Postconditioning via stuttering reperfusion limits myocardial infarct size in rabbit hearts: role of ERK1/2

Chad E. Darling,1* Rong Jiang,3* Michelle Maynard,1 Peter Whittaker,1,2 Jakob Vinten-Johansen,3 and Karin Przyklenk1,2

Departments of 1Emergency Medicine and 2Anesthesiology, University of Massachusetts Medical School, Worcester, Massachusetts; and 3Department of Cardiothoracic Surgery, Emory School of Medicine, Atlanta, Georgia

Submitted 18 January 2005; accepted in final form 31 May 2005

The present standard treatment for acute myocardial infarction is timely reperfusion. Although early reflow undoubtedly salvages myocytes and in turn reduces morbidity and mortality, it has been postulated that despite these undisputed benefits, restoration of flow may also have deleterious consequences, i.e., the controversial concept of lethal reperfusion injury (23, 34). Among the multiple strategies proposed during the past two decades to investigate and potentially attenuate reperfusion injury, Vinten-Johansen and colleagues (13, 39) have recently made the intriguing observation that in in vivo dog and rat models, infarct size was significantly reduced when restoration of flow was initiated in a stuttering manner vs. conventional full and abrupt reperfusion. Since these initial reports, there has been growing interest in both corroborating this concept of cardioprotection with stuttering reperfusion [termed “postconditioning” (post-C; Refs. 13, 39)] in additional models and species and elucidating the cellular mechanisms that may contribute to infarct size reduction with post-C (1, 6, 10, 12, 13, 21, 22, 25, 28, 35, 36, 39). Accordingly, our aims in the present study were to 1) assess the efficacy of infarct size reduction with post-C in an isolated buffer-perfused rabbit heart model of regional ischemia; and 2) test the hypothesis, using both pharmacological antagonists and direct assessment by standard immunoblotting, that activation of one or more classic survival kinases [in particular, phosphatidylinositol 3-kinase (PI3-kinase) and/or extracellular signal-regulated kinase (ERK)1/2] might play a critical role in this phenomenon.

METHODS

This study was approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School and was performed in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute of Laboratory Animals Resources (NIH Publication, Vol. 25, No. 28, Revised 1996).

Surgical Preparation

All experiments were conducted using the isolated buffer-perfused rabbit heart model of regional myocardial ischemia, which is a well-characterized preparation used routinely by our group and others (4, 8, 9, 15). In brief, 83 New Zealand White rabbits of 2.5–3.0 kg body wt (11–12 wk of age) were anesthetized with an injection of ketamine and xylazine (150 and 100 mg im, respectively). A tracheostomy was performed, and the animals were ventilated with room air. The heart was exposed via a left lateral thoracotomy, the pericardium was incised, and a dominant branch of the left circumflex coronary artery was ensnared with a 2.0 silk suture. The heart was then rapidly excised, placed in iced buffer, and immediately hung by the aortic root on a Langendorff apparatus for retrograde nonrecirculating buffer perfusion at a constant pressure of 85 mmHg. The buffer was composed of (in mM) 118 NaCl, 4.7 KCl, 24 NaHCO3, 1.2 KH2PO4, 1.2 MgSO4·7H2O, 11 glucose, and 2.5 CaCl2 anhydrous in distilled water (pH 7.4) and was continuously oxygenated with 95% O2-5% CO2. The perfusate was warmed to 37°C, and heart temperature was maintained at 37°C by immersion in a custom-made, water-jacketed chamber. An incision was made in the

Address for reprint requests and other correspondence: K. Przyklenk, Dept. of Emergency Medicine, Univ. of Massachusetts Medical School, 55 Lake Ave. North, Worcester, MA 01655 (e-mail: Karin.Przyklenk@umassmed.edu).
left atrium, and a latex balloon connected to a pressure transducer was positioned in the left ventricular (LV) cavity for continuous assessment of cardiodynamic function. The balloon was initially inflated to an end-diastolic pressure of 5–10 mmHg, and thereafter the balloon volume was held constant. All hearts were paced at 210 beats/min via electrodes positioned on the right ventricle.

