Reduction of infarct size with d-myo-inositol trisphosphate: role of PI3-kinase and mitochondrial K_{ATP} channels

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Przyklenk, Karin, Michelle Maynard, and Peter Whittaker. Reduction of infarct size with d-myo-inositol trisphosphate: role of PI3-kinase and mitochondrial K_{ATP} channels. Am J Physiol Heart Circ Physiol 290: H830–H836, 2006. First published September 23, 2005; doi:10.1152/ajpheart.00799.2005.—Prophylactic treatment with d-myo-inositol 1,4,5-trisphosphate hexasodium [d-myo-Ins(1,4,5)P_3], the sodium salt of the endogenous second messenger Ins(1,4,5)P_3, triggers a reduction of infarct size comparable in magnitude to that seen with ischemic preconditioning (PC). However, the mechanisms underlying d-myo-Ins(1,4,5)P_3-induced protection are unknown. Accordingly, our aim was to investigate the role of four archetypal mediators implicated in PC and other cardioprotective strategies (i.e., PKC, PI3-kinase/Akt, and mitochondrial and/or sarcosomal K_{ATP} channels) in the infarct-sparing effect of d-myo-Ins(1,4,5)P_3. Fifteen groups of isolated buffer-perfused rabbit hearts [5 treated with d-myo-Ins(1,4,5)P_3, 5 treated with PC, and 5 control cohorts] underwent 30 min of coronary artery occlusion and 2 h of reflow. One set of control, d-myo-Ins(1,4,5)P_3, and PC groups received no additional treatment, whereas the remaining sets were infused with chelerythrine, LY-294002, 5-hydroxydecanoate (5-HD), or HMR-1098 [inhibitors of PKC, PI3-kinase, and mitochondrial and sarcosomal ATP-sensitive K^+ (K_{ATP}) channels, respectively]. Infarct size (delineated by tetrazolium staining) was, as expected, significantly reduced in both d-myo-Ins(1,4,5)P_3- and PC-treated hearts versus controls. d-myo-Ins(1,4,5)P_3-induced cardioprotection was blocked by 5-HD but not HMR-1098, thereby implicating the involvement of mitochondrial, but not sarcosomal, K_{ATP} channels. Moreover, the benefits of d-myo-Ins(1,4,5)P_3 were abrogated by LY-294002, whereas, in contrast, chelerythrine had no effect. These latter pharmacological data corroborated by immunoblotting: D-insitol 1,4,5-trisphosphate (Ins(1,4,5)P_3) is a ubiquitous second messenger generated in parallel with diacylglycerol in response to activation of G protein-coupled receptors. Previous studies (10, 24) have shown that prophylactic administration of exogenous d-myo-Ins(1,4,5)P_3 hexasodium, the sodium salt of Ins(1,4,5)P_3, renders the rabbit heart resistant to a subsequent, sustained ischemic insult and initiates a reduction of infarct size similar in magnitude to that achieved with ischemic preconditioning (PC), the current “gold standard” of experimental cardioprotection. The mechanisms responsible for the infarct-sparing effect of exogenous d-myo-Ins(1,4,5)P_3 are, however, largely unknown; although recent evidence has identified Ins(1,4,5)P_3-receptor binding and communication via gap junctions/hemichannels as elements of the trigger phase of d-myo-Ins(1,4,5)P_3-induced protection (24), the distal signaling pathways contributing to the increased resistance to ischemia afforded by d-myo-Ins(1,4,5)P_3 have, to date, not been investigated. Four classic cellular mediators [PKC (in particular, the ε-isof orm of the kinase), phosphatidylinositol-3-kinase (PI3-kinase), as well as sarcosomal and mitochondrial ATP-sensitive K^+ (K_{ATP}) channels] have emerged as pivotal mechanistic components in multiple cardioprotective strategies, including, most notably, PC (9, 18, 31). Accordingly, our aim in the current study was to investigate the possible contribution of these four archetypal candidates in the reduction of infarct size achieved with prophylactic d-myo-Ins(1,4,5)P_3 treatment versus conventional ischemic PC.

MATERIALS AND 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 National Institutes of Health (NIH Publication No. 86-13, Revised 1996).

