α1-Adrenergic Agonists Preconditioning Rabbit Ischemic Myocardium Independent of Adenosine by Direct Activation of Protein Kinase C

Akihito Tsuchida, Yongge Liu, Guang S. Liu, Michael V. Cohen, James M. Downey

Abstract Ischemic preconditioning in the rabbit is initiated by adenosine A1-receptor stimulation, which activates protein kinase C (PKC). Additionally, α1-adrenergic agonists can similarly protect ischemic myocardium, but there has been confusion about the role adenosine receptors play in this protection. To characterize the interaction between adrenergic and adenosine receptors and to study the possible role of PKC in this protection, we used isolated rabbit hearts perfused with oxygenated Krebs’ buffer. All hearts were subjected to 30 minutes of regional myocardial ischemia and 2 hours of reperfusion. Infarct size was determined by triphenyltetrazolium staining. Pharmacologic preconditioning in hearts with a 5-minute phenylephrine (PE) infusion 10 minutes before the prolonged regional ischemia resulted in significantly smaller infarcts (9.7±1.3% of risk area) than in control hearts (31.0±2.6%, P<.05). This protection could be effectively blocked by administration of the α1-adrenergic blocker phenoxybenzamine. Methoxamine, an α1-selective agonist, failed to protect, whereas the α1-selective antagonist chlorothiazide aborted the protective effect of PE. Polymyxin B, an inhibitor of PKC, also blocked the protective effect of PE, implying that PKC has an important role in preconditioning.

Preconditioning the heart with 5 minutes of ischemia followed by 10 minutes of reperfusion renders it very resistant to ischemia from a subsequent ischemic insult. We have recently proposed that ischemic preconditioning is the result of an upregulation of protein kinase C (PKC) within the cardiac myocytes, probably through translocation of PKC into the membranes, where it can be activated by its cofactors. In this theory, occupancy of adenosine A1-receptors during the first coronary occlusion somehow upregulates PKC such that a PKC-mediated protective mechanism can be invoked during a second coronary occlusion. After a second episode of ischemia, adenosine receptors will again be populated with subsequent reactivation of previously upregulated PKC. Therefore, preconditioning appears to eliminate the expected time lag between receptor stimulation and protein phosphorylation when PKC is in this upregulated state. That PKC must phosphorylate protein very early in ischemia in order to afford protection is central to the hypothesis.

The adenosine receptor blocker 8-(p-sulfophenyl)theophylline (SPT) given at the same time as the PE infusion did not affect the protection, implying that an α1-agonist could initiate protection independent of adenosine, presumably by direct coupling to PKC. However, the protective effect of PE could be blocked if SPT were administered during the 30-minute regional ischemia. This observation suggested that adenosine receptor occupancy is necessary during long ischemia to reactivate PKC and mediate the protection. However, the addition of a second PE infusion beginning 5 minutes before and continuing throughout the long ischemic period restored the protective effect of PE despite the presence of SPT. Thus, as long as at least one of the receptors (α1-adrenergic or adenosine A1) is activated during long ischemia, protection will be realized. These data indicate that α1 receptors do not precondition through an adenosine intermediate but that α1-adrenergic and adenosine receptors activate parallel pathways within the myocyte that can trigger and mediate protection. (Circ Res. 1994;75:576-585.)

Key Words • adenosine • α1-adrenergic receptors • ischemic preconditioning • protein kinase C

An important ramification of the PKC upregulation theory is that any receptor that couples with PKC in the cardiocyte should be capable of preconditioning the heart. One is the α1-adrenergic receptor. In a previous study, we indeed found that liberation of norepinephrine in the heart by tyramine could mimic ischemic preconditioning. Because adenosine receptor blockade eliminated that protection, we hypothesized that norepinephrine was eliciting adenosine formation in the myocytes, which then in turn preconditioned the heart. In light of the PKC-upregulation theory, however, we now realize that norepinephrine may have acted to directly cause translocation of PKC, since α1-adrenergic receptors do couple directly with phospholipase C (the activator pathway for PKC). Therefore, adenosine may have been important in the tyramine study only during the sustained ischemic period, when, after the washout of norepinephrine, adenosine release and receptor occupancy would have been responsible for reactivating the PKC pathway.

In the present study, we further explore the link between α-adrenergic receptors and preconditioning by exposing isolated hearts to the α1-agonist phenylephrine hydrochloride (PE) and determining the differing effect on infarct size of a varying schedule of adenosine and specific α-adrenergic receptor blockers. The present data indicate that α1-adrenergic agonists can indepen-
dently precondition the heart and salvage ischemic myocardium without the interposition of adenosine. Furthermore, these observations provide additional evidence that PKC activation is a necessary step in the protection of preconditioning.

**Materials and Methods**

The present study was conducted in accordance with the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (publication No. [NIH] 85-23, revised 1985).

**Surgical Preparation of Animals**

New Zealand White rabbits (1.7 to 2.4 kg) of either sex were anesthetized with intravenous sodium pentobarbital (30 mg/kg). The neck was opened with a ventral midline incision, and a tracheotomy was performed. The rabbits were ventilated with a positive-pressure respirator (MD Industries) and 100% oxygen. Ventilation rate was 30 to 35 breaths per minute, and tidal volume was ~15 mL. A left thoracotomy was performed 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 heart was rapidly excised and mounted on a Langendorff apparatus by the aortic root. The heart was perfused with Krebs-Henseleit bicarbonate buffer consisting of (mmol/L) NaCl 118.5, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, CaCl2 2.5, NaHCO3 24.8, and glucose 10. A 95% O2/5% CO2 gas mixture was bubbled through the perfusate and perfusate temperature was maintained at 38°C. The coronary perfusion pressure was set at 75 mm Hg by adjusting the height of the reservoir. A saline-filled latex balloon connected by a polyethylene catheter to a pressure transducer was inserted into the left ventricle and inflated to produce an end-diastolic pressure of 5 mm Hg. Atrial pacing was performed at 200 beats per minute if the spontaneous rate was slower. Total coronary arterial flow was measured by timed collection of buffer exiting from the right heart. The heart was allowed to stabilize for 10 minutes before the experiment was begun.

