Published in final edited form as:

Basic Res Cardiol. 2008 September ; 103(5): 464–471. doi:10.1007/s00395-008-0737-9.

Acidosis, Oxygen, and Interference with Mitochondrial Permeability Transition Pore Formation in the Early Minutes of Reperfusion Are Critical to Postconditioning's Success

Michael V. Cohen^{1,2}, Xi-Ming Yang¹, and James M. Downey¹

¹Department of Physiology, University of South Alabama College of Medicine, Mobile, AL

²Department of Medicine, University of South Alabama College of Medicine, Mobile, AL

Abstract

Repetitive cycles of reflow/reocclusion in the initial 2 min following release of a prolonged coronary occlusion, i.e., ischemic postconditioning (IPoC), salvages ischemic myocardium. We have proposed that the intermittent ischemia prevents formation of mitochondrial permeability transition pores (MPTP) by maintaining an acidic myocardial pH for several minutes until survival kinases can be activated. To determine other requisites of IPoC, isolated rabbit hearts were subjected to 30 min of regional myocardial ischemia and 120 min of reperfusion. Infarct size was determined by staining with triphenyltetrazolium chloride. During the first 2 min of reperfusion the perfusate was either at pH7.4 following equilibration with 95% O₂/5% CO₂, pH6.9 following equilibration with 80% N₂/20% CO₂, or pH7.8 following equilibration with 100% O₂. Whereas acidic, oxygenated perfusate for the first 2 min of reperfusion was cardioprotective, protection was lost when acidic perfusate was hypoxic. However, the acidic, hypoxic hearts could be rescued by addition of phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator, to the perfusate. Therefore, both low pH and restoration of oxygenation are necessary for protection, and the signaling step requiring combined oxygen and H⁺ must be upstream of PKC. To gain further insight into the mechanism of IPoC, the latter was effected with 6 cycles of 10-sec reperfusion/10-sec reocclusion. Its protective effect was abrogated by either making the oxygenated perfusate alkaline during the reperfusion phases or making the reperfusion buffer hypoxic. Presumably the repeated coronary occlusions during IPoC keep myocardial pH low while the resupply of oxygen during the intermittent reperfusion provides fuel for the redox signaling that acts to prevent MPTP formation even after restoration of normal myocardial pH. Hearts treated simultaneously with IPoC and alkaline perfusate could not be rescued by addition to the perfusate of either PMA or SB216763 which inhibits GSK-3 β , the putative last cytoplasmic signaling step in the signal transduction cascade leading to MPTP inhibition. Yet cyclosporin A which also inhibits MPTP formation does rescue hearts made alkaline during IPoC. In view of prior studies in which the ROS scavenger N-2-mercaptopyrionyl glycine aborts IPoC's protection, our data reveal that IPoC's reperfusion periods are needed to support redox signaling rather than improve metabolism. The low pH, on the other hand, is equally necessary and seems to suppress MPTP directly rather than through upstream signaling.

Introduction

Ischemic preconditioning (IPC) [11] and postconditioning (IPoC) [23,24] have now repeatedly been demonstrated to salvage ischemic myocardium, although only the latter would appear to

have potential clinical appeal. Much of the intracellular signaling pathway for IPC has been identified [18] and appears to be in 2 parts: a pre-ischemic trigger phase and a mediator phase at reperfusion. Obviously in IPoC all of the protective signaling takes place only during the reperfusion phase. Although various signaling elements in IPoC have been identified such as adenosine A_{2b} receptors [15], protein kinase C (PKC) [14,15], mitochondrial K_{ATP} channels [14,23], extracellular signal-regulated kinase (ERK) [23], Akt [20,22], reactive oxygen species (ROS) [14], nitric oxide synthase [13,23], guanylyl cyclase [13,22], cGMP [13], and STAT3 [2], our understanding of the sequence of steps is more rudimentary than for IPC. Importantly involvement of the mitochondrial permeability transition pore (MPTP) has been implicated [1], and there is growing support for the suggestion that MPTP may be the end-effector of both IPC and IPoC. We have noted that acidosis must be maintained in the first 2 min of reperfusion if simulated IPoC is to be protective [3]. We postulated that the repeated coronary artery reocclusions of IPoC perpetuate acidosis which keeps MPTP from forming. We also proposed that the delivery of oxygen during the brief reperfusion promotes ROS formation which activates survival kinases through redox signaling. Those kinases keep MPTP closed after pH is allowed to normalize. Because of the potential usefulness of a postconditioning strategy in the treatment of patients presenting with acute myocardial infarction [17], we have further explored some of the required characteristics of IPoC. Because IPoC can currently be applied only in patients undergoing primary angioplasty, the many patients undergoing thrombolysis to restore flow to the ischemic myocardium can not benefit from this cardioprotective intervention. A better understanding of the requirements of IPoC might therefore provide insights into IPoC's exact mechanism.