**Experimental Protocols**

**Protocol 1: Reduction of infarct size with post-C.** Our aim in protocol 1 was to assess the efficacy of cardioprotection with post-C in our isolated rabbit heart model. Accordingly, 20 hearts underwent 30 min of sustained coronary artery occlusion followed by 2 h of reperfusion (Fig. 1). After the onset of ischemia, each heart was randomly assigned to receive either 1) abrupt and complete reperfusion (controls; \( n = 8 \)); or 2) post-C with stuttering reperfusion (post-C; \( n = 12 \)), which was composed of four alternating 30-s cycles of reperfusion and subsequent coronary reocclusion (total time, 4 min) implemented before full restoration of flow. Cardiodynamics were recorded at 1-min intervals throughout the protocol on a computerized data-acquisition system (Micro-Med; Louisville, KY). Coronary flow was measured by timed collection of effluent at baseline, during minutes 10 and 25 of occlusion, and at 15 min, 1 h, and 2 h after complete restoration of flow.

At the conclusion of the 2-h reperfusion period, the coronary artery branch was briefly reoccluded, and fluorescent polymer beads (2–9 μm in diameter; Duke Scientific; Palo Alto, CA) were injected into the coronary circulation to delineate the area at risk of infarction (AR). The heart was immediately removed from the apparatus, sliced into 5 or 6 transverse sections, illuminated under ultraviolet light, and digitally photographed. To delineate the area of necrosis (AN), the heart slices were then incubated in triphenyltetrazolium chloride for 15 min at 37°C (33), rephotographed, and stored in formalin.

**Protocol 2: Role of survival kinases in post-C-induced cardioprotection.**

**Protocol 2A: PI3-kinase.** To assess the potential contribution of PI3-kinase to infarct size reduction with post-C, an additional 19 hearts were instrumented and randomized to control and post-C groups as described for protocol 1. However, in this component of the study, all hearts were treated with LY-294002 (LY; LC Laboratories; Woburn, MA), which is a potent and selective inhibitor of PI3-kinase (16, 27). The antagonist was infused via a side port immediately proximal to the heart for a total of 30 min, beginning 25 min into sustained coronary occlusion, and maintained for the initial 25 min of postreperfusion (final concentration of 15 μM) (Fig. 1). Cardiodynamics, coronary flow, risk region, and infarct size were measured as in protocol 1.

**Protocol 2B: ERK1/2.** The design of protocol 2B was identical to that of protocol 2A except all hearts (a total of 14) were infused for 30 min with the ERK1/2 inhibitor PD-98059 (PD; final concentration of 10 μM; LC Laboratories; Fig. 1; Refs. 16, 36). Cardiodynamics, coronary flow, risk region, and infarct size were measured as described above.

**Protocol 3: Assessment of survival kinases.** A final 30 hearts were used to determine, by standard Western immunoblotting, whether the PI3-kinase and/or ERK1/2 pathways are preferentially upregulated during the early minutes after reperfusion in the salvaged subepicardium of postconditioned vs. control hearts. Hearts were randomized into five groups including nonischemic shams (\( n = 8 \)), two groups of controls (total, \( n = 11 \)), and two postconditioned groups (total, \( n = 11 \); Fig. 1). For the sham group, the coronary artery was ensnared, and hearts were hung on the Langendorff apparatus and perfused with buffer for 30 min. All other hearts underwent 30 min of sustained occlusion followed by either abrupt reperfusion for the control groups or four 30-s episodes of stuttering reflow as described in protocols 1 and 2 for the post-C groups. One pair of control and postconditioned groups was sampled at 5 min after initial restoration of flow [designated as control 5' (\( n = 4 \)) and post-C 5' (\( n = 7 \)), respectively]. For hearts in this post-C 5' cohort, the total “open-artery duration” was only 3 min (i.e., 2 min of stuttering reflow plus 1 min of full reperfusion). Thus to address the possibility that open-artery duration rather than total elapsed time may be an important determinant of survival kinase activity postreperfusion, two additional cohorts were enrolled including a control group sampled after 3 min of full reperfusion (control 3'; \( n = 7 \)) matched in terms of open-artery duration to the post-C 5' cohort, and a postconditioned group sampled at 7 min after initial restoration of flow (post-C 7'; \( n = 4 \)). With 2 min of stuttering reflow plus 3 min of full reperfusion and thus comparable in terms of open-artery duration to the control 5' cohort (Fig. 1).

