been shown to promote a sustained salvage, by that reasoning it seems likely that the A_{1}-induced salvage seen here will also be sustained. However, until a long-term reperfusion study can be performed with these drugs, we cannot eliminate the possibility that we are only delaying the evolution of infarction with these drugs.

These results reinforce our belief that endogenous adenosine released during the preconditioning ischemia activates A_{1}-receptors and triggers a metabolic change that protects the heart during the subsequent ischemia. Unfortunately, we still do not know the nature of that change. Several possible explanations for the protection seen here can be eliminated, however. Could the profound hypotension that accompanied all three drugs have caused the salvage? It is even possible that low perfusion pressure could have caused poor irrigation of the reperfused region and simply depressed the washout of enzymes and cofactor from otherwise irreversibly injured tissue. Two arms of the study would argue against that explanation. First, CGS 21680 caused comparable hypotension but did not limit infarct size. Second, when PIA was given just before reperfusion, causing an even more profound hypotension during reperfusion, protection was again absent. Indeed, Figure 4 shows that the success or failure of any of these interventions was unrelated to its effect on blood pressure. Because PIA continued to limit infarct size when bradycardia was prevented with pacing, the salvage appears to be unrelated to any change in heart rate either.

A_{1}-Adenosine agonists reduce cyclic AMP and contractility as well as slowing the heart rate, and the protection could be related to a reduced oxygen demand. Most investigators, however, have failed to see any limitation of infarct size when cyclic AMP was lowered with a β-blocker. Along these same lines, we found that the β-agonist dobutamine failed to increase infarct size even when it was given in massive dose. Finally, in a previous study, we were able to protect the heart with a transient infusion of intracoronary adenosine or PIA. In that study, the agonists were allowed to wash out for 10 minutes so that the heart could fully recover before the onset of ischemia.

The protective change in the myocardium may involve changes in ion channel activity. Adenosine A_{1}-receptors reportedly mediate the opening of the ATP-sensitive potassium channels. It is interesting that drugs that open that channel, such as nicorandil or pinacidil, are reported to be cardioprotective. Finally, in a recent abstract, Auchampach and Gross reported that glibenclamide, a blocker of the ATP-sensitive potassium channel, also blocks preconditioning in the dog heart.

Adenosine has recently been reported to protect the myocardium from infarction when given just before reperfusion. Our observations indicate that PIA was not effective when administered at this time point. Those results would suggest that the protective effect of adenosine A_{1}-receptor stimulation (and presumably preconditioning) is unrelated to the protection seen with adenosine given at reperfusion. Furthermore, the protective effect of adenosine at reperfusion is thought to be mediated by inhibition of neutrophil function, which is an A_{2} effect. The present data also indicate that the long-acting A_{2}-agonist CGS 21680 had no effect when given intravenously. A substantial level of CGS 21680 would have been present long after reperfusion in those rabbits, suggesting that any protection derived from late administration of adenosine may not be receptor mediated. Antineutrophil activity cannot be completely ruled out as the present mechanism, however, since Schrier et al have reported that some aspects of inflammation in the lung are inhibited by A_{1}-receptors; they suggest an altered blood vessel affinity as a possible mechanism.

In conclusion, we find that intravenous A_{1}-selective adenosine agonists limit the infarct size in rabbits that undergo 30 minutes of ischemia followed by 3 hours of reperfusion. The protection was related to neither the bradycardia nor the hypotension, and pretreatment was a requirement. These data suggest that pretreatment with an A_{1}-selective agent could be an effective means of conferring cardioprotection to patients with impending myocardial ischemia.

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