Infarct-sparing effect of myocardial postconditioning is dependent on protein kinase C signalling

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Abstract

Objective: Using non-selective and selective protein kinase C (PKC) \( \varepsilon \) and \( \delta \) isoform inhibitors, we tested the hypothesis that the cardioprotective phenotype invoked by postconditioning (postcon) is dependent on PKC signalling. Furthermore, we determined whether postconditioning alters pPKC\( \varepsilon \) and/or pPKC\( \delta \) in cytosolic and mitochondrial fractions.

Methods: Male Sprague–Dawley rats underwent 30 min left coronary artery (LCA) occlusion followed by 3 h of reperfusion. Rats were randomised to the following groups: Untreated, no intervention either before or after LCA occlusion; Postcon, 3 cycles of 10-s full reperfusion and 10-s re-occlusion, initiated immediately at the onset of reperfusion; Chelerythrine (non-selective PKC inhibitor, 5 mg/kg) \( T \) postcon; Rottlerin (PKC\( \delta \) inhibitor, 0.3 mg/kg) \( T \) postcon; KIE1-1 (PKC\( \varepsilon \) inhibitor, 3.8 mg/kg) \( T \) postcon. A subset of rats was employed to assess pPKC\( \varepsilon \) and/or pPKC\( \delta \) in sham, Isch/RP (30-min LCA occlusion followed by 30-min reperfusion), and postcon-treated hearts.

Results: Infarct size, expressed as area of necrosis as a percentage of the area at risk, AN/AAR (%), was significantly reduced by postcon compared to control (untreated) rats (39 ± 2% vs. 53 ± 1% in control, \( P < 0.001 \)). Treatment with chelerythrine alone or the PKC\( \varepsilon \) antagonist KIE1-1 alone at reperfusion had no effect on infarct size compared to control. In contrast, the infarct-sparing effect of postcon was abrogated by non-selective PKC inhibition and PKC\( \varepsilon \) antagonism (50 ± 2% and 50 ± 1%, respectively, \( P < 0.002 \)). Inhibition of PKC\( \delta \) reduced infarct size to values comparable to that in postcon group (36 ± 3% vs. 39 ± 2%). However, postcon in the presence of PKC\( \delta \) inhibitor did not enhance the infarct-sparing effects (38 ± 2%). In addition, pPKC\( \varepsilon \) in postcon hearts was significantly higher in the total cell homogenate (10338 ± 1627 vs. 4165 ± 608 in Isch/RP, arbitrary units), and pPKC\( \varepsilon \) translocation to mitochondria was significantly less (>2-fold decrease) compared to Isch/RP.

Conclusion: These data suggest that postcon modulates PKC during early reperfusion by increasing PKC\( \varepsilon \) expression and translocation to a site other than the outer mitochondrial membrane, and limits translocation of PKC\( \delta \) to mitochondria and associated deleterious signalling.

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Keywords: Postconditioning; Myocardial infarction; Mitochondria; Protein kinase C; Protein phosphorylation; Reperfusion injury

1. Introduction

Postconditioning (postcon) is a novel strategy to harness nature’s protection against myocardial ischaemia–reperfusion injury. Postconditioning is induced by brief repetitive mechanical interruptions of the early moments of reperfusion (i.e. a specified algorithm). The cardioprotective
phenotype invoked by postconditioning has been demonstrated to involve a reduction in infarct size, apoptosis as well as vascular injury. These physiological manifestations have been associated with a myriad of cellular and subcellular effects including attenuated generation of reactive oxygen species (ROS) and oxidant-mediated injury, endogenous activation of adenosine receptors, activation of K$_{\text{ATP}}$ channels, decreased intracellular and mitochondrial calcium accumulation and closure of the mitochondrial permeability transition pore (mPTP) (see review [1]). Indeed, postconditioning sets in motion triggers (adenosine, nitric oxide) and molecular signals (survival kinases and anti-apoptotic enzymes) during early reperfusion that are functionally linked to reduced cell death [1–5], provoking renewed interest in reperfusion injury, the molecular signals involved, and therapeutic strategies for treatment.