**Measurement of Infarct Size and Risk Area**

At the end of the study, the coronary branch was reoccluded, and fluorescent perfusion field of the occluded artery (the risk area). The heart was weighed, frozen, and then cut into 2-mm transverse slices. The heart slices were incubated for 20 minutes at 37°C in a 1% solution of triphenyltetrazolium chloride (TTC) in sodium phosphate buffer, pH 7.4. Viable tissue stained by TTC is deep red; necrotic tissue is unstained and appears tan. After staining, the slices were immersed in 10% formalin to enhance the contrast between stained and unstained tissue. The risk zone was identified by illuminating the slices with UV light. The areas of infarct and risk zone for each slice were measured by planimetry, and volumes were obtained by multiplying the area by the thickness of the section. Infarct size was normalized by expressing it as a percentage of the area at risk.

**Chemicals**

PE was obtained from Sigma Chemical Co. Phenoxbenzamine hydrochloride (PBZ), chloroethylenolidine dihydrochloride (CEC), and 8-(p-sulfophenyl)theophylline (SPT) were obtained from Research Biochemicals Inc. Polyoxymyxin (Poly B) was obtained from Calbiochem. PBZ and CEC were dissolved in ethanol (20 mg/mL), and PE was dissolved in water (0.1 mg/mL); each solution was then diluted with Krebs-Henseleit buffer. SPT and Poly B were dissolved in Krebs-Henseleit buffer directly.

**Experimental Protocols**

In all hearts, regional ischemia was produced by pulling the snare for 30 minutes, after which the snare was released, allowing reperfusion for 2 hours. Myocardial ischemia was confirmed by a decrease in left ventricular developed pressure and a fall in coronary flow as well as by the appearance of segmental wall motion abnormalities. Reperfusion was documented by resolution of these changes. The animals were divided into 17 groups. Fig 1 depicts the various drug and ischemic interventions. The first series of experiments was designed to test for an interaction between α1-adrenergic and adenosine stimulation of the myocardium: (1) The control group had only 30 minutes of ischemia and 120 minutes of reperfusion. (2) The ischemic preconditioning (PC) group received 5 minutes of global ischemia during cessation of aortic perfusion, followed by 10 minutes of reperfusion before 30 minutes of regional ischemia. (3) The low PE group received the α1-adrenergic receptor agonist PE (0.1 μmol/L) for 5 minutes, followed by a 10-minute drug-free interval before the 30-minute ischemic period. (4) The high (HI) PE group was identical to group 3 except that 10 μmol/L PE was infused. (5) The SPT early group received the adenosine

---

**Figure 1.** Experimental protocols depicting the timing of each intervention with respect to the prolonged 30-minute period of regional ischemia. PC indicates ischemic preconditioning; low/Hi PE, phenylephrine (PE) infusions of 0.1 and 10 μmol/L, respectively, infused for 5 minutes and ending 10 minutes before the 30-minute ischemia; PE late, PE (0.1 μmol/L) infused for 35 minutes beginning 5 minutes before and continuing through the 30-minute period of ischemia; MET, methoxamine; PBZ, phenoxybenzamine; CEC, chloroethylenolidine; SPT early and SPT late, 8-(p-sulfophenyl)theophylline infusion either for 15 minutes and ending 5 minutes before the 30-minute ischemia (early) or for 35 minutes beginning 5 minutes before and continuing through the 30-minute ischemia (late); and Poly B, polymyxin B.
TABLE 1. Baseline Hemodynamic Parameters and Coronary Flow

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>HR, bpm</th>
<th>LVDP, mm Hg</th>
<th>HR x LVDP, x100</th>
<th>Coronary Flow, mL/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>9</td>
<td>215±9</td>
<td>103±4</td>
<td>219±10</td>
<td>56±2</td>
</tr>
<tr>
<td>2. PC</td>
<td>8</td>
<td>223±8</td>
<td>110±7</td>
<td>245±17</td>
<td>59±3</td>
</tr>
<tr>
<td>3. Low PE</td>
<td>8</td>
<td>213±6</td>
<td>101±2</td>
<td>214±4</td>
<td>53±3</td>
</tr>
<tr>
<td>4. High PE</td>
<td>7</td>
<td>210±3</td>
<td>110±5</td>
<td>232±12</td>
<td>59±4</td>
</tr>
<tr>
<td>5. SPT early</td>
<td>5</td>
<td>218±9</td>
<td>95±5</td>
<td>208±17</td>
<td>57±5</td>
</tr>
<tr>
<td>6. SPT late</td>
<td>5</td>
<td>210±4</td>
<td>107±6</td>
<td>225±11</td>
<td>59±5</td>
</tr>
<tr>
<td>7. Low PE+SPT early</td>
<td>7</td>
<td>216±5</td>
<td>99±3</td>
<td>213±9</td>
<td>51±2</td>
</tr>
<tr>
<td>8. Low PE+SPT late</td>
<td>7</td>
<td>216±4</td>
<td>103±3</td>
<td>221±9</td>
<td>59±5</td>
</tr>
<tr>
<td>9. Low PE+PE late+SPT</td>
<td>7</td>
<td>213±3</td>
<td>106±3</td>
<td>225±9</td>
<td>59±2</td>
</tr>
<tr>
<td>10. PBZ</td>
<td>5</td>
<td>216±7</td>
<td>115±4</td>
<td>248±11</td>
<td>53±3</td>
</tr>
<tr>
<td>11. PC+PBZ</td>
<td>7</td>
<td>213±3</td>
<td>107±4</td>
<td>228±6</td>
<td>54±5</td>
</tr>
<tr>
<td>12. High PE+PBZ</td>
<td>7</td>
<td>202±3</td>
<td>109±4</td>
<td>220±9</td>
<td>61±6</td>
</tr>
<tr>
<td>13. MET</td>
<td>7</td>
<td>209±4</td>
<td>117±4</td>
<td>243±8</td>
<td>60±2</td>
</tr>
<tr>
<td>14. CEC</td>
<td>3</td>
<td>207±8</td>
<td>98±9</td>
<td>201±16</td>
<td>60±2</td>
</tr>
<tr>
<td>15. Low PE+CEC</td>
<td>7</td>
<td>218±3</td>
<td>105±3</td>
<td>228±8</td>
<td>61±4</td>
</tr>
<tr>
<td>16. Poly B</td>
<td>6</td>
<td>211±4</td>
<td>99±6</td>
<td>209±11</td>
<td>52±3</td>
</tr>
<tr>
<td>17. Low PE+Poly B</td>
<td>8</td>
<td>217±4</td>
<td>100±4</td>
<td>217±12</td>
<td>52±3</td>
</tr>
</tbody>
</table>

