Postconditioning: A Form of “Modified Reperfusion” Protects the Myocardium by Activating the Phosphatidylinositol 3-Kinase–Akt Pathway

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Brief intermittent episodes of ischemia and reperfusion, at the onset of reperfusion after a prolonged period of ischemia, confer cardioprotection, a phenomenon termed “ischemic postconditioning” (Postcond). We hypothesized that this phenomenon may just represent a modified form of reperfusion that activates the reperfusion injury salvage kinase (RISK) pathway. Isolated perfused rat hearts were subjected to: (a) 35 minutes of ischemia and 120 minutes of reperfusion, and infarct size was determined by tetrazolium staining; or (b) 35 minutes of ischemia and 7 minutes of reperfusion, and the phosphorylation states of Akt, endothelial NO synthase (eNOS), and p70S6K were determined. Postcond reduced infarct size from 51.2 ± 3.4% to 31.5 ± 4.1% (P < 0.01), an effect comparable with ischemic preconditioning (IPC; 27.5 ± 2.3%; P < 0.01). Of interest, the combined protective effects of IPC and Postcond were not additive (30.1 ± 4.8% with IPC + Postcond; P = NS). Inhibiting phosphatidylinositol 3-kinase (PI3K) at reperfusion using LY (n = 7) or Wortmannin (Wort) during the first 15 minutes of reperfusion completely abolished Postcond-induced protection (31.5 ± 4.1% with Postcond versus 51.7 ± 4.5% with Postcond + LY, P < 0.01; 56.2 ± 10.1% with Postcond + Wort; P < 0.01), suggesting that Postcond protects the heart by activating PI3K–Akt. Western blot analysis demonstrated that Postcond induced a significant increase in phosphorylation of Akt, eNOS, and p70S6K in an LY- and Wort-sensitive manner. In conclusion, we show for the first time that ischemic Postcond protects the myocardium by activating the prosurvival kinases PI3K–Akt, eNOS, and p70S6K in accordance with the RISK pathway.

The protection afforded by ischemic preconditioning (IPC), in which short periods of ischemia protect the myocardium against a subsequent lethal ischemic insult, can only be used if the IPC mimetic is applied before the index ischemic episode.1 The index ischemic episode is often unpredictable clinically, therefore, the ability to protect the heart by intervening at the time of reperfusion provides an approach that is more suited to the clinical scenario. The phenomenon of ischemic postconditioning (Postcond), first described by Vinten-Johansen’s group,2 in which brief intermittent repetitive interruptions to reperfusion at the onset of reperfusion after a prolonged period of ischemia reduced myocardial injury to an extent comparable to IPC, offers a novel approach to myocardial protection. Suggested mechanisms of protection include a reduction in neutrophil accumulation and decreased endothelial dysfunction, attenuation of oxidative stress, a reduction in apoptotic cell death, and attenuation of mitochondrial calcium accumulation.

Activation of the prosurvival kinase phosphatidylinositol 3-kinase (PI3K)–Akt and the mitogen-activated protein kinase p44/p42 extracellular signal-regulated kinase (ERK) 1/2 at the time of reperfusion together comprise the cardioprotective reperfusion injury salvage kinase (RISK) pathway.3 We hypothesize that ischemic Postcond protects the heart by activating the RISK pathway, specifically the PI3K–Akt pathway, in the first few minutes of reperfusion.

Methods

Male Sprague-Dawley rats (270 to 450 g; n = 103) were obtained from Charles River UK Limited (Margate, UK), and received humane care in accordance with the Home Office Guidance on the Operation of Animals (Scientific Procedures) Act of 1986 (Her Majesty’s Stationery Office, London, UK).