One family of signalling proteins commonly linked to the modulation of ischaemia–reperfusion injury is protein kinase C (PKC), in particular the PKC isozymes PKC$\varepsilon$ and PKC$\delta$. Importantly, PKC$\delta$ has been implicated as a key signalling element in myocardial and cerebral reperfusion injury processes [6–8]. PKC$\delta$ has been demonstrated to rapidly translocate to mitochondria within the first 5 min of reperfusion [9], and is associated with increased superoxide anion ($O_2^-$) generation, loss in mitochondrial function, as well as enhanced release of cytochrome c and downstream pro-apoptotic factors [9,10]. Conversely, translocation of PKC$\varepsilon$ has been determined to be pivotal in triggering cardioprotective effects of ischaemic and anaesthetic preconditioning [11–16]. Curiously, recent evidence indicates that volatile anaesthetics administered at reperfusion can also mimic postconditioning [17]. In addition, PKC$\varepsilon$ has been reported to exert cardioprotection by inhibiting the mPTP [18], which is also purportedly inhibited by postconditioning [19]. Accordingly, we postulated that postconditioning may trigger the activation of PKC$\varepsilon$ and/or limit PKC$\delta$ translocation, ultimately leading to a reduction in infarct size.

2. Materials and methods


2.1. In vivo rat surgical preparation

One hundred and thirty male Sprague–Dawley rats weighing 260–390 g were initially anaesthetised via intraperitoneal administration of sodium pentobarbital (40 mg kg$^{-1}$). A tracheotomy was performed, and the trachea was intubated with a cannula connected to a rodent ventilator (Harvard Rodent Ventilator Model 683, Holliston, MA). Rats were ventilated with $O_2$-enriched room air at 30–50 breaths per minute, with tidal volumes set to 1.0 ml/100 mg body weight. Arterial $pO_2$, $pCO_2$ and pH were monitored (Stat Profile M, Nova Biomedical, Waltham, MA) during the experiment and values were maintained within a normal physiological range (pH 7.35–7.45; $pCO_2$, 25–40 mm Hg, and $pO_2$, 80–110 mm Hg) by adjusting the ventilatory rate and tidal volume, or by intravenous administration of sodium bicarbonate. Body temperature was kept at a constant 37°C by using an adjustable heating pad.

The left carotid artery was cannulated with a 24-gauge angiocatheter connected to a fluid-filled pressure transducer to continuously monitor phasic and mean arterial pressure (MAP) and heart rate (HR) using the EMKA data acquisition and analysis system (EMKA Technologies Inc., Falls Church, VA). The right external jugular vein was cannulated for delivery of anaesthesia and drug treatments. The chest was opened via a left thoracotomy through the fourth or fifth intercostal space, and the ribs were gently retracted to expose the heart. After pericardiectomy, a 6-0 prolene (Ethicon, NJ) ligature was placed under the left coronary artery (LCA), and the ends of the suture were threaded through polyethylene tubing (PE-50) to form a snare for reversible LCA occlusion. Following surgical preparation (prior to LCA occlusion), a bolus dose of sodium heparin (100 U/kg) was administered to ensure heparinisation throughout the experiment. The LCA was occluded by tightening the snare using a lightweight hemostatic clamp. Ischaemia was confirmed by a transient decrease in blood pressure and cyanosis on the myocardial surface. Reperfusion was indicated by an epicardial hyperaemic response and rapid disappearance of cyanosis.

2.2. Experimental protocol for in vivo infarct size studies

Following stabilisation of haemodynamics and blood gases, rats were subjected to a 30-min LCA occlusion (index ischaemia) followed by 3 h of reperfusion with or without a postconditioning stimulus (3 cycles of 10-s reperfusion and 10-s re-occlusion) initiated at the onset of reperfusion. In appropriate groups, antagonists were administered as a slow infusion over a 5-min period starting 5 min prior to reperfusion. Rats were randomised to one of nine groups (Fig. 1): (1) Untreated (Control), no intervention either before or after LCA occlusion ($n=11$). (2) Vehicle control, dimethyl sulfoxide (DMSO, <300 l/kg) alone ($n=8$). (3) Postcon, initiated immediately at the onset of reperfusion, 3 cycles of 10 s full reperfusion and 10 s re-occlusion (total intervention time of 1 min) ($n=12$). (4) Chelerythrine (non-selective PKC inhibitor, 5 mg/kg) alone ($n=10$). (5) Chelerythrine + Postcon, postconditioning stimulus initiated in the presence of antagonist ($n=8$). (6) Rottlerin (PKC$\delta$ inhibitor, 0.3 mg/kg) alone ($n=10$). (7) Rottlerin + Postcon ($n=9$). (8) KIE1-1 (Tat conjugated PKC$\varepsilon$ inhibitor, 3.8 mg/kg; Tat is a cell-permeating carrier peptide) alone ($n=4$). (9) KIE1-1 + Postcon ($n=8$).
In preliminary studies, the efficacy of the Tat conjugated PKCε inhibitor, KIE1-1, was verified by administering the peptide in the absence and presence of preconditioning (precon). As mentioned previously, the cardioprotective effects of preconditioning involve the activation of PKCε [11–16]. To test KIE1-1 in precon, rats were assigned to one of the following groups: Precon, 5-min LCA occlusion followed by 10 min of reperfusion before index ischaemic event (n=10); Tat carrier control (cell-permeating carrier peptide, 3.8 mg/kg)+Precon, infused over a 5-min period starting 5 min prior to the precon stimulus (n=4); KIE1-1 alone (3.8 mg/kg), infused over a 5-min period starting 20 min prior to index ischaemia (n=4); KIE1-1+Precon (n=8).