n indicates number of rabbits; HR, heart rate; bpm, beats per minute; LVDP, left ventricular developed pressure; PC, ischemic preconditioning; low PE, 0.1 μmol/L phenylephrine; high PE, 10 μmol/L PE; SPT early, 8-p-(sulfophenyl)theophylline infused for 15 minutes ending 5 minutes before ischemia; SPT late, PE infused for 5 minutes before ischemia and for 30 minutes during ischemia; PE late, 0.1 μmol/L PE infused at the same schedule as SPT late; PBZ, phenoxymenzamine; MET, methoxamine; CEC, chloroethylclonidine; and Poly B, polymyxin B. Values are mean±SEM.

receptor antagonist SPT (100 μmol/L) for 15 minutes, followed by 5 minutes of washout before ischemia. (6) The SPT late group received an infusion of 100 μmol/L starting 5 minutes before ischemia and continuing until the end of the 30-minute ischemia. (7) The low PE+SPT early group was treated with both PE for 5 minutes as in group 3 and SPT for 15 minutes beginning 5 minutes before PE, then, as in the SPT early group, regional ischemia was initiated 5 minutes after cessation of this SPT infusion. (8) The low PE+SPT late group received low-dose PE as in group 3, but the SPT infusion was delayed until 5 minutes before coronary occlusion and then continued for the duration of the ischemia. (9) The low PE+PE late+SPT late group was treated with PE and SPT as in group 8 but also received an additional reinfusion of PE (0.1 μmol/L) concurrent with the SPT infusion. (10) The PBZ group was treated with the irreversible α1-receptor antagonist PBZ (10 μmol/L) for 10 minutes, followed by a 15-minute drug-free interval before coronary occlusion. (11) The PC+PBZ group was preconditioned with 5 minutes of ischemia as in group 2 but also pretreated with PBZ as in group 10; the PBZ infusion ended just before the 5 minutes of global ischemia. (12) In the high PE+PBZ group, infusions of high-dose PE and PBZ as in groups 4 and 10 were combined.

The second series of experiments was designed to identify the specific α1-adrenergic receptor subtype involved: (13) The MET group was treated with methoxamine (MET, 1 μmol/L), an α1-selective agonist, for 5 minutes, followed by a 10-minute drug-free period before 30 minutes of ischemia. (14) In the CEC group, CEC (50 μmol/L), an irreversible α1-selective receptor antagonist was added to the aortic perfusate for 10 minutes, ending 15 minutes before initiation of regional ischemia. (15) In the low PE+CEC group low-dose PE and CEC infusions were combined as in groups 3 and 14.

The third series of experiments was designed to test directly the relation between PKC activation and the cardioprotective action of α1-adrenergic agonists: (16) The Poly B group received Poly B (50 μmol/L), a specific PKC inhibitor, 20 minutes before and continuing to the end of ischemia. (17) In the low PE+Poly B group, low-dose PE and Poly B were infused together as outlined in groups 3 and 16.

We used two doses of PE in these studies, 10 and 0.1 μmol/L. We started the protocols with the high dose, and it was not until after the PBZ studies were completed that we realized that the 10-μmol/L dose was 100-fold higher than required for α1-receptor occupancy. Thereafter, we adjusted the dose downward with no loss of protection. The excessive dose of PE used in the PBZ studies does not change the interpretation.

Statistics

All values are given as mean±SEM. The significance of differences in hemodynamics after an intervention in any group was determined by ANOVA with replication and either the Newman-Keuls test or Scheffé's post hoc test.13 Differences in risk zone or infarct volumes or infarct-to-risk zone ratios between control and experimental groups were evaluated by unpaired Student's t test. A value of P<.05 was assumed to represent significance.