At the designated sampling time, the risk region was delineated in all control and postconditioned groups by brief reocclusion of the...
coronary artery and injection of fluorescent beads, and all hearts were stored in liquid nitrogen. To ensure that only viable, previously ischemic myocardium was used for analysis (5), subepicardial samples from within the center of the AR (i.e., devoid of fluorescence) were harvested from all control and postconditioned hearts, and comparable subepicardial samples were obtained in the matched region (distal to the suture) in the sham hearts. All samples were stored at −80°C until processing.

Endpoints and analysis. The primary endpoint of protocols 1 and 2 was infarct size. After 48 h of formalin fixation, right ventricular tissue was removed, and the LV slices were weighed. AR and AN in each heart slice were quantified from digital photographs using image-analysis software (SigmaScan Pro: Systat; Point Richmond, CA), corrected for tissue weight, and summed for each heart. AR was then expressed as a percent of the total LV weight, whereas AN was expressed as a percentage of the AR (8). All analyses of risk region and infarct size were done by an investigator who was blinded with regard to the group assignments.

Secondary endpoints for protocols 1 and 2 were cardiodynamics and coronary flow. LV pressures were tabulated for all hearts at baseline, at 10 and 25 min into sustained occlusion, and at 15 min, 1 h, and 2 h postreperfusion. For each time point, LV developed pressure was calculated as the difference between maximum LV systolic pressure and end-diastolic pressure.

For protocol 3, the primary endpoints were the expression of phospho-ERK1/2, phospho-Akt/PKB (downstream target of PI3-kinase), total ERK1/2, total Akt, and the phosphatase and tensin homolog on chromosome 10, PTEN [which is a negative regulator of PI3-kinase (20)] as assessed by standard Western immunoblotting. Frozen subepicardial samples (50–100 mg) were minced into fragments and homogenized in an extraction buffer that contained 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml of leupeptin- pepstatin A-protease. Homogenates were centrifuged at 16,000 g for 20 min, the supernatant was decanted, and protein concentration was determined using the BCA protein assay (Bio-Rad Laboratories; Hercules, CA). Extracted protein samples were reduced with 100 mM DTT and denatured at 95°C for 5 min. Proteins (70 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with primary antibodies for phospho-ERK1/2 (threonine 202/tirosine 204), phospho-Akt (serine 473), total ERK1/2, total Akt, and PTEN (Cell Signaling Technology; Beverly, MA and Santa Cruz Biotechnology; Santa Cruz, CA). Blots were washed and then incubated with goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology), and to enhance visualization of the immunoreactive bands, blots were subsequently incubated using the ECL detection system. Blots were exposed to ECL chemiluminescence film for 2–5 min and scanned, and bands of interest were quantified in a blinded manner using gel analysis software (LabImage; Kapelan; Halle, Germany). For each sample, data for total- and phospho-ERK1/2 represent the sum of the 42- and 44-kDa bands.

Statistics

Because protocols 1 and 2 were conducted sequentially rather than concurrently, separate statistical analyses were performed for each component of the study. Discrete variables (i.e., AR/LV, AN/AR) were compared between matched control and postconditioned groups by t-test. In addition, the extent of necrosis was further compared between groups by analysis of covariance (ANCOVA) incorporating risk region, which is well recognized as the major determinant of infarct size in this model, as the covariate (38). For variables measured repeatedly throughout the protocols (LV developed pressure, coronary flow), data were compared between matched control and postconditioned groups by two-factor ANOVA (for group and time) with repeated measures, and if significant F-values were obtained, post hoc pair-wise comparisons were made using the Newman-Keuls test. Comparisons of cardiodynamics were made using both absolute and relative (normalized to baseline) values; however, as both analyses yielded identical results, all data are for simplicity reported as percent of baseline. For protocol 3, the immunoreactivity of each protein of interest was normalized to nonischemic sham values and compared among the five study groups by ANOVA and subsequent Newman-Keuls post hoc test.

All data are reported as means ± SE, and P values ≤ 0.05 were considered statistically significant.

