Brief Definitive Communication

Protein Kinase C
Its Role in Ischemic Preconditioning in the Rat

M.E. Speechly-Dick, M.M. Mocanu, D.M. Yellon

Abstract The present study investigated whether protein kinase C (PKC) plays a role in ischemic preconditioning in the rat heart. Chelerythrine, a specific antagonist of PKC, and 1,2-dioctanoyl-sn-glycerol (DOG), a diacylglycerol analogue and specific antagonist of PKC, were used to determine whether preconditioning could be blocked or triggered, respectively. Sprague-Dawley rats were anesthetized and instrumented for coronary occlusion and reperfusion. All animals were subjected to 45 minutes of regional ischemia (ISC) followed by 2.5 hours of reperfusion. The preconditioning protocol consisted of 5 minutes of ischemia and then 10 minutes of reperfusion. There were six groups: (1) control (group C, n=5), (2) preconditioned and ISC (group PC, n=6), (3) chelerythrine given 2 minutes before ISC (group CC, n=5), (4) preconditioned and chelerythrine given 2 minutes before ISC (group PCC, n=6), (5) DOG (dissolved in dimethylsulfoxide [DMSO]) given 10 minutes before ISC (group CD, n=5), and (6) DMSO given 10 minutes before ISC (group DMSO, n=3). The end point was infarct size measured using triphenyltetrazolium chloride and expressed as a percentage of the volume at risk (I/R), measured with fluorescent particles. I/R was significantly reduced by preconditioning (group C, 58.6±5.0%; group PC, 32.7±6.3%; P<.01) and by the PKC agonist DOG, which reduced I/R to a similar extent as preconditioning (group C, 58.6±5.0%; group CD, 28.0±7.0%; P<.01). Inhibition of PKC with chelerythrine abolished protection, there being no significant difference in I/R between control and the chelerythrine-treated preconditioned group (group C, 58.6±5.0%; group PCC, 62.3±5.7%). Chelerythrine and the vehicle DMSO had no direct influence on infarct size (group CC, 63.2±2.2%; group DMSO, 60.7±9.9%). Thus, PKC stimulation with the diacylglycerol analogue DOG resulted in protection, and PKC inhibition with chelerythrine abolished protection. The finding that treatment with a PKC stimulator gives a similar degree of protection against infarction as that seen after ischemic preconditioning and that this protection can be blocked by a PKC inhibitor provides support for the hypothesis that PKC plays an important role in ischemic preconditioning. (Circ Res. 1994;75:586-590.)

Key Words • ischemic preconditioning • protein kinase C • myocardial protection • infarction

Ischemic preconditioning is the most powerful experimental strategy known for protecting the functioning heart from myocardial necrosis when it is subjected to ischemia.1

The mechanism underlying preconditioning is unknown and is currently under intense investigation. There is evidence in support of adenosine being the most important endogenous mediator of protection in some animals (rabbits and dogs),1 where it is thought to act via the A1 receptor to trigger preconditioning. This response can be blocked or reproduced by giving an adenosine antagonist or agonist, respectively. However, adenosine does not appear to play the same role in the rat, and although Liu and Downey2 have induced protection in the rat by giving adenosine or an analogue of adenosine, this effect could not be blocked by giving an adenosine antagonist. Other investigators, such as Li and Kloner3 and Cave et al.,4 have been unable to reproduce the effect of preconditioning in the rat with adenosine. This suggests that although adenosine may be involved in preconditioning in the rat, it is not the most important endogenous mediator, which may well be noradrenaline.5,6

Despite this apparent difference in the endogenous mediators of protection between species, when ischemia is used to precondition the myocardium, it has been clearly shown to be protective in all the species investigated so far.1 There is now some evidence for preconditioning in humans,7,8 and we suggest that there is a universal underlying mechanism for the phenomenon. Protein kinase C (PKC) is known to be a critical component in many intracellular signal transduction pathways,9 and a hypothesis has recently been proposed in which PKC plays a central role. Ytrehus et al.10 have suggested that endogenous ligands such as adenosine and noradrenaline initiate an intracellular pathway by acting on G-protein-linked receptors, which eventually leads to the activation of PKC. Activated PKC then phosphorylates a secondary effector protein, which in some way induces protection.