Results

Hemodynamic Parameters and Coronary Flow

One hundred fourteen rabbits were entered into the study (see Table 1 for number in each group). Hemodynamic parameters and coronary flow at baseline are summarized in Table 1. Heart rate (HR), left ventricular developed pressure (LVDP), and the product of HR and LVDP were comparable in each group at baseline. HR and LVDP after infusion of 10 μmol/L PE were significantly increased (HR, 243±6 beats per minute; LVDP, 133±6 mm Hg; P<.05 versus baseline), but the
lower dose of PE (0.1 μmol/L) and 1 μmol/L MET did not significantly change the hemodynamic parameters (HR, 213±4 beats per minute, and LVDP, 101±6 mm Hg, with 0.1 μmol/L PE; and HR, 209±4 beats per minute, and LVDP, 89±9 mm Hg, with MET). Both PBZ and CEC significantly reduced LVDP (89±8 mm Hg with PBZ and 72±3 mm Hg with CEC, each P<.05 versus baseline). PBZ completely abolished the hemodynamic changes induced by 10 μmol/L PE (HR, 203±2 beats per minute; LVDP, 101±6 mm Hg; P=NS versus baseline). However, LVDP was still low after combined treatment with CEC and 0.1 μmol/L PE infusion (78±4 mm Hg, P<.05 versus baseline). Poly B also decreased LVDP with and without PE (87±7 mm Hg in low PE+Poly B group and 79±8 mm Hg in rabbits with Poly B alone, each P<.05 versus predrug baseline). SPT did not significantly modify any of the hemodynamic parameters. During the drug-free interval after the 30-minute period of ischemia in animals treated with high-dose PE, PBZ, and CEC, all hemodynamics returned to predrug levels with the exception of the CEC+PE group. In the latter, LVDP had partially recovered to 90±5 mm Hg by the time of the long ischemia. In the two groups of rabbits treated continuously with Poly B before and during the 30-minute ischemia, LVDP continued to be mildly depressed during the infusion, but the changes were comparable in both groups.

Baseline coronary flow was also comparable in each group (Table 1). PE (0.1 and 10 μmol/L) and MET (1 μmol/L) infusions all significantly reduced coronary flow (44±2 mL/min in low PE, 50±1 mL/min in high PE, and 32±3 mL/min in MET, which were 83%, 85%, and 53% of baseline, respectively). But the coronary flow reduction by high PE was completely blocked by PBZ (57±4 mL/min). CEC reduced coronary flow to 68% of baseline (P<.05), and this level was unchanged in the group treated with combined CEC and PE. SPT also caused a modest 20% fall in coronary flow. Poly B did not modify coronary flow. Although PE, MET, CEC, and SPT all caused temporary decreases in coronary flow, flow returned to baseline levels after discontinuation of drug infusion. No reactive hyperemia as is typically seen after myocardial ischemia was observed with cessation of any of these drugs.

**Infarct Size Data**

Body weights, heart weights, and volumes of risk zone were similar in the 17 groups (Table 2). Infarct sizes are also indicated in Table 2, and individual points are presented in Figs 2 through 5.

**Effect of α1-Adrenergic Agonists and Interaction With Adenosine Receptors**

Fig 2 reveals that infarct size in control rabbits averaged 31.0±2.6% of the risk zone. As expected, ischemic preconditioning significantly reduced infarction to 9.2±1.5% of the myocardium at risk (P<.05). Both doses of PE had equivalent effects. A brief 5-minute infusion of this α1-adrenergic agonist was able to successfully substitute for the 5-minute ischemic interval of the ischemic preconditioning protocol and elicit as much protection as the latter. PBZ, which itself had no direct effect on infarct size, aborted the protective effect of high-dose PE (23.4±2.1% infarction). However, PBZ was unable to block the protective effect

---

**Table 2. Infarct Size Data**

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight, kg</th>
<th>Heart Weight, g</th>
<th>Risk Zone, cm&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Infarct, cm&lt;sup&gt;3&lt;/sup&gt;</th>
<th>I/R, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>1.8±0.1</td>
<td>8.1±0.5</td>
<td>0.718±0.032</td>
<td>0.233±0.024</td>
<td>31.0±2.6</td>
</tr>
<tr>
<td>2. PC</td>
<td>1.9±0.1</td>
<td>8.1±0.5</td>
<td>0.732±0.061</td>
<td>0.069±0.015*</td>
<td>9.2±1.5*</td>
</tr>
<tr>
<td>3. Low PE</td>
<td>2.0±0.1</td>
<td>8.6±0.3</td>
<td>0.680±0.017</td>
<td>0.065±0.008*</td>
<td>9.7±1.3*</td>
</tr>
<tr>
<td>4. High PE</td>
<td>2.0±0.1</td>
<td>8.4±0.2</td>
<td>0.741±0.071</td>
<td>0.068±0.009*</td>
<td>9.2±1.1*</td>
</tr>
<tr>
<td>5. SPT early</td>
<td>2.1±0.1</td>
<td>8.8±0.5</td>
<td>0.838±0.027</td>
<td>0.240±0.033</td>
<td>28.6±3.8</td>
</tr>
<tr>
<td>6. SPT late</td>
<td>1.9±0.1</td>
<td>8.1±0.5</td>
<td>0.721±0.078</td>
<td>0.206±0.033</td>
<td>27.9±2.6</td>
</tr>
<tr>
<td>7. Low PE+SPT early</td>
<td>1.9±0.0</td>
<td>7.8±0.2</td>
<td>0.762±0.046</td>
<td>0.094±0.015</td>
<td>12.5±1.9*</td>
</tr>
<tr>
<td>8. Low PE+SPT late</td>
<td>1.9±0.1</td>
<td>8.4±0.5</td>
<td>0.734±0.041</td>
<td>0.179±0.033</td>
<td>23.7±3.5</td>
</tr>
<tr>
<td>9. Low PE+PE late+SPT late</td>
<td>2.0±0.0</td>
<td>7.9±0.1</td>
<td>0.699±0.023</td>
<td>0.070±0.009*</td>
<td>10.0±1.1*</td>
</tr>
<tr>
<td>10. PBZ</td>
<td>1.9±0.0</td>
<td>7.2±0.1</td>
<td>0.704±0.065</td>
<td>0.180±0.033</td>
<td>24.7±2.3</td>
</tr>
<tr>
<td>11. PC+PBZ</td>
<td>1.9±0.0</td>
<td>7.9±0.1</td>
<td>0.748±0.100</td>
<td>0.058±0.025*</td>
<td>7.9±1.8*</td>
</tr>
<tr>
<td>12. High PE+PBZ</td>
<td>2.1±0.1</td>
<td>9.1±0.3</td>
<td>0.788±0.050</td>
<td>0.191±0.029</td>
<td>23.4±2.1</td>
</tr>
<tr>
<td>13. MET</td>
<td>2.2±0.0</td>
<td>8.5±0.2</td>
<td>0.772±0.055</td>
<td>0.257±0.053</td>
<td>31.5±5.6</td>
</tr>
<tr>
<td>14. CEC</td>
<td>1.9±0.0</td>
<td>8.8±0.3</td>
<td>0.730±0.058</td>
<td>0.188±0.046</td>
<td>24.7±4.3</td>
</tr>
<tr>
<td>15. Low PE+CEC</td>
<td>2.0±0.0</td>
<td>8.3±0.4</td>
<td>0.744±0.031</td>
<td>0.182±0.027</td>
<td>24.1±3.0</td>
</tr>
<tr>
<td>16. Poly B</td>
<td>2.0±0.0</td>
<td>7.7±0.5</td>
<td>0.642±0.024</td>
<td>0.184±0.038</td>
<td>27.7±4.8</td>
</tr>
<tr>
<td>17. Low PE+Poly B</td>
<td>2.0±0.1</td>
<td>8.5±0.2</td>
<td>0.816±0.066</td>
<td>0.283±0.054</td>
<td>32.9±3.8</td>
</tr>
</tbody>
</table>