Methods

The present study was performed in accordance with the Guide for the Care and Use of Laboratory Animals [12] and approved by the Institutional Animal Care and Use Committee.

Isolated Rabbit Heart Model

New Zealand white rabbits were anesthetized with sodium pentobarbital, intubated, and ventilated with 100% oxygen. A branch of the left coronary artery was surrounded by a balloon occluder. Excised hearts were mounted on a Langendorff apparatus and perfused with modified Krebs-Henseleit bicarbonate buffer that contained (in mM) 118.5 NaCl, 25 NaHCO₃, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 2.5 CaCl₂, and 10.0 glucose. The buffer was equilibrated with 95% O₂/5% CO₂ to create perfusate pH 7.4.

To create acidotic buffer (pH 6.9) the buffer was equilibrated with a 20% CO₂ gas mixture. To make the buffer alkaline (pH 7.8) it was equilibrated with 100% oxygen. Because alkalotic pH precipitated a Ca⁺⁺ salt in the buffer, the CaCl₂ concentration was lowered to 1.5mM. This low Ca⁺⁺ buffer had no independent effect on infarct size [3].

Control hearts underwent regional coronary occlusion by inflation of the coronary balloon occluder for 30 min and reperfusion for 2 h (Fig. 1). A second control group also underwent 30 min of regional occlusion, but as the balloon on the occluder was deflated retrograde perfusion of the aorta was stopped for 2 min thus creating global ischemia. The perfusion territory of the instrumented coronary artery was, therefore, ischemic for 32 min. Thereafter normal perfusion of the heart occurred for 118 min. In group 3 after 30 min of regional ischemia the heart was reperfused with hypoxic, acidic perfusate for the initial 2 min of the reperfusion period. As described above the perfusate had been equilibrated with 20% CO₂ gas mixture to make the perfusate acidic. The other gas was 80% N₂ to make the perfusate hypoxic. The pO₂ of the perfusate was reduced from 600 mmHg in the standard perfusate equilibrated with 95% O₂/5% CO₂ to 25 mmHg. After 2 min of reperfusion the perfusate was switched to standard buffer equilibrated with 95% O₂/5% CO₂. In a fourth group the heart was also perfused

for the first 2 min of the reperfusion period with hypoxic and acidic buffer as in group 3. Additionally 0.05nM phorbol 12-myristate 13-acetate (PMA) was added to the perfusate from 1 min before to 5 min after the onset of reperfusion. In group 5 IPoC was included so that after the 30-min regional ischemia 6 cycles of 10-sec reperfusion/10-sec reocclusion were initiated. At the same time these hearts were made hypoxic as above. In groups 6 and 7 the effect of infusions of 1 μ M SB216763 starting 1 min before reperfusion and continuing for either 6 or 16 min was evaluated, resp. In the next 2 groups IPoC was initiated immediately after reperfusion and alkalotic perfusate equilibrated with 100% O₂ was substituted for the standard perfusate from 1 min before to 3 min after reperfusion. Additionally either 0.05 nM PMA was added to the perfusate from 1 min before to 5 min after reperfusion or 1 μ M SB216763 was added from 1 min before to 15 min after the onset of reperfusion. Finally in the tenth group hearts were postconditioned and made alkalotic as above. In this group cyclosporin A (CsA) was infused only during the reperfusion phases of the postconditioning cycles of reperfusion/ischemia. This group has already been reported [3], but is included for comparison with the other groups.

At the beginning of each experiment pH, pCO₂, and pO₂ were measured in the buffer equilibrated with the various gas mixtures with an ABL-5 blood gas analyzer (Radiometer, Copenhagen, Denmark).