2.3. Determination of infarct size

Upon completion of the experiment, the LCA was reoccluded and the AAR was delineated by injecting 1.0 ml of 20% Unisperse blue dye via the external jugular vein. The heart was rapidly excised and placed into 0.9% saline. An individual blinded to the protocol subsequently separated the LV from remaining tissue and thinly (~2 mm) cross-sectioned the LV before separating the AAR (unstained) from blue stained non-ischaemic zone. The AAR was incubated for 10 min in a phosphate-buffered 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution at 37 °C, enabling a clear differentiation of necrotic tissue (area of necrosis, AN). AN and AAR were determined by gravimetric analysis. Infarct size was expressed as a percentage of the AAR (AN/AAR).

2.4. Isolation of mitochondria from rat heart

A subset of rats was randomly assigned to one of three groups: (1) Sham, subjected to surgical manipulation without LCA occlusion (n=3); (2) Isch/RP, 30-min LCA occlusion and 30-min reperfusion (n=6); (3) Postcon, postconditioning stimulus (3 cycles of 10-s reperfusion and 10-s re-occlusion) initiated at the onset of reperfusion following index ischaemia (n=6). Cytosolic and mitochondrial fractions were isolated by differential centrifugation as described by Sayen et al. [20]. Preliminary Western blot studies (data not shown) determined that our mitochondrial isolation process resulted in <6% sarcoplasmic reticulum contamination and negligible sarcoplasmic reticulum contamination (contamination in mitochondria was calculated as a percentage of total cell lysate). Briefly, the AAR was delineated with blue dye at the end of the experimental protocol, the hearts were rapidly excised and placed into ice cold MSE buffer [in mmol/l: 220 mannitol, 70 sucrose, 2 EGTA, 5 MOPS (pH 7.4), 2 taurine supplemented with 0.1% fatty acid-free bovine serum albumin (BSA) and protease inhibitor cocktail]. Following separation of the AAR from the non-ischaemic zone, ~200 mg of minced AAR tissue was homogenised in 3 ml of MSE buffer with a Polytron-type tissue grinder at 11,000 RPM for 2.5 s, followed by 2 quick strokes at 500 RPM with a loose fitting Potter-Elvehjem tissue grinder. The homogenate was then transferred to a 15-ml conical polypropylene tube and centrifuged twice at 600×g for 5 min each (4 °C). The pellet was discarded and the supernatant was then centrifuged for 10 min at 3000×g (4 °C) to pellet
mitochondria. The supernatant (crude cytosol) was collected and further purified by centrifugation at 100,000×g for 1 h, at 4 °C. The mitochondrial pellet was then rinsed and gently re-suspended in 1 ml MSE buffer before repeating the 3000×g spin for 10 min. The final mitochondrial pellet was rinsed and re-suspended in 200 μl of MSE buffer. Cytosolic and mitochondrial aliquots were frozen until Western blot analysis was performed. Protein concentration was determined using the Bradford assay, and for all experiments, an equal amount of mitochondrial and cytosolic protein was loaded on gels.