RESULTS

Protocol 1: Reduction of Infarct Size with Post-C

Cardiodynamics. Baseline values of LV developed pressure and coronary flow averaged 85 mmHg and 75 ml/min, respectively, for the 20 hearts enrolled in protocol 1, with no differences between hearts later assigned to the control and postconditioned groups. As expected, both coronary flow and LV developed pressure were decreased during sustained ischemia (i.e., to 41–49% of their respective baseline values) and showed partial recovery during the 2 h of reperfusion. Although there was a modest trend toward greater postischemic improvement in LV developed pressure after relief of ischemia (P = 0.28), there were no significant differences in either LV function or coronary flow between control and postconditioned groups at any time during the protocol (Table 1).

Risk region and infarct size. AR values were comparable in control and postconditioned groups and averaged 35–36% of total LV weight (Fig. 2). Mean infarct size (AN/AR) in the control cohort was 45 ± 6% of the risk region. In contrast, AN/AR was significantly reduced with post-C to a mean of 28 ± 4% (P < 0.05 vs. controls; Fig. 2). Cardioprotection with post-C was further confirmed by ANCOVA incorporating risk region as the covariate (P < 0.05; data not shown).

Protocol 2: Role of Survival Kinases in Post-C-Induced Cardioprotection

Protocol 2A: PI-3 kinase. Cardiodynamics. As in protocol 1, LV function and coronary flow were comparable at baseline in hearts later assigned to the control and postconditioned LY-treated groups (Table 2). There was a trend toward better preservation of LV developed pressure in the postconditioned group (P = 0.08); however, there were no statistically signif-

Table 1. Cardiodynamics of protocol 1

<table>
<thead>
<tr>
<th></th>
<th>Baseline, %</th>
<th>End Occlusion, %</th>
<th>Reperfusion, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>15 min</td>
</tr>
<tr>
<td>LV developed pressure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control hearts</td>
<td>100</td>
<td>47±6</td>
<td>62±12</td>
</tr>
<tr>
<td>Postconditioned hearts</td>
<td>100</td>
<td>41±5</td>
<td>79±6</td>
</tr>
<tr>
<td>Coronary flow</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control hearts</td>
<td>100</td>
<td>48±4</td>
<td>61±6</td>
</tr>
<tr>
<td>Postconditioned hearts</td>
<td>100</td>
<td>49±4</td>
<td>68±4</td>
</tr>
</tbody>
</table>

Values are means ± SE, expressed as percentage of baseline. LV, left ventricular. *P < 0.01 over time and P = 0.28 (nonsignificant) between groups; †P < 0.01 over time and P = 0.73 (nonsignificant) between groups by two-factor ANOVA with replication.
significant differences in cardiodynamics or coronary flow between the LY-treated cohorts at any time during the protocol.

RISK REGION AND INFARCT SIZE. AR/LV averaged 38 ± 4 and 44 ± 3% in the control and postconditioned LY-treated groups, respectively (Fig. 2).

Table 2. Cardiodynamics of protocol 2A

<table>
<thead>
<tr>
<th></th>
<th>Baseline, %</th>
<th>End Occlusion, %</th>
<th>15 min</th>
<th>1 h</th>
<th>2 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV developed pressure*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control + LY</td>
<td>100</td>
<td>30±5</td>
<td>48±4</td>
<td>44±5</td>
<td>36±6</td>
</tr>
<tr>
<td>Postconditioned + LY</td>
<td>100</td>
<td>32±7</td>
<td>60±5</td>
<td>55±4</td>
<td>46±4</td>
</tr>
<tr>
<td>Coronary flow†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control + LY</td>
<td>100</td>
<td>57±5</td>
<td>72±7</td>
<td>62±6</td>
<td>54±6</td>
</tr>
<tr>
<td>Postconditioned + LY</td>
<td>100</td>
<td>51±2</td>
<td>80±6</td>
<td>59±5</td>
<td>48±5</td>
</tr>
</tbody>
</table>

Values are means ± SE, expressed as percentage of baseline. LY, LY-294002.