Infarct size measurement

After 2 h of reperfusion the coronary artery was reoccluded and 2-9 μ m diameter fluorescent microspheres (Microgenics Corp., Freemont, CA) were injected into the perfusate. The risk zone was nonfluorescent. Hearts were weighed, frozen, and sliced transversely into 2-mm thick pieces. Slices were incubated for 8 min at 37°C in buffered 1% triphenyltetrazolium chloride which stains noninfarcted myocardium brick red and then fixed in 10% formalin. Infarct and risk zone regions were traced on overlying clear acetate sheets and areas determined by planimetry. Volumes were calculated by multiplying areas by slice thickness and summing them for each heart. Infarct size is expressed as a percentage of risk zone.

Chemicals

PMA was obtained from Sigma Aldrich Chemical Co. (St. Louis, MO), while SB216763 and CsA were purchased from Tocris Cookson, Inc. (Ellisville, MO).

Statistics

Data are presented as mean $\pm$ SEM. One-way ANOVA with Student-Newman-Keuls post hoc test was used to evaluate differences in baseline hemodynamics and infarct size between groups. ANOVA for repeated measures with the Tukey post hoc test examined temporal differences in hemodynamics in any given group. For infarct size-risk zone volume plots the significance of differences between regression lines was tested by ANCOVA. The difference was significant if p was <0.05.

Results

Hemodynamics

There were no differences in hemodynamics at baseline among the groups (Table 1). Developed left ventricular pressure and coronary flow fell predictably during coronary occlusion with partial rebound during reperfusion. None of the interventions appeared to have any significant effect on hemodynamics.

Infarct Size

Infarct size data are presented in Table 2. Importantly, average risk zone sizes ranged from 1.06 to 1.48 cm³ with no significant differences among them. Infarct size in control hearts averaged $34.4 \pm 2.2\%$ of the risk zone, and was unchanged when 2 min of global ischemia immediately followed the 30-min period of regional ischemia (Fig. 2). We previously demonstrated that reperfusion with an acidic pH for 2 min limited infarct size by an amount similar to that seen with IPoC ($15.0 \pm 2.6\%$ infarction) [2] and we include that historical data here for comparison. To determine whether restoration of oxygen was also required for the acidotic perfusate to trigger protection in early reperfusion, we combined myocardial-sparing acidosis with hypoxia. This combination aborted the protection ($35.7 \pm 3.0\%$ infarction). These experiments demonstrated that protection from simulated IPoC with an acidic perfusate was also dependent on the presence of oxygen. We extended these observations to IPoC itself. The protection from IPoC is also dependent on the presence of oxygen during the reperfusion cycles since making the standard buffer hypoxic during IPoC also aborted its protection ($32.8 \pm 1.4\%$ infarction) implying that both low pH and reoxygenation were needed to limit infarction. However, in hearts made both acidic and hypoxic in the early minutes of reperfusion, and, therefore, destined to have large infarct, salvage of ischemic myocardium was still possible if PKC were activated by simultaneous exposure of the hearts to PMA. PMA lowered infarct size to $12.2 \pm 2.2\%$.

We next focused on GSK-3 β which has been proposed to reside near the very end of the protective signaling cascade. We wanted to see how short the infusion could be and still protect. While a 6-min infusion of SB216763 was not protective, a 16-min infusion was ($20.0 \pm 1.8\%$ infarction) (Fig. 3).

In a previous study we reported that alkalotic perfusate during IPoC abrogates IPoC's protection ($34.8 \pm 2.5\%$ infarction) (Fig. 4) [3]. We reasoned that any acidosis produced during the occlusion cycles must have been neutralized by the alkaline perfusate thus promoting MPTP formation. We were curious if any of the previously identified protective interventions could restore protection to postconditioned hearts reperfused with alkaline buffer. Therefore, we tried to salvage hearts postconditioned with alkalotic buffer by simultaneously infusing PMA. As shown in Fig. 4 PMA was unable to salvage these hearts. Next we tried a 16-min infusion of SB216763 but it also failed to salvage hearts made alkalotic during IPoC (Fig. 4). By comparison cyclosporin A (CsA) (0.75 μ M) added to postconditioned hearts receiving alkalotic perfusate during the reperfusion phases of the reperfusion/ischemia cycles did limit infarction ($15.1 \pm 1.9\%$ infarction) [3] (reproduced in Fig. 4) suggesting that direct inhibition of MPTP with CsA is more potent than attempts to prevent MPTP formation by initiating previously identified protective cell signaling.