2.5. Western immunoblotting

2.5.1. Translocation

pPKCδ and pPKCc levels were assessed using 60 μg of mitochondrial and cytosolic protein resolved on 8% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Hybond ECL; Amersham, Piscataway, NJ). After transfer, the membranes were washed three times in TBS-T (0.1% Tween-20) and then blocked for 1 h at room temperature with 5% milk in TBS-T with 0.1% azide. Membranes were then probed with the following primary antibodies: pPKCδ (Ser 643) polyclonal rabbit antibody (1:1000 in 5% BSA in TBS-T, Cell Signalling Technology), pPKCc (Ser 729) polyclonal rabbit antibody (1:1000 in 3% milk in TBS-T, Upstate) or tPKCc polyclonal rabbit antibody (1:1000 in 3% milk in TBS-T, Upstate). Antibodies: pPKCδ (C-YGRKKRRQRRR) to enhance cellular uptake [27]. Membranes were subsequently incubated on wet ice for 30 min and then were run on the same gel. Antiporin (anti-VDAC) polyclonal rabbit antibody (1:2000 in 3% milk in TBS-T, Calbiochem) and anti-actin monoclonal mouse antibody (1:5000 in 3% milk in TBS-T, Sigma) were used to determine equal loading and to verify fraction purity (mitochondrial and cytosolic, respectively). Membranes were subsequently washed four times in TBS-T and then subjected to the appropriate horseradish peroxidase conjugated secondary antibody for 1 h at room temperature. Protein bands were visualised using a chemiluminescence system (ECL kit; Amersham, Piscataway, NJ) and densitometry was quantitated using NIH ImageJ software. Individual pPKC isoform densities were expressed as a ratio of their respective actin or VDAC control and multiplied by 100. To accurately measure translocation, cytosolic and mitochondrial fractions from the same heart were run on the same gel. Translocation was calculated by dividing the normalized mitochondria density by the normalized cytosolic density and multiplying by 100 (mito/cyt0 ratio).

2.5.2. Detection of pPKCc in total cell homogenate

Additional rat experiments (same protocol as described in Section 2.4) were undertaken to extract total proteins from the AAR tissue: Sham (n=1), Isch/R (n=4) and Postcon (n=4). 100 mg heart tissue was placed in 500 μl 1× lysis buffer (Cell Signalling; Beverly, MA) with added PMSF (1 mM), and homogenised at 4 °C for 20 s. Samples were subsequently incubated on wet ice for 30 min and then centrifuged at 16,000×g for 10 min at 4 °C. Aliquots were stored at –80 °C until Western blot analysis was performed. Following protein concentration determination using the Bradford assay, 50 μg of total protein was resolved on 8% SDS-polyacrylamide gel and standard Western blot procedures were carried out as described above.

2.6. Chemicals

Rottlerin was purchased from BIOMOL International (Plymouth Meeting, PA) and the PKCδ inhibitor, KIE1-1 was kindly supplied by KAI Pharmaceuticals (South San Francisco, CA). Protease inhibitor cocktail was obtained from Roche Applied Science (Indianapolis, IN) and MOPS buffer from Mediatech (Herndon, VA). All other drugs were purchased from Sigma/RBI (St. Louis, MO). Chelerythrine and KIE1-1 were dissolved directly in 0.9% saline, whereas Rottlerin was dissolved in <300 μl/kg DMSO. Previous studies from our lab have shown that this concentration of DMSO has no effect on haemodynamics or infarct size in the rat model [21], confirmed by results below.

Chelerythrine was employed as it acts as a selective PKC antagonist, effectively targeting the catalytic site of all PKC isoforms [22]. We used chelerythrine at a dose (5 mg/kg) that has previously been demonstrated to inhibit the translocation of PKCδ [15] and PKCc [23] and associated cardioprotection [15,23,24]. We additionally used the selective PKCδ inhibitor, rottlerin (0.3 mg/kg) which has been reported to prevent PKCδ translocation in preconditioned animals [25] and the highly selective PKCc antagonist, KIE1-1 (3.8 mg/kg) (C-EAVSLKPT) [26] which was conjugated to the cell permeable peptide, Tat (C-YGRKKRRQRRR) to enhance cellular uptake [27].

2.7. Statistical analysis

All values are reported as mean±S.E.M. Data were analysed using SigmaStat 2.0 for Windows statistical software package (SPSS, Inc., Chicago, IL). One-way analysis of variance (ANOVA) with a Student–Newman–Keuls post-hoc test was employed to determine if any significant differences existed between groups for individual parameters. Statistical analysis of infarct size was performed over all groups simultaneously; data may be regrouped for a more logical presentation. In all tests, P<0.05 was considered indicative of statistical significance.