Table 2. Cardiodynamics of protocol 2A

AN/AR in the control LY-treated cohort was 51 ± 6%, which is similar to the mean infarct size of 45% observed in control hearts in protocol 1. Moreover, cardioprotection with post-C was maintained despite administration of LY; AN/AR was 29 ± 4%, which is comparable to the value of 28% obtained with post-C in protocol 1 [P < 0.05 and P < 0.01 vs. LY-treated controls by t-test (Fig. 2) and ANCOVA, respectively].

Protocol 2B: ERK1/2. Cardiodynamics. There were no significant differences in cardiodynamics or coronary flow at baseline and no differences in recovery of LV developed pressure and coronary flow after relief of ischemia in control vs. postconditioned PD-treated hearts (Table 3).

RISK REGION AND INFARCT SIZE. AR/LV averaged 46 ± 47% in the control and postconditioned PD-treated groups (Fig. 2).

Mean infarct size in the control PD-treated hearts, 46 ± 8% of the risk region, was consistent with the values of 45 and 51% observed for control groups in protocols 1 and 2A. However, in contrast with the cardioprotective effects of post-C seen in protocols 1 and 2A, the infarct-sparing effect of
post-C was abrogated in hearts that received PD during stuttering reflow; i.e., AN/AR averaged 63 ± 9% (Fig. 2) with no significant difference by t-test or ANCOVA between PD-treated control vs. postconditioned groups.

Protocol 3: Assessment of survival kinases. Total ERK1/2 and total Akt did not differ among the five study groups (data not shown).

There was no increase in the immunoreactivity of phospho-Akt, which is an index of PI3-kinase activity, in control or postconditioned groups vs. sham hearts, or more notably, between control vs. postconditioned cohorts (Fig. 3). Moreover, although there was a reduction in total PTEN in all hearts sampled after relief of ischemia when compared with nonischemic sham hearts (P < 0.05), immunoreactivity of PTEN was comparable among control and postconditioned groups (Fig. 3). In contrast, post-C was associated with a significant 50% increase in the expression of phospho-ERK vs. both sham and control cohorts (Fig. 3).

DISCUSSION

In this study, we demonstrate significant cardioprotection with post-C in the isolated buffer-perfused rabbit heart model of regional ischemia. Second, we report that infarct size reduction with post-C was abrogated by the ERK1/2 inhibitor PD but not by the PI3-kinase antagonist LY. These findings are consistent with our observation of increased immunoreactivity of phospho-ERK1/2 but not phospho-Akt during the initial minutes after relief of ischemia in postconditioned vs. control hearts. Taken together, these data implicate the involvement of ERK1/2 signaling rather than PI3-kinase/Akt in the reduction of infarct size achieved with post-C in isolated rabbit heart.

Reperfusion Injury, Modified Reperfusion, and the Concept of Post-C

Although there is no question that reperfusion is essential to salvage ischemic myocardium, it has been postulated that restoration of flow per se may have potentially deleterious consequences (2, 3, 31). Multiple adverse effects have been attributed to reperfusion including exacerbation of microvascular injury, myocardial stunning, reperfusion arrhythmias, and perhaps most notably, irreversible myocyte death (37). Moreover, it is generally believed that the harmful effects of reperfusion are manifest rapidly (within seconds to minutes) after reflow is initiated (29); as a result, this brief period in early reflow represents a critical window in which cardioprotective strategies aimed at attenuating reperfusion injury need to be implemented.

Although definitive proof of the existence of reperfusion injury has been elusive, arguably the most compelling evidence in support of this phenomenon is the purported ability of physiological or pharmacological modifications, applied at the onset of reperfusion, to decrease cardiomyocyte death (18, 23, 37). In this regard, the concept of post-C, or stuttering reperfusion, has generated considerable recent interest. The term “postconditioning” was first introduced by Vinten-Johansen and Zhao in 2003 (39), and in many respects it is a logical extension of the longstanding concept of gentle or graded reperfusion that has been shown previously to be cardioprotective (19, 24, 30). Specifically, anesthetized open-chest dogs underwent 60 min of left anterior descending artery occlusion, and upon relief of sustained ischemia, received either full and abrupt reflow (controls) or three repeated cycles of 30 s of reperfusion and 30 s of reocclusion before full restoration of flow (postconditioned group). Post-C was profoundly protective, evoking a significant ~45% reduction of infarct size, which is comparable in magnitude to that achieved with the well-described phenomenon of ischemic preconditioning (39).