Unfortunately the absolute size of the risk zone is an independent determinant of the percentage of the risk zone that infarcts in the rabbit heart. Thus rabbits with small risk zones will give the appearance of protection when none exists. This potential artifact can be eliminated by plotting infarct size against risk zone size. Although there were no significant differences in average risk zone volume between groups (Table 2), the range was still wide. The 16-min infusion of SB216763 significantly shifted the relationship down and to the right as did acidosis alone and CsA (Fig. 5A). However, the regression lines for the treatment of alkalotic postconditioned hearts with either PMA or SB216763 were not different than the control line (data not shown). Additionally regression lines for hearts treated with hypoxic perfusate and either IPoC or acidic buffer were similar to the control line (Fig. 5B).

Discussion

Coronary occlusion kills myocardial tissue. Although reperfusion is required to salvage ischemic tissue, reperfusion injury contributes to some of the myocardial infarction. IPoC effectively targets the reperfusion component of cell killing. But IPoC is not applicable to coronary revascularization in settings other than the catheterization suite thus encouraging investigation of its mechanism with the hope of being able to apply the strategy in those being reperfused with thrombolytic agents. The current investigation has enabled us to make at least 3 important observations. Firstly IPoC's salvage of ischemic myocardium following release of a coronary occlusion is critically dependent on both maintenance of myocardial acidosis during the initial 2 min of reperfusion and the simultaneous resupply of oxygen. Furthermore, the effects of reoxygenation on signaling occur upstream of PKC. Secondly salvage of myocardium when an ischemic heart is reperfused with an alkaline perfusate cannot be achieved by triggering the cardioprotective signaling cascade at either the level of protein kinase C or GSK-3 β , the latter being the most downstream cytoplasmic signaling element so far identified, but rescue is still possible with CsA suggesting that CsA is the more potent inhibitor of MPTP. Finally GSK-3 β suppression at reperfusion when the ischemic heart is reperfused with pH 7.4 buffer is effective in reducing myocardial infarction, but GSK-3 β antagonism must persist for somewhere between 5 and 15 min of the initial reperfusion period.

After Zhao *et al.* [24] published their seminal observation that IPoC would diminish the size of myocardial infarcts in dogs, initial skepticism that several brief coronary occlusions after a longer occlusion would somehow have a salutary effect faded as investigators confirmed the cardioprotective ability of IPoC in other species [7,8,20,23]. However, how alternate reperfusion and reocclusion could protect the myocardium remained a mystery even after investigators provided evidence that IPoC like IPC was also dependent on familiar signaling elements [13-15,20,22,23]. We later found that cardiac perfusion with oxygenated acidotic buffer for only the first 2 min of reperfusion was just as protective as an IPoC protocol [3]. We postulated that the acidic environment blocked MPTP formation long enough so that signaling could be triggered leading to endogenous attenuation of MPTP formation which would continue to block MPTP formation even after correction of myocardial pH. This hypothesis provided an explanation of the need for the reocclusion phases of the IPoC cycles of reperfusion/reocclusion. What was needed during reperfusion? The present observations suggest that O₂ is as important during the first 2 min of reperfusion as acidosis. Whereas oxygenated acidic perfusate reduced infarction, hypoxic acidic perfusate did not. And finally IPoC's cardioprotection was aborted when the standard neutral buffer perfusing the previously ischemic as well as normal myocardium during the reperfusion phases of the postconditioning cycles was hypoxic.

Clearly oxygen is the critical ingredient, but why is it important? During the index ischemia all of the components of the preconditioning mechanism would have been activated up to the step where redox signaling activates PKC. At reperfusion we postulated that reintroduction of oxygen led to ROS production which then activated PKC which in turn increased the sensitivity of adenosine A_{2b} receptors to initiate the signaling cascade [18]. Penna and colleagues [14] have shown that the ROS scavenger N-2-mercaptopropionyl glycine can abort IPoC's protection. And we have demonstrated that the same ROS scavenger will block the protection of acidotic perfusate during the first 2 min of reperfusion [3]. Therefore we suggest that the coronary reocclusions of IPoC inhibit MPTP by maintaining an acidic milieu while the reperfusion periods reintroduce oxygen, a necessary ingredient to generate the signaling ROS.

In a recent study of preconditioning we found that when a free radical scavenger was present only during preconditioning's ischemic cycle, protection was unaffected. However, if the scavenger were present during the reperfusion cycle protection was completely blocked [4].