There is some experimental evidence for the role of PKC in preconditioning in the rabbit and the rat. Ytrehus et al.10 were able to block protection with two PKC inhibitors (staurosporine and polymyxin B, each of which act on a different binding site on PKC) in an in vivo rabbit model of preconditioning. They also demonstrated the ability of PKC stimulation to precondition in an isolated buffer-perfused rabbit heart model by treating with a phorbol ester (4β-phorbol 12-myristate 13-acetate [PMA]) and a diacylglycerol (1-oleyl-2-acetylglycerol). In a study of postischemic functional recovery in isolated rat hearts, Mitchell et al.11 were able to

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inhibit preconditioning with PKC antagonists but were unable to simulate preconditioning with a stimulator.

The rat myocardium appears to respond differently to some of the endogenous mediators of protection seen in other species described above. If PKC has a central role in preconditioning in the rat, we would have evidence for the hypothesis that the intracellular mechanism of preconditioning is common to all species despite the variability in extracellular triggers. Accordingly, we chose specifically to study the role of PKC in the rat. We compared the effects of both a highly selective PKC antagonist and a highly selective agonist on infarct size after regional ischemia in a well-described in vivo rat model of preconditioning.12,13

Materials and Methods

Surgical Preparation

All the male Sprague-Dawley rats, 300 to 340 g, were obtained from a single source and were then kept under identical conditions. They were handled in accordance with the Guidance on the Operation of the Animals (Scientific Procedures) Act 1986, published by Her Majesty’s Stationery Office, London, England.

Anesthesia was induced with sodium pentobarbital (40 mg/kg IP). A midline incision was made in the neck, and a tracheostomy was performed. Positive pressure ventilation was instituted through the tracheostomy with 100% oxygen at a rate of 30 to 35 cycles per minute and a tidal volume of ~5 mL. (MD Industries ventilator). The respiratory rate was varied to maintain the blood pH within the normal range. Anesthesia was maintained with repeated doses of sodium pentobarbital (6 mg hourly or as required). Cannulation of a carotid artery allowed the blood pressure and arterial blood gases to be measured and drugs to be administered. Leads were connected to the chest and limbs to obtain a single-channel ECG, which was used to continuously monitor heart rate, arrhythmias, and ST-segment changes.

A left paramedian thoracotomy was performed, the pericardium was incised, and the heart was exposed. A 3-0 silk suture (Mersilk W506) on an atraumatic needle was placed around the left coronary artery at a point two thirds of the way between its origin near the pulmonary conus and the cardiac apex. The ends of the thread were passed through a short length of plastic tubing to form a snare. Ischemia was induced by pulling the snare taut and clamping it in position. Myocardial ischemia was confirmed by the presence of ST-segment elevation on the ECG and by the visual assessment of regional cyanosis and dyskinesis of the relevant segment of the left ventricle. Releasing the snare resulted in reperfusion, confirmed by conspicuous reactive hyperemia and the return of the ST segments to normal.

The carotid cannula was flushed regularly with heparinized saline (2.5 U/mL) throughout the experiment. A bolus of 250 U heparin sodium (1000 U/mL) was given immediately before coronary occlusion and before removing the hearts.

Experimental Protocol

All groups were subjected to a long period of sustained ischemia (45 minutes) followed by 2 hours and 30 minutes of reperfusion. In the preconditioned group, this long ischemia was preceded by a preconditioning protocol consisting of 5 minutes of ischemia followed by 10 minutes of reperfusion. There was a 5-minute stabilization period before the experimental protocol in all groups, and this was extended to 15 minutes in nonpreconditioned groups, so that both nonpreconditioned and preconditioned groups entered the long ischemia after a similar period of time. There were six experimental groups: (1) control rats subjected to long ischemia and reperfusion (group C, n=5), (2) preconditioned rats subjected to preconditioning before long ischemia (group PC, n=6), (3) control rats given a bolus of chelerythrine 2 minutes before long ischemia (group CC, n=5), (4) preconditioned rats given a bolus of chelerythrine 2 minutes before long ischemia (group PCC, n=6), (5) control rats given a bolus of 1,2-diacyl-sn-glycerol (DOG) 10 minutes before long ischemia (n=5), and (6) control rats given a bolus of the vehicle dimethylsulfoxide (DMSO) 10 minutes before long ischemia (group DMSO, n=3).