Isolated Perfused Rat Heart

Hearts were excised rapidly and mounted on a Langendorff perfusion system. All hearts were subjected to 35 minutes of regional ischemia and 120 minutes of reperfusion as described previously.4 Hearts were assigned randomly to 1 of the following groups: (1) control (n = 11) or control with 0.02% dimethyl sulfoxide (DMSO) vehicle (n = 4) for the first 15 minutes of reperfusion; (2) IPC (n = 11), comprising 2 cycles of 5 minutes of global ischemia followed by 10 minutes of reperfusion before the index ischemia; (3) Postcond (n = 10), comprising 6 cycles of 10 seconds of reperfusion followed by 10 seconds of global ischemia immediately after the index ischemia; (4) IPC + Postcond (n = 7); (5) Postcond + LY294002 (LY; n = 13); hearts with the PI3K inhibitor LY (15 μmol/L),7 given for the first 15 minutes of reperfusion; (6) Control + LY (n = 5), hearts with LY given for the first 15 minutes of reperfusion; (7) Postcond with the PI3K inhibitor Wortmannin (Wort; 100 μmol/L; n = 7),7 given for the first 15 minutes of reperfusion; and (8) Control + Wort (n = 5), hearts with Wort given for the first 15 minutes of reperfusion.

Western Blot Analysis

Hearts were subjected to 35 minutes of regional ischemia followed by 7 minutes of reperfusion. Hearts (n = 5 per group) were assigned randomly to treatment groups 1, 3, and 5 to 8, as described previously. At the end of reperfusion, the ventricular tissue at risk was excised and freeze-clamped in liquid nitrogen before being stored at −80°C. Phosphorylation states of Akt (phospho-Akt, serine [Ser] 473), endothelial NO synthase (eNOS; phospho-eNOS, Ser 1177), p70s6 kinase (phospho-p70s6k, Thr 389), and total levels of Akt, eNOS, and p70S6K proteins were analyzed by SDS-PAGE.

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Inhibiting PI3K at Time of Reperfusion Abrogates Protection Induced by Ischemic Postcond

Infarct size, represented as a percentage of the area at risk, was significantly reduced in the IPC group (control 51.2 ± 3.4% versus IPC 27.5 ± 2.3%; \( P < 0.01 \); Figure 2). Postcond similarly reduced infarct size (control 51.2 ± 3.4% versus Postcond 31.5 ± 4.1%; \( P < 0.01 \)). The protection induced by the combination of both IPC and Postcond did not differ compared with either alone (IPC + Postcond 30.1 ± 4.8% versus 27.5 ± 2.3% with IPC and 31.5 ± 4.1% with Postcond; \( P = \text{NS} \)). However, the infarct reduction afforded by Postcond was abolished completely in the presence of the PI3K inhibitor LY or Wort given during the first 15 minutes of reperfusion (31.5 ± 4.1% with Postcond versus 51.7 ± 4.5% with Postcond + LY, \( P < 0.01 \); 56.2 ± 10.1% with Postcond + Wort, \( P < 0.01 \); Figure 2); 0.02% DMSO did not influence infarct size in control or Postcond groups (control + DMSO 49.6 ± 9.2%; Postcond + DMSO 33.2 ± 4%). LY or Wort did not influence infarct size in control groups (control + LY 56.8 ± 10.5%; control + Wort 52.3 ± 13.3%).

Discussion

We demonstrate for the first time in the isolated perfused rat heart that ischemic Postcond protects the heart against ischemia reperfusion injury by activating the prosurvival kinase PI3-Akt and its downstream targets eNOS and p70S6K. These data suggest that Postcond may protect the heart by recruiting the RISK pathway in the same way as insulin, bradykinin, atorvastatin, and urocortin when given during the first few minutes of reperfusion.\(^5\) Therefore, these agents may be considered to protect by “pharmacological Postcond.”

Interestingly, the combined protective effects of IPC and Postcond do not appear to be additive, which is in disagreement with the findings of Downey’s group that combined IPC and Postcond in the in vivo rabbit heart induced additive protection.\(^8\) This discrepancy may be attributable to the different models of ischemia reperfusion injury used.

Ischemic Postcond, as originally described in the in vivo dog model,\(^2\) has been observed in the in vivo rabbit\(^7\) and rat heart\(^8\) and in rat cardiomyocytes.\(^4\) The first study investigating this phenomenon implicated a reduction in neutrophil accumulation and an improvement in endothelial function as

**Results**

**Ischemic Postcond Activates Akt, eNOS, and p70S6K at Time of Reperfusion**

Ischemic Postcond induced Akt phosphorylation (in arbitrary units; from 36.6 ± 6.8 in vehicle control to 83.0 ± 3.3 with Postcond; \( P < 0.01 \), eNOS (from 8.9 ± 2.1 in vehicle control to 40.2 ± 12.5 with Postcond; \( P < 0.01 \)), and p70S6K (from 11.0 ± 3.6 in vehicle control to 79.6 ± 7.7 with Postcond; \( P < 0.01 \)). When the PI3K inhibitor LY or Wort was given for the first 15 minutes of reperfusion, the Postcond-induced phosphorylation was abolished: Akt (15.3 ± 3.5), eNOS (13 ± 7.6) and p70s6K (3.8 ± 2.1) with LY, and Akt (38.2 ± 3.8), eNOS (14 ± 5.4), and p70s6K (46.1 ± 7) with Wort (Figure 1a through 1c).