Ischemic preconditioning with perfusion of the heart with hypoxic buffer during the preconditioning ischemia/reperfusion cycle also blocked preconditioning's protection and actually increased infarct size. Thus protective redox signaling clearly occurs only during periods when oxygen is plentiful. The same explanation likely pertains to the present study. Furthermore, as predicted by our hypothesized signaling paradigm, activation of PKC by PMA rescued hypoxic and acidic hearts which were unable to produce ROS. Thus PKC activation is downstream of the oxygen-dependent signaling step. Although not addressed in this study, we hypothesize that the ROS generated by reintroduction of O₂ directly activate PKC [10].

All of the steps of the cardioprotective signaling cascade at reperfusion have not yet been fully identified, but both PKC and GSK-3 β have been implicated [18]. In a comprehensive investigation Juhaszova *et al.* [9] demonstrated that multiple diverse protective signals converge on inhibition of GSK-3 β , and several investigators have demonstrated that pharmacological antagonism of GSK-3 β is quite cardioprotective [6,19]. Previous investigators have either given SB216763 intravenously to *in situ* preparations at reperfusion [6] or as extended infusions to isolated hearts [5] to antagonize GSK-3 β and protect the ischemic heart. Additionally PKC is a critical kinase in the signaling cascade triggered by cardioprotective interventions at reperfusion. Philipp *et al.* [15] demonstrated that a brief infusion of PMA at reperfusion was very protective. Our findings suggest that reperfusion with alkaline buffer opposes IPoC's protection so strongly that it could not be reversed by either activation of PKC with PMA or blockade of GSK-3 β phosphorylation with SB216763. Interestingly, the hearts could be rescued by CsA suggesting that CsA's direct inhibition of MPTP formation is more potent than that caused by activating the cell signaling cascade at upstream sites. The reason for this differing potency is not yet understood.

In prior publications we have noted that various signaling elements, including adenosine receptors [16,21] and Akt [16], must be activated for prolonged periods following reperfusion to yield cardioprotection. We postulated that support for up to 1 h of viable but affected cells following reperfusion is required until sufficient repair is completed to permit the cells to survive without support of protective signaling. Surprisingly attenuation of GSK-3 β activity must occur for a much briefer period. The critical threshold for protection is somewhere between only 5 and 15 min. These observations again suggest that the ischemically injured myocardium must be supported for a period of time following reperfusion in order to survive.

Protective interventions at the time of myocardial revascularization for acute coronary occlusion could potentially be used in patients presenting with acute myocardial infarction. While IPoC holds promise for promoting salvage in primary angioplasty, a pharmacological approach would be needed for patients undergoing thrombolytic therapy. The present experiments broaden our knowledge of the mechanisms involved in IPoC and should help us to design a potent therapy for protecting the reperfused heart.

Acknowledgment

This study was supported in part by a grant from the Heart, Lung, and Blood Institute of the National Institutes of Health, HL-20468.

References

1. Argaud L, Gateau-Roesch O, Raissy O, Loufouat J, Robert D, Ovize M. Postconditioning inhibits mitochondrial permeability transition. *Circulation* 2005;111:194–197. [PubMed: 15642769]
2. Boengler K, Buechert A, Heinen Y, Roeskes C, Hilfiker-Kleiner D, Heusch G, Schulz R. Cardioprotection by ischemic postconditioning is lost in aged and STAT3-deficient mice. *Circ Res* 2008;102:131–135. [PubMed: 17967780]