Materials

Chelerythrine (LC Laboratories) was dissolved in distilled water (5 mg in 3 mL) and was given at a dose of 5 mg/kg body wt (a volume of 0.9 mL). DOG (Sigma Chemical Co) was dissolved in DMSO (25 mg DOG in 0.5 mL), and the volume was increased to 5 mL by adding distilled water to achieve a concentration of 5 mg/mL. A single bolus of 8 mg/kg body wt DOG (=0.5 mL) was administered over 2 minutes, 10 minutes before long ischemia. One group was given DMSO alone (0.5 mL in 4.5 mL distilled water) at a dose of 0.5 mL, administered as a slow bolus over 2 minutes, 10 minutes before the long ischemia.

Infarct Size Measurement

At the end of the long reperfusion period, the hearts were rapidly excised and retrogradely perfused with heparinized saline. The snare was tightened to reocclude the branch of the left coronary artery, and fluorescent particles (1 to 10 μm in diameter, Duke Scientific Corp) suspended in saline were infused retrogradely through the aorta. This delineated the ischemic area of the myocardium as a nonfluorescent perfusion defect. The heart was weighed and frozen overnight. The next morning, the heart was sliced into 1-mm-thick transverse sections from apex to base and incubated in triphenyl tetrazolium chloride solution (1% in phosphate buffer, pH 7.4) in a water bath at 37°C for 20 minutes. The slices were then kept in formalin (10% solution) for 6 hours. At the end of these procedures, the viable tissue was stained red, and the infarcted tissue remained pale.14 The volume of myocardium at risk and the infarct volume were calculated by using computerized planimetry.

Arrhythmias

The present study was not designed primarily to examine arrhythmias. However, arrhythmias were monitored by ECG as described above. Ventricular tachycardia (VT) and ventricular fibrillation (VF) were defined according to the Lambeth Conventions.15 Ventricular premature beats were not quantified when they occurred in isolation or in salvos of less than four beats.

Statistical Analysis

Continuously distributed variables are presented as group means with the corresponding SEM. Statistical differences between groups were evaluated by one-way ANOVA with Fisher’s protected least significant difference post hoc test.16 A value of P≤.05 was considered significant. Arrhythmia incidences were compared by two-tailed Fisher’s exact probability test.17

Results

Four of the initial 34 animals used in the study were excluded: one in group C, which was excluded when the coronary ligature tore; one in group CC and another in group PCC, which died shortly after reperfusion; and one in group CD, which died of uncontrollable hemorrhage during ischemia.
The reduction in $P<.01$).

**Hemodynamics**

Hemodynamic data are presented in Fig 1 as the rate-pressure product (RPP). There was no significant difference in RPP between the groups. There was a gradual fall in RPP with time, as would be expected. The administration of drugs had no significant effect on RPP or on blood pressure or heart rate when these were examined independently (see Table 1).

**Risk Volume and Infarct Size**

There were no significant differences in body weight, heart weight, or risk volume between the groups (see Table 2). Results for I/R, where I is the infarct volume and $R$ is the volume of myocardium at risk, are shown in Fig 2. I/R was significantly reduced by preconditioning (group C, 58.6±5.0%; group PC, 32.7±6.3%; $P<.01$). I/R was reduced to the same extent by the PKC agonist DMSO (group C, 58.6±5.0%; group CD, 28±7.0%; $P<.01$). The reduction in I/R induced by preconditioning was completely abolished when the PKC inhibitor chelerythrine was administered during the 10-minute reperfusion period (group PCC, 62.3±5.7%), so that I/R in group PCC was not significantly different from that in group C. Neither chelerythrine nor DMSO (vehicle) treatment had any influence on infarct size (group CC, 63.2±2.2%; group DMSO, 60.7±9.9%).