3. Results

3.1. Haemodynamic data

The haemodynamic data are summarised in Table 1. There were no significant differences observed between control and treatment groups for any variable [HR, MAP or...
rate-pressure product (RPP) at baseline or at the end of ischaemia. Significant differences in HR were observed at the end of reperfusion between control and postcon hearts treated with rotterlin or KIE1-1. Significant differences in HR were also detected between control (306 ± 10 bpm) and hearts subjected to precon (383 ± 12), precon + Tat carrier control (381 ± 11), KIE1-1 (preisch) (390 ± 19) and precon + KIE1-1 (394 ± 12) (P<0.05). No significant differences in MAP or RPP were observed at the end of reperfusion.

3.2. Efficacy of the Tat conjugated PKCε inhibitor, KIE1-1 on preconditioning

Preliminary studies revealed that the Tat conjugated PKCε inhibitor, KIE1-1, at a concentration of 3.8 mg/kg, had no effect on infarct size when administered as a pretreatment alone (51 ± 1% vs. 53 ± 1% in control), however when given as a pretreatment before the precon stimulus it abolished the infarct size reduction with precon (44 ± 2% vs. 34 ± 2% in precon, P<0.007). Importantly, the Tat carrier control (peptide vehicle) did not have an effect on precon cardioprotection (35 ± 1% vs. 34 ± 2% in precon).

3.3. Infarct size

The area placed at risk by LCA occlusion, expressed as a percent of left ventricular mass (AAR/LV), was comparable among groups (~38–42%) (Table 2). Infarct size, expressed as a percentage of area at risk (AN/AAR), was significantly reduced in the postcon group (39 ± 2%) compared with the control or vehicle rats (53 ± 1% or 54 ± 3%, respectively, P<0.001) (Table 2, Fig. 2). Reperfusion treatment with chelerythrine (non-selective PKC inhibitor, 5 mg/kg) and KIE1-1 (selective PKCε antagonist, 3.8 mg/kg) alone had no effect on infarct size compared to control (Table 2, Fig. 2A). However, chelerythrine and the PKCε inhibitor KIE1-1 abrogated postcon infarct size reduction (50 ± 2% and 50 ± 1%, respectively, P<0.002) (Table 2, Fig. 2A). Interestingly, rotterlin (selective PKC δ blocker, 0.3 mg/kg) alone resulted in similar protection as observed in postcon hearts (36 ± 3% vs. 39 ± 2% in postcon) (Table 2, Fig. 2B). However, the infarct-sparing effect of postcon was not enhanced by rotterlin (38 ± 2%), suggesting that postcon may involve modulation of PKCδ during early reperfusion (Table 2, Fig. 2B).

3.4. Western blot analysis

In order to investigate the significance of the molecular pathways inferred by the infarct size data, western blot analysis was performed on total cell homogenate, cytosolic and mitochondrial fractions obtained from sham, Isch/RP and postcon hearts harvested at 30-min reperfusion. Compared to Isch/RP (4165 ± 608, arbitrary units), postcon resulted in significantly higher total pPKCε levels, similar to sham (10,338 ± 1627 vs. 10,044 in sham, arbitrary units) (Fig. 3). However, despite the observation that postcon resulted in significantly higher pPKCε in the total cell homogenate (Fig. 3), we did not detect any significant differences in pPKCε between groups in the cytosolic or mitochondrial fractions (Table 2).}

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline Heart rate</th>
<th>25-min ischaemia Heart rate</th>
<th>180-min reperfusion Heart rate</th>
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<tbody>
<tr>
<td></td>
<td>MAP</td>
<td>RPP</td>
<td>MAP</td>
</tr>
<tr>
<td>Control</td>
<td>368 ± 13</td>
<td>91 ± 3</td>
<td>33 ± 2</td>
</tr>
<tr>
<td>Postcon</td>
<td>348 ± 12</td>
<td>88 ± 3</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>Chelerythrine</td>
<td>356 ± 25</td>
<td>82 ± 7</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>Postcon + Chelerythrine</td>
<td>347 ± 11</td>
<td>92 ± 3</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>Vehicle</td>
<td>381 ± 16</td>
<td>83 ± 6</td>
<td>32 ± 4</td>
</tr>
<tr>
<td>Rottlerin</td>
<td>389 ± 9</td>
<td>88 ± 4</td>
<td>34 ± 2</td>
</tr>
<tr>
<td>Postcon + Rottlerin</td>
<td>402 ± 9</td>
<td>96 ± 6</td>
<td>39 ± 2</td>
</tr>
<tr>
<td>KIE1-1</td>
<td>409 ± 14</td>
<td>104 ± 14</td>
<td>43 ± 7</td>
</tr>
<tr>
<td>Postcon + KIE1-1</td>
<td>366 ± 13</td>
<td>82 ± 3</td>
<td>30 ± 2</td>
</tr>
</tbody>
</table>

MAP, mean arterial pressure; RPP, rate-pressure product/1000.
* P<0.05 vs. values in control group.