3. Cohen MV, Yang X-M, Downey JM. The pH hypothesis of postconditioning: staccato reperfusion reintroduces oxygen and perpetuates myocardial acidosis. *Circulation* 2007;115:1895–1903. [PubMed: 17389262]
4. Dost T, Cohen MV, Downey JM. Redox signaling triggers protection during the reperfusion rather than the ischemic phase of preconditioning. *Basic Res Cardiol*. 2008in press
5. Förster K, Paul I, Solenkova N, Staudt A, Cohen MV, Downey JM, Felix SB, Krieg T. NECA at reperfusion limits infarction and inhibits formation of the mitochondrial permeability transition pore by activating p70S6 kinase. *Basic Res Cardiol* 2006;101:319–326. [PubMed: 16604438]
6. Gross ER, Hsu AK, Gross GJ. GSK3 β inhibition and K_{ATP} channel opening mediate acute opioid-induced cardioprotection at reperfusion. *Basic Res Cardiol* 2007;102:341–349. [PubMed: 17450314]
7. Heusch G, Büchert A, Feldhaus S, Schulz R. No loss of cardioprotection by postconditioning in connexin 43-deficient mice. *Basic Res Cardiol* 2006;101:354–356. [PubMed: 16568250]
8. Iliodromitis EK, Georgiadis M, Cohen MV, Downey JM, Bofilis E, Kremastinos DT. Protection from postconditioning depends on the number of short ischemic insults in anesthetized pigs. *Basic Res Cardiol* 2006;101:502–507. [PubMed: 16830072]
9. Juhaszova M, Zorov DB, Kim S-H, Pepe S, Fu Q, Fishbein KW, Ziman BD, Wang S, Ytrehus K, Antos CL, Olson EN, Sollott SJ. Glycogen synthase kinase-3 β mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. *J Clin Invest* 2004;113:1535–1549. [PubMed: 15173880]
10. Korichneva I, Hoyos B, Chua R, Levi E, Hammerling U. Zinc release from protein kinase C as the common event during activation by lipid second messenger or reactive oxygen. *J Biol Chem* 2002;277:44327–44331. [PubMed: 12213816]
11. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986;74:1124–1136. [PubMed: 3769170]
12. National Research Council. Guide for the Care and Use of Laboratory Animals. Vol. 7th ed.. National Academy Press; Washington, D.C.: 1996.
13. Penna C, Cappello S, Mancardi D, Raimondo S, Rastaldo R, Gattullo D, Losano G, Pagliaro P. Post-conditioning reduces infarct size in the isolated rat heart: role of coronary flow and pressure and the nitric oxide/cGMP pathway. *Basic Res Cardiol* 2006;101:168–179. [PubMed: 16132172]
14. Penna C, Rastaldo R, Mancardi D, Raimondo S, Cappello S, Gattullo D, Losano G, Pagliaro P. Post-conditioning induced cardioprotection requires signaling through a redox-sensitive mechanism, mitochondrial ATP-sensitive K⁺ channel and protein kinase C activation. *Basic Res Cardiol* 2006;101:180–189. [PubMed: 16450075]
15. Philipp S, Yang X-M, Cui L, Davis AM, Downey JM, Cohen MV. Postconditioning protects rabbit hearts through a protein kinase C-adenosine A_{2b} receptor cascade. *Cardiovasc Res* 2006;70:308–314. [PubMed: 16545350]
16. Solenkova NV, Solodushko V, Cohen MV, Downey JM. Endogenous adenosine protects preconditioned heart during early minutes of reperfusion by activating Akt. *Am J Physiol* 2006;290:H441–H449.
17. Thibault H, Piot C, Staat P, Bontemps L, Sportouch C, Rioufol G, Cung TT, Bonnefoy E, Angoulvant D, Aupetit J-F, Finet G, André-Fouët X, Macia JC, Raczk F, Rossi R, Itti R, Kirkorian G, Derumeaux G, Ovize M. Long-term benefit of postconditioning. *Circulation* 2008;117:1037–1044. [PubMed: 18268150]
18. Tissier R, Cohen MV, Downey JM. Protecting the acutely ischemic myocardium beyond reperfusion therapies: are we any closer to realizing the dream of infarct size elimination? *Arch Mal Coeur Vaiss* 2007;100:794–802. [PubMed: 18033009]
19. Tong H, Imahashi K, Steenbergen C, Murphy E. Phosphorylation of glycogen synthase kinase-3 β during preconditioning through a phosphatidylinositol-3-kinase-dependent pathway is cardioprotective. *Circ Res* 2002;90:377–379. [PubMed: 11884365]
20. Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM. Postconditioning: a form of “modified reperfusion” protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. *Circ Res* 2004;95:230–232. [PubMed: 15242972]
21. Xu Z, Downey JM, Cohen MV. Timing and duration of administration are crucial for antiinfarct effect of AMP 579 infused at reperfusion in rabbit heart. *Heart Dis* 2003;5:368–371. [PubMed: 14633317]

22. Yang X-M, Philipp S, Downey JM, Cohen MV. Postconditioning's protection is not dependent on circulating blood factors or cells but involves adenosine receptors and requires PI3-kinase and guanylyl cyclase activation. *Basic Res Cardiol* 2005;100:57–63. [PubMed: 15614590]
23. Yang X-M, Proctor JB, Cui L, Krieg T, Downey JM, Cohen MV. Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways. *J Am Coll Cardiol* 2004;44:1103–1110. [PubMed: 15337225]
24. Zhao Z-Q, Corvera JS, Halkos ME, Kerendi F, Wang N-P, Guyton RA, Vinten-Johansen J. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. *Am J Physiol* 2003;285:H579.