Table 3. Cardiodynamics of protocol 2B

<table>
<thead>
<tr>
<th></th>
<th>Baseline, %</th>
<th>End</th>
<th>Occlusion, %</th>
<th>15 min</th>
<th>1 h</th>
<th>2 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV developed pressure*</td>
<td>100</td>
<td>34±11</td>
<td>51±5</td>
<td>48±4</td>
<td>37±4</td>
<td></td>
</tr>
<tr>
<td>Control + PD</td>
<td>100</td>
<td>35±5</td>
<td>61±7</td>
<td>51±6</td>
<td>41±5</td>
<td></td>
</tr>
<tr>
<td>Postconditioned + PD</td>
<td>100</td>
<td>54±4</td>
<td>77±9</td>
<td>62±5</td>
<td>51±7</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE, expressed as percentage of baseline. PD, PD-98059. *P < 0.01 over time and $P = 0.54$ (nonsignificant) between groups; †P < 0.05 vs. control 3 by two-factor ANOVA with replication.

Fig. 3. Immunoreactivity in subepicardial samples including representative immunoblots (right). A: phospho-Akt; B: phosphatase and tensin homolog on chromosome 10 (PTEN). C: phospho-ERK1/2. Mean values for sham, control, and postconditioned hearts were normalized to sham values. *P < 0.05 vs. sham; †P < 0.05 vs. control 3; ‡P < 0.05 vs. control 5'.
In the present study, we found an ∼40% reduction of infarct size with post-C. These data compare favorably with the initial results that Zhao et al. (39) and Halkos et al. (10) obtained using the canine model, with subsequent reports of cardioprotection with post-C in in vivo rabbit preparations and isolated buffer-perfused rat hearts (1, 21, 22, 28, 36), and with the 40–50% reduction of infarct size seen in preconditioned rabbit hearts vs. control hearts in concurrent studies conducted by our group. However, despite the emerging consensus that hearts vs. control hearts in concurrent studies conducted by our group. However, despite the emerging consensus that hearts vs. control hearts in concurrent studies conducted by our group.

Potential Mechanisms of Post-C

Comparison with gentle reperfusion. Perhaps the most compelling but as-yet largely unresolved issue regarding post-C is the elucidation of the cellular mechanisms responsible for this phenomenon. Initial studies focused in part on candidates implicated to play a role in lethal reperfusion injury including neutrophil-mediated inflammation and production of oxygen-derived free radicals upon relief of ischemia (13, 39). In this regard, Zhao, Vinten-Johansen, and colleagues have shown evidence of attenuated oxygen radical production with post-C in in vivo dog and rat models (13, 39). The consistent observation of infarct size reduction with post-C in isolated buffer-perfused heart models described previously by our group (6), shown in the present study, and reported by others (12, 21, 26, 35) argues against the concept that neutrophils (or other circulating blood-borne components) are the sole mediators of this phenomenon but does not rule out a role for oxygen radicals generated from non-neutrophil sources (i.e., the coronary vascular endothelium).

Is post-C simply a variation of gentle reperfusion, or are these distinct phenomena? As recently reviewed by Vinten-Johansen et al. (32), there are at least two phenotypic differences between post-C and gentle reperfusion: 1) although infarct size is reduced with both strategies, protection of the coronary vascular endothelium is achieved with stuttering reflow but was not seen with graded reperfusion; and 2) gentle reperfusion was associated with an increase in neutrophil influx, whereas neutrophil accumulation is attenuated with post-C (32). Regarding mechanisms, most studies investigating gentle reperfusion focused on the possible role of hemostatic parameters, neutrophil influx, acidosis, and oxygen-derived free radicals. Although as mentioned previously, neutrophils and oxygen radicals are also under investigation in the setting of post-C, present emphasis has largely shifted to cellular signaling. Thus the degree of redundancy between the two phenomena is unclear.