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body weight (g)</th>
<th>LV weight (mg)</th>
<th>AAR/LV (%)</th>
<th>AN/AAR (%)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>297 ± 13</td>
<td>587 ± 26</td>
<td>39 ± 2</td>
<td>53 ± 1†</td>
</tr>
<tr>
<td>Postcon</td>
<td>12</td>
<td>324 ± 14</td>
<td>588 ± 30</td>
<td>41 ± 3</td>
<td>39 ± 2*</td>
</tr>
<tr>
<td>Chelerythrine</td>
<td>10</td>
<td>335 ± 7</td>
<td>620 ± 15</td>
<td>40 ± 3</td>
<td>48 ± 1†</td>
</tr>
<tr>
<td>Postcon + Chelerythrine</td>
<td>8</td>
<td>327 ± 8</td>
<td>661 ± 11</td>
<td>42 ± 3</td>
<td>50 ± 2†</td>
</tr>
<tr>
<td>Vehicle</td>
<td>8</td>
<td>369 ± 6</td>
<td>698 ± 15</td>
<td>38 ± 3</td>
<td>54 ± 3*</td>
</tr>
<tr>
<td>Rottlerin</td>
<td>10</td>
<td>344 ± 5</td>
<td>685 ± 13</td>
<td>42 ± 2</td>
<td>36 ± 3*</td>
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<td>Postcon + Rottlerin</td>
<td>9</td>
<td>339 ± 9</td>
<td>642 ± 20</td>
<td>40 ± 1</td>
<td>38 ± 2*</td>
</tr>
<tr>
<td>KIE1-1</td>
<td>4</td>
<td>367 ± 6</td>
<td>738 ± 46</td>
<td>39 ± 1</td>
<td>50 ± 1†</td>
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<tr>
<td>Postcon + KIE1-1</td>
<td>8</td>
<td>335 ± 7</td>
<td>664 ± 19</td>
<td>40 ± 2</td>
<td>50 ± 1†</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.E.M. All treatment groups were assessed simultaneously using one-way ANOVA for individual parameters.
* P<0.05 vs. values in control and vehicle group.
† P<0.05 vs. values in post-con group.
mitochondrial fractions (Fig. 4B and C). Although pPKCε was present at very low levels, tPKCε was detected in all groups including sham, consistent with localisation on the outer membrane of the mitochondria. Importantly, no differences in tPKCε were observed between sham, Isch/RP or postcon in the cytosolic and mitochondrial fractions.

With regard to PKCδ, there was a trend for pPKCδ levels in Isch/RP to decrease in the cytosolic fraction (72 ± 9 vs. 101 ± 2 in sham) while being significantly increased in the mitochondrial fraction (23 ± 4 vs. 7 ± 1 in sham, P < 0.03) during early reperfusion (Fig. 5B). Moreover, when expressed as a percent translocation between the mitochondrial and cytosolic fractions (mito/cyto), Isch/RP was associated with a 5-fold increase in pPKCδ translocation to the mitochondria relative to sham (35 ± 8 vs. 7 ± 1 in sham, P < 0.04) (Fig. 5C). Importantly, our data revealed that postcon hearts exhibited significantly less pPKCδ translocation to mitochondria (>2-fold decrease) relative to hearts
subjected to Isch/RP alone (16 ± 2 vs. 35 ± 8 in Isch/RP, P < 0.05) (Fig. 5C).