I/R indicates percentage of risk zone infarcted; PC, ischemic preconditioning; low PE, 0.1 μmol/L phenylephrine; high PE, 10 μmol/L PE; SPT early, 8-p-(sulphophenyl)theophylline infused for 15 minutes ending 5 minutes before ischemia; SPT late, SPT infused for 5 minutes before ischemia and for 30 minutes during ischemia; PE late, 0.1 μmol/L PE infused at the same schedule as SPT late; PBZ, phenoxymenzamine; MET, methoxamine; CEC, chloroethyliclonidinone; and Poly B, polymyxin B. Values are mean±SEM.

*P<.05 vs control.
of ischemic preconditioning (7.9±1.8% infarction, P<.05 versus control). These results indicate that although α-receptor stimulation can successfully precondition myocardium, α-receptors themselves do not participate in the ischemic preconditioning phenomenon.

Although pharmacologic preconditioning was successful with both doses of PE, it was not clear whether this result was an independent effect of the α₁-adrenergic agonist or whether it was dependent on increased adenosine production as previously suggested.⁵ Fig 3 demonstrates the continuing effectiveness of low-dose PE in the presence of SPT, a nonspecific adenosine antagonist. Because SPT remains in the extracellular space, it is an effective blocker only during the time of infusion, with the blockade waning rapidly during the subsequent drug-free perfusion. In the absence of PE, neither the SPT early nor the SPT late protocol caused infarct size to differ from that seen in the control group. However, timing of SPT administration clearly affected the PE-induced protection. If low-dose PE and SPT early were coadministered before regional ischemia, then only 12.5±1.9% of the risk zone became infarcted (P<.05 versus control). Therefore, despite adenosine receptor blockade, α₁-receptor activation successfully initiated the protective process. However, when adenosine receptors were blocked during the 30-minute ischemic period with the SPT late protocol, PE-induced protection was aborted (23.7±3.5% infarction, P=NS versus control). Therefore, protection was dependent not only on α₁-receptor occupancy before ischemia but also on adenosine receptor stimulation during the ischemia. Note that the requirement for the late adenosine receptor occupancy could in turn be obviated by also infusing low-dose PE during the coronary occlusion. In this latter situation, low PE+PE late+SPT late, infarction averaged only 10.0±1.1% of the risk zone, indicating the return of protection (P<.05 versus control).

**Characterization of the α₁-Adrenergic Subtype**

As already noted, the nonselective α₁-adrenergic agonist PE protects ischemic myocardium, and the effect is aborted by treatment with PBZ. But in Fig 4, infusion of 1 μmol/L MET, an α₁-selective adrenergic agonist,¹⁴ can be seen to have no protective effect (31.5±5.6% infarction). CEC is a selective α₂-adrenergic receptor blocker, which was found to have no independent effect.
on infarct size. When CEC was infused with PE, the protective effect of PE was abolished (24.1±3.0% infarction, P=NS versus control). The above results indicate that the δ_{0.1}-adrenergic receptor subtype rather than the δ_{0.2} subtype is the one responsible for PE-induced protection.

**Effect of Protein Kinase C Inhibition**

As depicted in Fig 5, Poly B, a specific inhibitor of the enzyme activity of PKC, had no specific effect on infarct size. However, when Poly B was infused during the time of coronary occlusion in the low PE+Poly B group, all protection induced by PE was abolished (32.9±3.8% infarction).

**Effect of Risk Zone Volume on Infarct Size**

It is known that risk zone dimensions in rabbits have a direct influence on the percent of the risk zone that becomes infarcted. Therefore, to demonstrate that smaller infarcts in protected groups were the result of the treatment rather than merely smaller risk zones, infarct size was plotted against risk zone volume in Fig 6 for all animals used in the present study. All filled-in circles represent rabbits in groups without observed protection, whereas open circles denote animals in groups experiencing small infarct-to-risk zone ratios after a successful preconditioning stimulus. It is obvious that two very different populations exist with little overlap. Hence, the smaller infarcts in the groups with successful preconditioning are not the result of smaller risk zones.