Figure 1.

Experimental protocols. Abbreviations: CsA = cyclosporin A; PMA = phorbol 12-myristate 13-acetate

Figure 2.

Infarct size as a % of risk zone. Individual points are represented by open circles and group means by filled-in circles. SEM is indicated for each group. Infarction was comparable in control hearts with only 30 min of regional ischemia and those with an additional 2 min of global ischemia. Whereas perfusion with an acidic buffer for the first 2 min of reperfusion was very protective, protection was abrogated if the acidic perfusate was also made hypoxic. Yet the latter hearts could be salvaged by simultaneous infusion of phorbol 12-myristate 13-acetate (PMA). Ischemic postconditioning (IPoC) with 6 cycles of 10-sec reperfusion/10-sec coronary reocclusion also decreased infarction, and this protection was similarly lost when the perfusate was made hypoxic. The acidosis and IPoC groups are reproduced from an earlier study [3] for the purpose of comparison. * $p < 0.001$ vs control

Figure 3.

Infarct size as a % of risk zone. Individual points are represented by open circles and group means by filled-in circles. SEM is indicated for each group. Although SB216763 added to the perfusate from 1 min before to 5 min after reperfusion (SB-6') was not cardioprotective, it was when the infusion was extended to 15 min following reperfusion (SB-16'). * $p < 0.001$ vs control

Figure 4.

Infarct size as a % of risk zone. Individual points are represented by open circles and group means by filled-in circles. SEM is indicated for each group. Ischemic postconditioning (IPoC) with 6 cycles of 10-sec reperfusion/10-sec coronary reocclusion was quite protective, but this protection was blocked if the perfusate administered during the reperfusion phases of the postconditioning cycles was alkaline. IPoC's protection could not be restored by addition to the alkaline perfusate of either phorbol 12-myristate 13-acetate (PMA) or SB216763 (SB-16'), but could be by addition of cyclosporin A (CsA). The CsA group is reproduced from an earlier study [3] for the purpose of comparison. * $p < 0.001$ vs control

Figure 5.

A) Plot of absolute infarct size against risk zone volume for individual hearts from control, SB216763 (SB)-16', and ischemic postconditioning (IPoC) + alkalosis + cyclosporin A (CsA) (reproduced from [3]) groups and their respective regression lines. The regression lines for the SB-16' and CsA groups are displaced downwards and are significantly different from the control regression line ($p < 0.05$). The regression lines for the other two groups are not different than the regression line for the control group. B) Plot of absolute infarct size against risk zone volume for individual hearts from control, acidosis + hypoxia and ischemic postconditioning (IPoC) + low P_{O2} groups and their respective regression lines. The regression lines for the three groups are not different.