4. Discussion

The results of the current investigation suggest that the cardioprotective effects of postconditioning are dependent on PKC signalling. Our data demonstrate that the infarct-sparing effects of postconditioning are reversed by selective PKCα inhibition, suggesting that postconditioning may be triggered by endogenous activation of PKCα. This finding was also corroborated by our Western blot data, which demonstrate that postconditioning is associated with significantly higher levels of pPKCα in total cell homogenate relative to the untreated Isch/RP group, an effect that appeared independent of its translocation to mitochondria. In addition, our data support previous observations that reperfusion stimulates PKCα translocation to mitochondria and that inhibition of PKCα during reperfusion substantially reduces infarct size [9,28]. Importantly, we extend these findings by revealing that the cardioprotective effects of postconditioning are not enhanced by PKCα inhibition, and that postconditioning prevents significant reperfusion-induced PKCα translocation to mitochondria. Taken together, these data suggest that postconditioning modulates PKC during early reperfusion by increasing PKCα expression and translocation to a site other than the outer mitochondrial membrane, and limits translocation of PKCα to mitochondria and associated deleterious signalling. The concept of a signalling role for PKC during reperfusion is consistent with studies showing that endogenous activators of PKC such as adenosine [21] and opioids [29] are involved in the cardioprotective effects of postconditioning.

4.1. Evidence for a role of PKCα activation in postconditioning cardioprotection

PKCα is an essential component involved in the triggering process of ischaemic preconditioning [11,14,15,30–34]. Importantly, PKCα is activated not only by a preconditioning stimulus, but also by treatments that mimic preconditioning such as adenosine [35], nitric oxide (NO•) [36], volatile anaesthetics [12,13,16,37], and exogenously applied PKCα agonists [38]. Interestingly, many of these same triggers (adenosine, NO•) and signals (survival kinases and anti-apoptotic enzymes) that are implicated in preconditioning and the activation of PKCα have also been identified to play a role in postconditioning [2–5,19,21,39]. A recent example is the finding that isoflurane, a well-known anaesthetic preconditioning agent that mediates cardioprotection via activation of PKCα [12,40,41], also invokes a postconditioning-like effect when administered at the onset of reperfusion [17]. Therefore, we hypothesise that PKCα may also play a role in postconditioning-mediated protection. In the present study, we demonstrated that selective PKCα inhibition (at a concentration that inhibited preconditioning) reversed the infarct-sparing effects of postconditioning, thus providing functional evidence for the involvement of endogenously activated PKCα in the modification of reperfusion injury processes.

Only one other study has examined the effects of PKCα activation on reperfusion injury. Inagaki et al. demonstrated in Langendorff perfused rat hearts that exogenous activation of PKCα at the onset of reperfusion did not improve contractile function or reduce creatine kinase (CK) activity [6]. The major difference between this and the current study, apart from the end points measured and the model employed, is the use of exogenous versus endogenous activation of PKCα to modify reperfusion injury processes. Indeed, it is entirely possible that endogenous activation of PKCα is functionally involved in cardioprotection, and that exogenously applied PKCα...
activators are not as effective in eliciting the same degree of protection. Additional studies are needed to reconcile the importance of both endogenous and exogenous PKC\(\varepsilon\) specifically during reperfusion.

Since cardioprotection mediated by activation of PKC\(\varepsilon\) has been reported to redistribute to mitochondria with ischaemic and pharmacological preconditioning [15,42] where it is thought to orchestrate protection, we attempted to elucidate if postconditioning triggers PKC\(\varepsilon\) translocation to mitochondria. Indeed, Baines et al. have shown that cardiac mitochondria constitutively express PKC\(\varepsilon\) where it can interact with (1) MAPK to form protective signalling modules [43] and (2) mPTP to inhibit pore opening in the heart [18]. As mentioned previously, accumulating evidence suggest that the postconditioning cardioprotection involves inhibition of mPTP opening [19,44]. However, while our data show that PKC\(\varepsilon\) is localised on the outer membrane of mitochondria, and that postconditioning resulted in significantly higher pPKC\(\varepsilon\) expression in total cell homogenate compared to Isch/RP, we did not detect any differences in pPKC\(\varepsilon\) in the cytosolic or mitochondrial fraction. There are several explanations why we did not observe any translocation of pPKC\(\varepsilon\) to mitochondria with postconditioning. Firstly, it is possible that PKC\(\varepsilon\) translocates to the inner mitochondrial membrane to activate protective downstream signalling, rather than translocating to the outer mitochondrial membrane. Indeed, very recent evidence by Costa et al. indicates that PKC\(\varepsilon\) plays a critical role in transmitting a cardioprotective signal from activated PKG (located on the cytosolic interface) to mitochondrial K\(\text{ATP}\) channels (located on the inner mitochondrial membrane) [45]. Secondly, PKC\(\varepsilon\) may translocate to a different subcellular location not identified in this study. Uecker et al. identified that preconditioning promotes translocation of PKC\(\varepsilon\) to sarcolemma, while PKC\(\delta\) redistributes to mitochondria [46]. The latter case remains to be determined for postconditioning. Thirdly, timing after ischaemia–reperfusion may be critical to the levels of PKC measured in area at risk myocardium. We harvested hearts after 30 min of reperfusion, based on the fact that we detected significantly higher pPKC\(\varepsilon\) levels in postconditioned hearts compared to untreated Isch/RP hearts at this time. However, if postconditioning results in transient rather than sustained translocation of PKC\(\varepsilon\), then we may have missed the critical window of opportunity in detecting an increase in PKC\(\varepsilon\). Indeed, there is some evidence to indicate that preconditioning results in transient PKC\(\varepsilon\) translocation, [47] but the time course of PKC\(\varepsilon\) expression during postconditioning is not known. Nonetheless, our finding that selective PKC\(\varepsilon\) inhibition reverses the infarct-sparing effects of postconditioning in conjunction with our observation that postconditioning results in higher pPKC\(\varepsilon\) levels in total cell homogenate provides initial evidence for a role for PKC\(\varepsilon\) in postconditioning cardioprotection.