**Discussion**

The present study has shown that δ_{0.1}-adrenergic agonists can precondition the heart against infarction and that this protective effect is not dependent on endogenous adenosine production or adenosine receptor occupancy. Furthermore, it appears that it is the δ_{0.1}-adrenergic receptor subtype that is mainly responsible for this protective effect. Finally, the protection derived from δ_{0.1} stimulation could be blocked with a PKC inhibitor. These observations further cement the central role of PKC in the mechanism of both ischemic and pharmacologic preconditioning. The δ_{0.1}-receptor is known to couple with PKC in the myocyte, and the PKC theory of ischemic preconditioning would predict that activation of those receptors should mimic ischemic preconditioning.

**Effect of Changes in Hemodynamic Conditions and Differences in Risk Zones on Infarct Size**

As noted above, high-dose PE modestly increased LVDP, whereas PBZ, CEC, and Poly B lowered it. However, changes related to PE, PBZ, and CEC largely disappeared during the drug-free interval before the 30-minute coronary occlusion, with return to predrug hemodynamics. Only in the CEC+PE group did there continue to be a mildly lower LVDP. Administration of Poly B caused a mild fall in LVDP for the duration of the infusion, and the decrease was comparable in rabbits receiving this drug alone or with PE. These changes would be expected to have little effect on infarct size. If anything, a mild decrease in contractility should diminish infarction, whereas both Poly B and CEC blocked the protective effect of PE. In fact, in a prior investigation Van Winkle et al dramatically varied loading conditions of the isolated perfused rabbit heart without producing any significant changes in the volume of infarcted tissue. Therefore, changes in the infarct-to-risk zone ratio can confidently be attributed to direct effects of the given intervention.

The influence of risk zone on infarction has recently been described in the rabbit. As noted in Table 2, risk zones were comparable in all groups, although there was a tendency for risk zones to be larger in rabbits in the SPT early group and smaller in animals treated with Poly B. However, Fig 6 demonstrates that differences in risk zone volumes cannot account for differences in infarct volumes between protected and unprotected groups.

**Effect of δ_{0.1}-Adrenergic Agonist on Protection of Ischemic Myocardium and Interaction With Adenosine**

A previous study from this laboratory has demonstrated that inducing norepinephrine release from adrenergic
nerve terminals by intravenous tyramine made the heart as resistant to infarction as did ischemic preconditioning. Bankwala et al. have also observed that norepinephrine release by tyramine as well as direct infusion of norepinephrine could salvage ischemic myocardium. This protection could be abolished by pretreatment with BE 2254, a specific \(\alpha_1\)-adrenergic antagonist, or by PD 115,199, a nonselective long-acting adenosine-receptor blocker. It was concluded that catecholamine-induced protection required both adenosine receptor activation as well as \(\alpha_1\)-adrenergic receptor stimulation, which argued against a direct link between the \(\alpha_1\)-receptor and the signaling pathways responsible for the protection. In a recent preliminary report, Hale and Klomer have also shown a protective effect of PE in an in vivo rabbit model, although no link to adenosine release or receptor activation was evaluated.

Previous studies have asserted that adrenergic receptor stimulation acts to increase adenosine production by the myocardium in guinea pigs and dogs. Furthermore, Kitakaze and colleagues reported that ischemic preconditioning in dogs increased adenosine production by stimulating \(5'\)-nucleotidase activity. They proposed that \(\alpha_1\) activation was responsible for that increase. The \(\alpha_1\)-receptor antagonist prazosin attenuated both the increased enzyme activity and adenosine production and blunted cardioprotection. Conversely, the \(\alpha_1\)-agonist MET increased \(5'\)-nucleotidase activity and limited infarct size.

The observations made in the present experiments in part confirm our prior data but also provide additional information, which allows a more accurate interpretation. Exposure to PE can effectively be substituted for a brief period of ischemia and can precondition the myocardium, resulting in salvage of ischemic tissue. This salutary effect was aborted by the irreversible blockade of \(\alpha_1\)-adrenergic receptors with PBZ. That confirmed our previous observation that blockade of \(\alpha_1\)-receptors with BE 2254 prevented the cardioprotective action of tyramine-induced norepinephrine release. However, results of adenosine receptor blockade were quite different in the two studies. In our previous study, we administered the adenosine blocker PD 115,199 intravenously and studied the heart in situ. Therefore, adenosine receptor blockade was present both before and during the period of regional ischemia. Thus, the critical time for adenosine receptor blockade was unknown. In the present study, we used an isolated heart preparation with a nonrecirculating perfusate. In this preparation, we could turn the blockade on and off at will. Blocking adenosine receptors only during the early PE infusion had no effect on protection, indicating that \(\alpha_1\)-receptor stimulation directly initiated the protective effects and acted independent of adenosine receptors (see Fig 3). But when the adenosine receptors were blocked during the later coronary occlusion, the protective effect of PE was completely lost, indicating that activation of adenosine receptors by endogenous adenosine during the prolonged ischemic period was necessary even when the preconditioning was initiated with \(\alpha_1\)-agonists.

These observations might still be compatible with the theory of Kitakaze et al. if it were proposed that protection is derived from augmented adenosine production specifically during the 30-minute ischemic period. Arguing against that interpretation, however, is our observation that the protective effect of PE could be restored if PE infusion were resumed during the ischemic period along with the adenosine receptor blockade (low PE + PE late + SPT late). Clearly, \(\alpha_1\)-receptors can couple directly with the protective pathway and do not require adenosine as an intermediary.