Table 1

Hemodynamics

	Baseline			29' Occlusion			30' Occlusion			3-5' or 15' Reperfusion *			30' Reperfusion		
	HR	LVDP	CF	HR	LVDP	CF	HR	LVDP	CF	HR	LVDP	CF	HR	LVDP	CF
Control	231 ±6	104 ±3	9.8 ±0.4				209 ±8 [†]	46 ±4 [†]	4.2 ±0.3 [†]				199 ±8 [†]	68 ±6 [†]	6.7 ±0.3 [†]
2' global ischemia	221 ±8	109 ±5	9.4 ±0.3				210 ±16	40 ±8 [†]	4.3 ±0.1 [†]				215 ±20	83 ±1 [§]	6.7 ±0.2 [†]
Acidosis + Low PO ₂	213 ±12	112 ±3	9.8 ±0.3				208 ±10	38 ±6 [†]	4.5 ±0.3 [†]				218 ±10	82 ±5 [†]	7.1 ±0.3 [†]
IPoC + Low PO ₂	224 ±6	116 ±2	9.4 ±0.2				206 ±12	41 ±4 [†]	4.3 ±0.3 [†]				223 ±6	87 ±6 [†]	7.3 ±0.2 [†]
SB216763-6'	218 ±11	120 ±4	10.7 ±0.6	204 ±14	73 ±3 [†]	5.0 ±0.2 [†]	204 ±14	68 ±5 [†]	4.6 ±0.3 [†]	196 ±11	85 ±12 [§]	6.3 ±0.4 [†]	221 ±9	79 ±8 [§]	6.9 ±0.9 [†]
SB216763-16'	202 ±7	122 ±3	9.8 ±0.2	183 ±6	40 ±4 [†]	5.3 ±0.3 [†]	177 ±4	37 ±3 [†]	4.6 ±0.2 [†]	204 ±14	78 ±6 [†]	6.3 ±0.3 [†]	195 ±11	71 ±7 [†]	6.1 ±0.1 [†]
IPoC + Alkalosis + PMA	198 ±6	110 ±5	9.5 ±0.2	191 ±12	44 ±2 [†]	4.1 ±0.2 [†]	172 ±10	39 ±3 [†]	3.6 ±0.2 [†]	181 ±17	66 ±6 [†]	5.7 ±0.2 [†]	200 ±9	79 ±6 [†]	6.8 ±0.3 [†]
IPoC + Alkalosis + SB216763-16'	210 ±9	118 ±3	9.5 ±0.2	203 ±13	44 ±6 [†]	5.4 ±0.2 [†]	191 ±12	37 ±5 [†]	4.7 ±0.3 [†]	210 ±7	73 ±6 [†]	6.5 ±0.2 [†]	213 ±8	70 ±8 [†]	6.9 ±0.2 [†]
IPoC + Alkalosis + C _s A	213 ±7	106 ±3	9.5 ±0.3	210 ±15	42 ±4 [†]	4.5 ±0.3 [†]	200 ±10	40 ±5 [†]	4.0 ±0.2 [†]	188 ±9	70 ±4 [†]	6.5 ±0.3 [†]	201 ±6	77 ±5 [†]	8.1 ±0.5 [§]
Acidosis + Low PO ₂ + PMA	217 ±5	118 ±3	9.8 ±0.2	202 ±6	51 ±4 [†]	5.6 ±0.2 [†]	189 ±10	36 ±5 [†]	4.5 ±0.2 [†]	153 ±4 [†]	27 ±5 [†]	5.7 ±0.2 [†]	211 ±8	87 ±6 [†]	7.1 ±0.2 [†]

Mean ± SEM

Abbreviations: CF = coronary flow (ml/min/gm); C_sA = cyclosporin A; HR = heart rate (B/min); IPoC = ischemic postconditioning; LVDP = left ventricular developed pressure (mmHg); PMA = phenol 12-myristate 13-acetate; SB216763-6' and -16' = 6- and 16-min infusions of SB216763

Statistical significance of difference between baseline and any other time point in a given group:

* Hemodynamics measured after 3-5 min of reperfusion in SB216763-6', IPoC + alkalosis + PMA, IPoC + alkalosis + C_sA, and Acidosis + Low PO₂ + PMA groups and after 15 min of reperfusion in SB216763-16' and IPoC + alkalosis + SB216763-16' groups

[†] p<0.001

[‡] p<0.01

[§] p<0.05

Table 2

Infarct data

	n	Body Weight (kg)	Heart Weight (g)	Risk zone (cm ³)	Infarct size (cm ³)	I/R (%)
Control	9	2.1±0.1	7.0±0.1	1.24±0.10	0.43±0.04	34.4±2.2
2' global ischemia	4	2.1±0.0	6.7±0.1	1.25±0.10	0.49±0.04	39.4±2.7
Acidosis + Low P _{O2}	6	2.0±0.0	6.6±0.2	1.28±0.15	0.47±0.08	35.7±3.0
IPoC + Low P _{O2}	6	2.2±0.1	7.0±0.1	1.48±0.09	0.49±0.03	32.8±1.4
SB216763-6'	4	2.3±0.1	6.9±0.1	1.21±0.09	0.39±0.03	32.0±2.1
SB216763-16'	6	2.4±0.0	7.0±0.1	1.44±0.09	0.29±0.03	20.0±1.8*
IPoC + Alkalosis + PMA	6	2.1±0.0	6.9±0.1	1.36±0.09	0.44±0.06	31.4±2.1
IPoC + Alkalosis + SB216763-16'	6	2.3±0.0	6.9±0.1	1.48±0.16	0.53±0.06	35.5±1.7
IPoC + Alkalosis + CsA	6	2.0±0.1	6.8±0.1	1.06±0.08	0.16±0.02 [§]	15.1±1.9*
Acidosis + Low P _{O2} + PMA	6	2.5±0.1	7.1±0.1	1.33±0.09	0.17±0.04 [§]	12.2±2.2*

Mean ± SEM

Abbreviations: see Table 1; I/R = infarct/risk zone ratio; n = number of hearts

Statistical significance of difference between control and other groups:

* p<0.001

[§] p<0.005