4.2. Postconditioning’s cardioprotective phenotype involves decreased translocation and phosphorylation of PKC\(\delta\) to mitochondria

Accumulating evidence suggests that endogenous activation of PKC\(\delta\) during early reperfusion is responsible for initiating a deleterious signalling cascade [6,7,9,10,48–50]. Not only has PKC\(\delta\) been shown to rapidly translocate to mitochondria within the first 5 min of reperfusion following ischaemia [9], but also selective PKC\(\delta\) antagonism limits reperfusion injury [6,7,48] and associated adverse signalling events [9,10,50]. Our data corroborate the aforementioned studies by confirming that translocation of PKC\(\delta\) to mitochondria was observed during reperfusion and that infusion of the PKC\(\delta\) inhibitor, rottlerin, 5 min prior to reperfusion successfully reduced infarct size in the in vivo rat model. These data not only verify that PKC\(\delta\) is instrumental in mediating reperfusion injury processes, but also suggest that inhibition of PKC\(\delta\) induced during early reperfusion can impact upon the outcome of late reperfusion injuries. This is entirely consistent with Inagaki et al. findings that intracoronary treatment with PKC\(\delta\) inhibitor peptide for 1 minute only at the onset of reperfusion efficiently reduced cardiac damage induced by reperfusion in the in vivo porcine model of acute myocardial infarction [7]. Notably, this also draws attention to the possibility that postconditioning, which is also invoked during the early moments of reperfusion, may involve modulation of PKC\(\delta\). To this end, postconditioning in the presence of PKC\(\delta\) inhibition did not result in additive infarct-sparing effects. One interpretation is that postconditioning may mediate cardioprotection via negative modulation of PKC\(\delta\). Alternatively, the infarct size reduction achieved with PKC\(\delta\) inhibition may be maximal, therefore not providing any additional protection with postconditioning in combination with PKC\(\delta\) inhibition. However, this appears unlikely since remote postconditioning in the same model used in the current study confers greater protection than native postconditioning [51]. To resolve this question, we performed Western blot analysis and probed for pPKC\(\delta\) in cytosolic and mitochondrial fraction. Our novel data reveal that postconditioning significantly limits reperfusion-induced translocation of PKC\(\delta\) to mitochondria. We speculate that postconditioning may reduce ROS as a stimulus for PKC\(\delta\) activation. Indeed, PKC\(\delta\) rapidly translocates to mitochondria in hearts treated with H\(_2\)O\(_2\), the by-product of O\(_2^-\) [9]. Observations that postconditioning significantly reduces O\(_2^-\) generation and antioxidant-mediated injury [44,52,53], suggest that postconditioning may inhibit oxidant-induced PKC\(\delta\) translocation to mitochondria.

4.3. Conclusion

Collectively, the data presented demonstrate that the infarct-sparing effect of postconditioning is dependent on...
PKC signalling. The protection observed appears to involve activation of PKCe, since selective isoform inhibition prevented the infarct size reduction produced by postconditioning and postconditioning was associated with significantly higher pPKCe levels in area at risk myocardium. In addition, our data reveal that postconditioning limits reperfusion-induced PKCδ translocation to mitochondria.

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