It was also apparent that either \(\alpha_1\) or adenosine receptor activation must occur at two distinct times (both before and during ischemia) to produce protection. Thornton et al. have recently reported that the protection of ischemic preconditioning can be blocked by administering an adenosine receptor antagonist before the brief preconditioning ischemia or just before the prolonged ischemic interval, also demonstrating that protection of ischemic myocardium requires adenosine receptor occupancy during the initial brief ischemia and again during the prolonged ischemic insult.

**Effect of PKC on the Protection of PE-Induced Myocardial Preconditioning**

Although stimulation of \(\alpha\)-adrenergic and adenosine receptors could each trigger protection, it was initially unclear what, if anything, these very different receptors had in common. But, in fact, both \(\alpha_1\)-adrenergic and adenosine receptors initiate inositol phosphate metabolism, which in turn activates PKC. PKC is an important cellular kinase responsible for phosphorylation of key cellular elements including ion channels. Recent evidence from this laboratory has demonstrated that PKC is involved in the protection of ischemic preconditioning as well. Whereas PKC inhibitors Poly B and staurosporine blocked protection, PKC activators 4\(\beta\)-phorbol 12-myristate 13-acetate and oleoyl acetyl glycerol could independently protect the heart. As demonstrated in Fig 5, Poly B, which competes with the cofactor phosphatidylserine for binding at the allosteric control site of PKC, thus selectively inhibiting the kinase activity of the enzyme, completely blocked the ability of PE to salvage ischemic myocardium. These data directly demonstrate the role of PKC in the protection of \(\alpha_1\)-adrenergic agonists and complement the prior observations that PKC is involved in ischemic preconditioning. Myocardial protection can also be conferred on ischemic myocardium by the cholinergic \(M_2\)-agonists acetylcholine and carbachol. Because carbachol also increases inositol trisphosphate in cardiac tissues, one would expect the protective effect of this drug to be mediated by activated PKC as well. In theory, then, any receptor that couples with PKC in the myocyte should be capable of preconditioning.

We believe that the dependence on two periods of adenosine or \(\alpha_1\)-receptor stimulation occurs because preconditioning involves an upregulation of the PKC pathway. The initial receptor occupancy would begin the upregulation process, perhaps by initiating translocation. The second exposure seems to more quickly activate the kinase moiety of the enzyme and initiate phosphorylation of critical cellular constituents, resulting in protection of ischemic myocardium. Apparently, substantial injury results if there is a significant delay between receptor activation and actual phosphorylation of proteins by PKC as would occur in a heart in which PKC had not been upregulated.
We have previously proposed that the upregulation of PKC that seems to be present in preconditioned myocardium may be related to physical translocation of the enzyme into the membranes, a critical step in the PKC activation process. Others have documented the ability of \( \alpha_1 \)-adrenergic agonists, including PE, to translocate PKC from the cytosol to the cell membrane\(^{26,30,36,37} \) and to initiate phosphorylation by this enzyme.\(^{10} \) In our model the early PE infusion was terminated 10 minutes before the onset of regional ischemia. Therefore, PKC upregulation, and hence the preconditioned state, would have to persist at least 10 minutes. Although Bogoyevitch et al\(^{32} \) have shown in rat ventricular myocytes that phorbol esters can induce PKC translocation lasting up to 1 hour, the effect of epinephrine\(^{37} \) and norepinephrine\(^{36} \) are more transitory and may wane after even 5 minutes. However, the time course of translocation may be more prolonged in intact rabbit myocardium. In addition, probably only one of the many known isoforms of PKC may be involved and thus would likely be overlooked in the standard biochemical assay that measures total PKC activity. Hopefully, the exact mechanism of this upregulation will be revealed in future studies.

**Exogenous Versus Endogenous Catecholamines in Induction of Protection**

Whereas a redundant signaling pathway appears to exist for PKC-mediated protection in the heart, the data would also indicate that at least in the rabbit heart only adenosine is released in sufficient quantity to induce ischemic preconditioning. PBZ could not block the protection of ischemic preconditioning in the present study (Fig 2), whereas adenosine receptor blockade completely eliminated the protection.\(^{38} \) If PE is supplied exogenously during both the preconditioning period and the ischemic period, then protection can be realized independent of adenosine receptors. This redundant pathway becomes more interesting because ischemia is known to trigger local norepinephrine release in the heart.\(^{39} \) Therefore, in the rabbit, although catecholamines may be capable of initiating protection, not enough is released during a 5-minute period of ischemia to trigger protection. In some species or under differing stresses, it is conceivable that sufficient norepinephrine could be released to reach threshold for preconditioning. That could explain why neither adenosine receptor blockade\(^{40,41} \) nor \( \alpha_1 \) blockade with BE 2254 (authors' unpublished observation) could block the anti-infarct effect of preconditioning in rats. A combination of both blockers has not, to our knowledge, been tested in rat heart. Ischemic preconditioning in the dog also appears to be much more dependent on \( \alpha \)-adrenergic receptor stimulation.\(^{25-27} \) The density of \( \alpha \)-adrenergic receptors in the left ventricle of rats is significantly higher than in other species,\(^{11,42} \) and the ratio of \( \alpha_1 \)-to-\( \beta \)-receptors is \( \approx 9 \) times higher in the rat than in the dog and 13 times higher than in the rabbit.\(^{8} \) Furthermore, the composition of adrenergic receptor subtypes varies with species.\(^{43} \) Differing receptor populations in the ventricle may lead to varying physiological responses among the species.