**Figure 1.** A through C, Representative Western blots demonstrating phosphorylated and total protein levels. Densities (in arbitrary units [AU]) show that inhibiting PI3K using LY or Wort abolishes phosphorylation of Akt (A), eNOS (B), and p70S6K (C) induced by ischemic Postcond (n=5/group; \( ^*P < 0.01 \)). Cont indicates control; Post, Postcond.

Immunoelectrophoresis using antibodies obtained from New England Biolabs as described previously.\(^7\) Levels of phosphorylated proteins were normalized to their total protein levels. Relative densitometry was determined using a computerized software package (NIH Image 1.63).\(^7\)

**Statistical Analysis**

All values are expressed as mean ± SEM. Infarct size and Western blot results were analyzed by 1-way ANOVA and Fisher protected test of least significant difference. Differences were considered statistically significant when \( P < 0.05 \).

**Figure 2.** Inhibiting PI3K using LY or Wort for the first 15 minutes of reperfusion abrogates the reduction in infarct-risk volume ratio induced by ischemic Postcond (\(^*P < 0.01 \)). Cont indicates control; Post, Postcond.
possible mechanisms of protection. Subsequent studies, including the study we are reporting here, have demonstrated that protection can occur in the absence of blood constituents, suggesting that Postcond may exert a direct effect on the myocyte.

In this regard, Vinten-Johansen’s group demonstrated using the in vivo rat heart that Postcond attenuated the production of reactive oxygen species (ROS) immediately at reperfusion and that ischemic Postcond protection was lost if instituted after 1 minute of reperfusion. The same group demonstrated that “hypoxic Postcond” of neonatal rat myocytes resulted in less necrotic cell death, attenuated ROS production, and reduced mitochondrial calcium loading.

Recent data have also demonstrated that the presence of either the mitogen-activated protein kinase kinase (MEK)1/2 inhibitor PD98059 or the NOS inhibitor L-NAME during Postcond in in vivo rabbit hearts can abrogate protection, suggesting that the MEK1/2–ERK1/2 cascades and NOS may be required for protection. However, in this study, it was not demonstrated that Postcond actually activated either ERK1/2 or NOS directly.

Opening of the mitochondrial permeability transition pore (mPTP) during the first few minutes of reperfusion has been demonstrated to mediate cell death, and inhibiting its opening is cardioprotective. We postulate that Postcond protects the heart by inhibiting mPTP opening through activation of Akt and eNOS (Figure 3).

The term ischemic Postcond is probably a misnomer because the word “conditioning” implies that the process prepares the myocardium for the ischemic event, as perceived in IPC. Ischemic Postcond may simply constitute a variation of controlled reperfusion, which previous studies have demonstrated to be cardioprotective. Therefore, ischemic Postcond may be considered a “passive process” that modifies reperfusion injury rendering the cell and mitochondria more resistant to the biochemical and metabolic perturbation that occurs in the transition from ischemia to reperfusion (Figure 3). We show that ischemic Postcond may also be an “active process” by activating prosurvival kinases such as the PI3K–Akt pathway (Figure 3) in accordance with the RISK pathway.

In conclusion, our study demonstrates that ischemic Postcond significantly reduces infarct size in isolated perfused rat hearts and that the effects of IPC and Postcond combined are not additive. In addition, we show for the first time that protection is mediated via the PI3K–Akt prosurvival signaling cascade and its downstream targets, namely, eNOS and p70s6K. Interventions such as ischemic Postcond, which target the first few minutes of reperfusion, may offer greater opportunity for protection, clinically such as at the time of thrombolysis, angioplasty, and cardiac surgery.

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References

**Key Words:** ischemia • cardioprotection • reperfusion • postconditioning • kinases
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