Role of survival kinases. Our group and others (22, 28, 35, 36) have reasoned that the benefits of post-C may depend critically upon the rapid and preferential upregulation of the classic survival kinases PI3-kinase and/or ERK1/2 (11, 14, 16, 17, 27) at the onset of reperfusion in postconditioned vs. control hearts. Although all studies to date support a role for survival kinases, disparate conclusions have been drawn with regard to the specific kinase(s) involved; i.e., both ERK1/2 (36) and PI3-kinase (22) have been reported in separate protocols from the same laboratory to contribute to infarct size reduction with post-C in the in vivo rabbit model, whereas PI3-kinase has been implicated in isolated buffer-perfused rat (28) and rabbit (35) hearts. In contrast with previous data obtained from isolated heart preparations, our present results favor the involvement of ERK1/2 rather than PI3-kinase in the reduction of infarct size achieved with post-C in isolated buffer-perfused rabbit hearts subjected to regional ischemia-reperfusion.

Comparison with Data Obtained in Rats. It could be argued that the robust evidence for the role of PI3-kinase obtained by Tsang et al. (28) in isolated rat hearts vs. our data implicating the involvement of ERK1/2 in the rabbit model may simply reflect differences among species. There are, however, noteworthy differences between the two protocols. First, despite the integrated experimental design (use of pharmacological PI3-kinase inhibitors together with immunoblotting), it must be emphasized that Tsang et al. confined their analysis to PI3-kinase and did not quantify phospho-ERK1/2 immunoreactivity or assess the effects of pharmacological ERK1/2 inhibition. Thus the possibility cannot be excluded that both PI3-kinase and ERK1/2 signaling may play roles in post-C-induced cardioprotection in rat heart. Second, our myocardial samples obtained for the assessment of phospho-Akt and phospho-ERK were from viable, previously ischemic subepicardium, whereas Tsang et al. appear to have sampled the full transmural thickness of the myocardium at risk (28); i.e., presumably, a heterogeneous mixture of necrotic and viable myocardium with in all likelihood a greater proportion of viable tissue present in samples harvested from postconditioned vs. control hearts.

To investigate the possible importance of subepicardial vs. transmural sampling, we conducted an ancillary analysis to assess the effects of sampling site on phospho-Akt and phospho-ERK immunoreactivity in our model. For six of the hearts enrolled in protocol 3 (control 3’ group; n = 3 and post-C 5’ group; n = 3), we obtained samples from the viable subepicardium (as per our study design; data utilized in protocol 3), samples from the subendocardial half of the LV wall (the region containing the infarct), as well as transmural samples.
We found that for phospho-Akt, the sampling site was indeed an important determinant of outcome; although we observed no difference between control and postconditioned groups in phospho-Akt in the viable subepicardium, phospho-Akt was increased in transmural samples harvested from postconditioned hearts vs. transmural samples obtained from control hearts (Fig. 4). This latter observation is consistent with data reported in the rat model (28) and may, as hypothesized, be explained by the presumably greater proportion of necrotic tissue in transmural samples obtained from control vs. postconditioned hearts. In contrast, we found that immunoreactivity of phospho-ERK1/2 was increased, regardless of the site of sampling, in postconditioned vs. control hearts (Fig. 4).

Additional detailed analyses will be required to understand the specific features of the undoubtedly complex temporal relationship between the development of myocardial necrosis and the loss of phospho-Akt and phospho-ERK immunoreactivity, and, specifically, to determine the time after cardiomyocyte death at which the immunoreactivity of each of these kinases wanes. Nonetheless, the present results show that conclusions regarding kinase activity can be influenced by the use of subepicardial vs. transmural samples, and in the viable subepicardium, expression of phospho-ERK but not phospho-Akt was increased in postconditioned rabbit hearts vs. control hearts.

COMPARISON WITH DATA OBTAINED IN RABBITS. Differences among studies using rabbit hearts are more difficult to reconcile (22, 35). However, no previous reports using the rabbit model, including the in vivo study implicating the involvement of ERK1/2, interrogated the role(s) of both PI3-kinase and ERK1/2 in the same cohorts of animals. In addition, all studies in rabbits have relied exclusively on the use of pharmacological inhibition (22, 35, 36). Finally, both previous protocols focusing on the role of PI3-kinase in rabbit heart utilized wortmannin rather than LY as the antagonist (22, 35), which thereby raises the possibility that irreversible and thus sustained inhibition of PI3-kinase (rather than the reversible inhibition achieved by the LY compound) or inhibition of other kinases such as PI4-kinase and myosin light chain kinase by wortmannin (22, 35) may have influenced the outcome.