It has recently been reported that catecholamine depletion with reserpine blocked the ability of ischemic preconditioning to protect the rabbit heart,\(^{44,45} \) which would indicate that adrenergic receptors do play a role in ischemic preconditioning. One possible explanation, however, is that reserpine may sufficiently depress myocardial metabolism so that not enough adenosine is generated during the preconditioning ischemia to populate enough receptors to initiate the preconditioning phenomenon. This suggestion is supported by the observation that administration of a \( \beta \)-adrenergic agonist to reserpine-treated animals can restore the protection of a brief ischemic interval.\(^{45} \)

Recently Iwamoto et al\(^{46} \) attempted to precondition the canine heart with stellate ganglion stimulation. Although the present data would suggest that the norepinephrine released by such a maneuver should protect the heart, they were unable to demonstrate protection. The most logical explanation is that insufficient norepinephrine was released to trigger the protection. Another possibility is that dogs may differ fundamentally from rabbits.

**Influence of \( \alpha_1 \)-Adrenergic Receptor Subtypes on Cardioprotection**

The ability of \( \alpha_1 \)-adrenergic agonists to protect ischemic myocardium is not a universal property of such agents. As depicted in Fig 4, MET was ineffective. This observation excludes the possibility that simple myocardial ischemia related to coronary vasoconstriction was initiating the protective effect of PE, since MET depressed coronary flow much more than PE. This seemingly anomalous response of MET is clarified when one considers that there may be up to four \( \alpha \)-receptor subtypes,\(^{11} \) \( \alpha_1 \), \( \alpha_2 \), \( \alpha_3 \), and \( \alpha_4 \). The first two being the best characterized, CEC irreversibly alkylates the \( \alpha_2 \) subtype and clearly does not affect \( \alpha_1 \)-receptors and has been used as a pharmacologic tool to distinguish between subtypes. As seen in Fig 4, when the nonselective \( \alpha \)-agonist PE is administered to hearts pretreated with CEC, the protective properties of PE are eliminated. Conversely, MET is a selective \( \alpha_1 \)-agonist.\(^{14} \) Therefore, it is likely that stimulation of the \( \alpha_1 \)-as opposed to the \( \alpha \)-adrenergic receptor subtype is responsible for the PE-induced protection of ischemic myocardium.

The differential effects of adrenergic receptor subtypes on PKC activation are controversial. \( \alpha_1 \)-Adrenergic agonists activate PKC via phospholipase C, which, when activated, breaks down phosphatidylinositol 4,5-diphosphate in the membrane, yielding inositol 1,4,5-trisphosphate and diacylglycerol. The former releases \( \mathrm{Ca}^{2+} \) from nonmitochondrial intracellular stores and increases contractility, whereas the latter activates PKC. Both Knowlton et al\(^{47} \) and del Balzo et al\(^{48} \) have demonstrated in neonatal rat ventricular myocytes that the \( \alpha_1 \)- and not the \( \alpha_2 \)-adrenergic receptor is responsible for activation of PKC. Thus, it is surprising that protection in the present study appears to be via an \( \alpha_2 \)-receptor.

On the other hand, Han et al\(^{49} \) showed in rat vas deferens that only \( \alpha_2 \)-receptor subtypes caused inositol phospholipid hydrolysis with presumed release of \( \mathrm{Ca}^{2+} \) from the endoplasmic reticulum, whereas the \( \alpha_1 \) subtype had no effect on inositol phospholipid metabolism but controlled the opening of dihydropyridine-sensitive membrane channels to allow influx of extracellular \( \mathrm{Ca}^{2+} \). Michel et al\(^{50} \) in studies of rat cerebral cortex,
Cotecchia et al. in studies of a hamster smooth muscle cell line, and Takanashi et al. in studies of rabbit ventricle, have also confirmed that α₁-adrenergic receptor stimulation is closely coupled to inositol phosphate generation and that α₁-receptor activation has no effect. Therefore, it is not yet clear which receptor subtypes activate PKC, particularly in rabbit heart.

Kitakaze et al. recently reported that they could protect the dog heart with MET pretreatment. Those data are obviously discrepant with the present results. We suspect, however, that the MET in that study was administered at a sufficiently high concentration to not be selective for the receptor subtype. In the present study, the dose was carefully chosen to be one that would likely be selective for the α₁-receptor.

Conclusions

The present investigations reveal that α₁-adrenergic agents and adenosine agonists are equivalent in their ability to trigger the preconditioning phenomenon, most likely because both receptors can activate PKC in the myocyte. The data also reveal that two periods of receptor activation are required to protect the heart, one before ischemia and another concurrent with the onset of ischemia. As long as at least one of the receptor types is activated during each period, protection will occur. PKC appears to be part of a common pathway initiated by diverse receptor agonists released during myocardial ischemia but always ending in protection. This realization should provide an important impetus for uncovering a clinically useful agent that can stimulate PKC either directly or indirectly to salvage myocardium in individuals with ischemic heart disease.

Acknowledgments

This study was supported in part by grants from the National Institutes of Health, Heart, Lung, and Blood Institute, HL-29232 and HL-50688.

References


41. Li Y, Kloner RA. The cardioprotective effects of ischemic “pre-conditioning” are not mediated by adenosine receptors in rat hearts. Circulation. 1993;87:1642-1648.


43. Takanashi M, Norota I, Endoh M. Potent inhibitory action of chloroethylclonidine on the positive inotropic effect and phosphoinositol hydrolysis mediated via myocardial α1-adrenoceptors in the rabbit ventricular myocardium. Naunyn Schmiedebergs Arch Pharmacol. 1991;343:669-673.


49. Han C, Abel PW, Minneman KP. α1-Adrenoceptor subtypes linked to different mechanisms for increasing intracellular Ca2+ in smooth muscle. Nature. 1987;329:333-335.


alpha 1-adrenergic agonists precondition rabbit ischemic myocardium independent of adenosine by direct activation of protein kinase C.
A Tsuchida, Y Liu, G S Liu, M V Cohen and J M Downey

Circ Res. 1994;75:576-585
doi: 10.1161/01.RES.75.3.576

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1994 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/75/3/576