**Arrhythmias**

Table 3 shows the incidence of ventricular arrhythmias, both VF and VT, in all groups during long ischemia, and Fig 3 illustrates the percent incidence of these arrhythmias. There was a significant reduction in the incidence of VF in preconditioned groups PC and PCC when compared with group C (group C versus group PC, $P<.01$; group C versus group PCC, $P<.04$), but there was no significant difference between the other groups (group C versus groups CC, CD, and DMSO). Preconditioning alone resulted in a reduction in the incidence of VT (group C versus group PC, $P<.01$), but there was no significant difference between the other groups and group C (group C versus groups CC, PC, CD, and DMSO). There were no ventricular arrhythmias in any group during either the 5-minute preconditioning period or during the long reperfusion period. There was no significant difference between groups in the time to onset of ventricular arrhythmias during the 45-minute period of ischemia (group C, 6.8±0.5 minutes; group CC, 7.0±0.9 minutes; group PCC, 7.0±0.8 minutes; group CD, 6.4±0.5 minutes; and group DMSO, 6.7±0.3 minutes).

**Table 1. Heart Weight, Body Weight, and Risk Volume**

<table>
<thead>
<tr>
<th>Group</th>
<th>Risk Volume, cm$^3$</th>
<th>Body Weight, g</th>
<th>Heart Weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.16±0.05</td>
<td>319±4</td>
<td>1.27±0.04</td>
</tr>
<tr>
<td>PC</td>
<td>0.18±0.04</td>
<td>314±7</td>
<td>1.27±0.03</td>
</tr>
<tr>
<td>CC</td>
<td>0.21±0.03</td>
<td>320±3</td>
<td>1.27±0.03</td>
</tr>
<tr>
<td>PCC</td>
<td>0.17±0.03</td>
<td>316±3</td>
<td>1.24±0.03</td>
</tr>
<tr>
<td>CD</td>
<td>0.21±0.01</td>
<td>323±6</td>
<td>1.19±0.04</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.19±0.01</td>
<td>306±7</td>
<td>1.18±0.06</td>
</tr>
</tbody>
</table>

C indicates control; PC, preconditioned; CC, control+chelerythrine; PCC, preconditioned+chelerythrine; CD, control+1,2-dioctanoyl-sn-glycerol; and DMSO, control+dimethylsulfoxide. Values are mean±SEM.

There was no significant difference between groups.

**Table 2. Heart Weight, Body Weight, and Risk Volume**

<table>
<thead>
<tr>
<th>Group</th>
<th>Stabilization</th>
<th>Preisch</th>
<th>End Ischemia</th>
<th>60-min Reperfusion</th>
<th>120-min Reperfusion</th>
<th>150-min Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>HR, bpm</td>
<td>BP, mm Hg</td>
<td>HR, bpm</td>
<td>BP, mm Hg</td>
<td>HR, bpm</td>
<td>BP, mm Hg</td>
</tr>
<tr>
<td>PC</td>
<td>368±6</td>
<td>105±8</td>
<td>360±9</td>
<td>97±4</td>
<td>342±12</td>
<td>111±14</td>
</tr>
<tr>
<td>CC</td>
<td>358±12</td>
<td>120±10</td>
<td>355±5</td>
<td>107±13</td>
<td>345±7</td>
<td>105±15</td>
</tr>
<tr>
<td>PCC</td>
<td>348±22</td>
<td>116±14</td>
<td>336±17</td>
<td>105±16</td>
<td>354±17</td>
<td>121±19</td>
</tr>
<tr>
<td>CD</td>
<td>355±12</td>
<td>110±13</td>
<td>350±6</td>
<td>117±13</td>
<td>355±9</td>
<td>130±12</td>
</tr>
<tr>
<td>DMSO</td>
<td>348±7</td>
<td>124±14</td>
<td>348±7</td>
<td>118±13</td>
<td>336±6</td>
<td>130±9</td>
</tr>
</tbody>
</table>

HR indicates heart rate; bpm, beats per minute; BP, blood pressure; C, control; PC, preconditioned; CC, control+chelerythrine; PCC, preconditioned+chelerythrine; CD, control+1,2-dioctanoyl-sn-glycerol; and DMSO, control+dimethylsulfoxide. Values are mean±SEM.