To address the concept that our use of LY rather than wortmannin may have influenced our conclusions, we performed supplemental post hoc experiments in which control and postconditioned rabbit hearts, prepared as described in protocols 1 and 2, were treated with wortmannin at a concentration of 100 nM, beginning 5 min before the onset of reperfusion and maintained for 20 min [i.e., dose and timing of treatment identical to that reported by Yang et al. (35) to block infarct size reduction with post-C in the isolated buffer-perfused rabbit heart model of regional ischemia]. We observed persistent cardioprotection with post-C despite administration of 100 nM wortmannin; infarct sizes averaged 32 ± 9% vs. 63 ± 7% of the risk region in wortmannin-treated postconditioned hearts (n = 5) vs. wortmannin-treated control hearts (n = 4; P < 0.05). These data corroborate our finding of continued reduction of infarct size with post-C in LY-treated hearts but are at variance with the results of Yang and colleagues (35).

We cannot definitively reconcile these seemingly discrepant outcomes; however, two factors appear noteworthy. First, the remaining major difference in our protocol vs. the study of Yang et al. (35) is the post-C algorithm that was employed, namely, four cycles of 30-s reperfusion plus 30-s regional reocclusion in our study vs. six cycles of 10-s reperfusion plus 10-s global ischemia used by Yang and colleagues (35). Second, in the study by Yang et al. (35), recovery of coronary flow during the post-C period was apparently compromised in wortmannin-treated hearts. The authors report that in the absence of inhibitor treatment, coronary flow increased from 4.2 to 10.2 ml·min⁻¹·g of tissue⁻¹ immediately after the six cycles of post-C, whereas in wortmannin-treated hearts, coronary flow was 4.0 at the end of occlusion.
and 3.0 ml·min⁻¹·g of tissue⁻¹ immediately after the post-C regimen (35).

Although logic would dictate that by definition, intermittent reperfusion must be established to initiate post-C, no data are presently available regarding the magnitude of reflow that is required. This raises the intriguing possibility that in the study of Yang et al. (35), wortmannin did not block protection by inhibiting PI3-kinase/Akt; rather, protection failed because of reduced reperfusion flow rates and hence an inadequate post-C stimulus. That is, in the setting of wortmannin treatment, the speed and/or magnitude of reflow may have been sufficiently blunted during the short, 10-s intervals of intermittent reperfusion such that the post-C algorithm was rendered suboptimal, whereas with the 30-s intervals of intermittent reflow employed in our protocols, the as-yet undefined threshold in the magnitude and/or duration of reflow was maintained, and persistent cardioprotection with post-C was seen despite administration of wortmannin. Additional comprehensive experiments, preferably in large animal models in which coronary artery blood flow can be continuously monitored and precisely controlled by application of micromanometer occluders, are required to support or refute this concept.

In summary, we report significant cardioprotection with post-C in isolated buffer-perfused rabbit heart. Although our results support a pivotal role for ERK1/2 but not PI3-kinase/Akt signaling, in the reduction of infarct size obtained with stuttering reperfusion, both the optimal post-C stimulus as well as precise details concerning the cellular mechanisms of this intriguing phenomenon remain to be elucidated. Second, with the exception of one published abstract that shows persistent post-C-induced cardioprotection in a rabbit model of hypercholesterolemia (7), the potentially crucial question of whether the efficacy of post-C is maintained in the presence of clinically relevant comorbidities (i.e., hypertension, diabetes, etc.) has to date not been explored. Finally, additional studies are required to establish the safety, efficacy, and potential clinical relevance of post-C as a therapeutic strategy in the setting of reperfusion for acute myocardial infarction.

GRANTS

This work is supported in part by The Worcester Foundation for Biomedical Research and National Heart, Lung, and Blood Institute Grant R01 HL-63713 (to K. Przyklenk).

REFERENCES


35. Yang XM, Downey JM, and Cohen MV. Post-conditioning’s protection is not dependent on circulating blood factors or cells but requires PI3-kinase and guanylyl cyclase activation. *Basic Res Cardiol* 100: 57–63, 2005.