*P<.05 for group C vs group PCC.
**Discussion**

The present study shows that ischemic preconditioning in the rat heart can be blocked if PKC is inhibited with a selective antagonist (chelerythrine), with infarct size used as the end point in an in vivo rat model. It also demonstrates that stimulation of PKC with a selective agonist (DOG) can precondition the same model. The present study provides evidence for the hypothesis that PKC activation is a pivotal step in the intracellular signaling pathway of ischemic preconditioning in the rat.

PKC has been recognized as a critical component of intracellular signal transduction pathways for a decade. It is known to have a vital role in cellular regulation, tumor promotion, and probably onogenesis. It has also been proposed that PKC plays a central role in ischemic preconditioning according to the following mechanism: an endogenous ligand (such as adenosine in the rabbit) binds to the adenosine A1 receptor on the myocyte surface, which then activates phospholipase C via a G protein; activated phospholipase C then causes the breakdown of phosphatidylinositol 4,5-diphosphate and phosphatidylcholine to produce diacylglycerol and inositol-1,4,5-trisphosphate; increased levels of diacly-...
interaction is needed between the lipid and the binding site on PKC for its activation.20 The phorbol ester PMA has been used to simulate the effect of preconditioning on infarct size in the isolated rabbit heart10 but did not have this effect in the isolated rat heart, where functional recovery was the end point.11 However, a more specific diacylglycerol agonist did mimic preconditioning in this rat model. Phorbol esters are hardly degraded once administered and therefore may abnormally extend the cellular response to the preconditioning stimulus and distort the sequence of events.20 Accordingly, it would seem to be preferable to use a diacylglycerol when investigating the mechanism of preconditioning rather than the nonspecific phorbol esters.

Molecular cloning techniques have revealed that PKC exists in at least eight forms with distinct physiological substrates. These forms can be further subdivided into either calcium-dependent or -independent groups.8 In support of the hypothesis of the involvement of PKC in preconditioning, Strasser et al22 have experimental evidence for the direct activation of PKC by cardiac ischemia in the isolated rat heart. There have also been studies into PKC isotype expression, such as by Bogoyevitch et al,22 who have characterized the expression of PKC isotypes in the adult rat ventricular myocyte. They found that the major isotype present was PKC-ε and that it was activated and moved from the cytosol to the membrane by phorbol esters, adrenaline, and endothelin. A more recent study23 used Western blotting with subtype-specific peptide antibodies to determine which PKC subtypes are involved in preconditioning in isolated rat hearts subjected to different periods of ischemia. That study showed that α, β, ε, and ζ are the predominant isoforms of PKC in the rat heart. It also showed that a brief period of ischemia (2.5 minutes), such as that used to precondition, caused a rapid translocation to the membrane of the calcium-independent subtypes δ, ε, and ζ and the calcium-dependent subtype α. Prolonged ischemia (30 or 60 minutes) led to the induction of calcium-independent forms of PKC δ and ε in the cytosol. Weinbrenner et al23 surmised from that study that different mechanisms are involved in the response of the myocyte to short and long episodes of ischemia.

Our results show a loss of the protection against infarction conferred by preconditioning if PKC is inhibited and a protective effect against infarction when the enzyme is activated. Ischemic preconditioning is also known to protect against arrhythmias,3 and in the present study this held true for both VF and VT. Interestingly, despite DOG being able to protect against infarction, it had no protective effect against arrhythmias, which occurred with the same incidence with and without treatment with DOG. Furthermore, protection was seen against VF in the PCC group, suggesting that a PKC inhibitor could not inhibit the protective effect against arrhythmias induced by preconditioning. These observations would be consistent with the anti-infarct and antiarrhythmic effects of preconditioning being mediated by different signaling pathways.

The findings of the present study support a role for PKC in preconditioning against ischemia in the rat and provide an important link with previous work looking at PKC activation and the translocation of specific isoforms in rat hearts in response to ischemia. Although the endogenous ligands and receptors involved may differ between species, the observation that PKC activation is a common step suggests that the intracellular signaling pathway of ischemic preconditioning may be common to all species.

Acknowledgments
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