Surgical Preparation

Experiments were conducted by using the isolated buffer-perfused rabbit heart model of regional ischemia (2, 6, 10, 24). In brief, New Zealand White rabbits weighing 2.5–3.5 kg were anesthetized with an intramuscular injection of ketamine plus xylazine (150 mg and 100 mg, respectively). A tracheostomy was performed, the animals were ventilated with room air, the hearts were exposed via a left lateral thoracotomy, and the pericardium was incised. In all animals, a dominant anterior branch of the left circumflex coronary artery was ensnared with a 2-0 silk suture for later occlusion/reperfusion. The hearts were then excised and placed in an ice bath, and, after rapid cannulation of the aortic root, retrograde perfusion (nonrecirculating) was initiated at a pressure of 85 mmHg. The buffer was composed of (in mM) 118 NaCl, 4.7 KCl, 24 NaHCO_3, 1.2 KH_2PO_4, 1.2 MgSO_4-7H_2O, 11 glucose, and 2.5 CaCl_2 anhydrous in distilled water at a pH of 7.4 and was continuously oxygenated with 95% O_2-5% CO_2. The perfusate was warmed to 37°C, and heart temperature was maintained at 37°C by immersion in a water-jacketed chamber. An incision was made in the left atrium, and a latex balloon connected to a pressure transducer was positioned in the left ventricular (LV) cavity for continuous assessment of hemodynamic function. The balloon was initially inflated to an end-diastolic pressure of 5–10 mmHg, and thereafter the balloon volume was held constant. All...
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D-myo-Ins(1,4,5)P₃-induced cardioprotection, control, D-myo-Ins(1,4,5)P₃-treated groups as described above (n = 4 hearts/group). At the time corresponding to the start of sustained ischemia in protocol 1, the artery was ligated, fluorescent polymer beads were injected to delineate the ischemic territory, and the hearts were rapidly frozen in liquid nitrogen (Fig. 1). All hearts were stored at −80°C until processed.

End points. To assess the activation/translocation of PKC-ε and PI3-Kinase in D-myo-Ins(1,4,5)P₃-treated hearts.

Study design. Previous studies have shown that PC-induced cardioprotection is associated with subcellular redistribution (and thus, presumably, activation) of PKC-ε (22, 23) and increased activity of PI3-kinase/Akt (11, 27) during the initial minutes of the sustained ischemic insult. Accordingly, our aim in protocol 2 was to determine, by standard Western blot analysis, whether hearts treated with exogenous D-myo-Ins(1,4,5)P₃ display a similar upregulation of these kinases at the onset of the prolonged coronary occlusion. An additional 12 hearts were randomized into control, PC-, and D-myo-Ins(1,4,5)P₃-treated groups as described above (n = 4 hearts/group). At the time corresponding to the start of sustained ischemia in protocol 1, the artery was ligated, fluorescent polymer beads were injected to delineate the ischemic territory, and the hearts were rapidly frozen in liquid nitrogen (Fig. 1). All hearts were stored at −80°C until processed.

End points. To assess the activation/translocation of PKC-ε (i.e., the redistribution of the isoform from the cytosol to membranes and mitochondria), LV samples harvested from the center of the risk region were processed for the preparation of cytosolic and particulate fractions as

Hearts were paced at 210 beats/min via electrodes positioned on the right ventricle. After a 15-min equilibration period, baseline hemodynamic data (described in Protocol 1: Study design) were obtained, and baseline coronary flow was measured by the timed collection of coronary effluent.

Ins(1,4,5)P₃ treatment was shown in early pilot studies to trigger optimal perfusate was 6 μM; Duke Scientific, Palo Alto, CA), dissolved in 5 ml buffer and administered over 1 min, beginning 25 min before the onset of coronary artery occlusion, via a sidearm located immediately proximal to the heart (final concentration in perfusate was 6 μM) (10, 24). This dose and timing of D-myo-Ins(1,4,5)P₃ treatment was shown in early pilot studies to trigger optimal protection in this model (data not shown). Five groups of hearts received ischemic PC, initiated by the standard stimulus of 5 min of brief coronary artery occlusion followed by 10 min of reflow. In the remaining five control groups, uninterrupted buffer perfusion was maintained throughout the intervention phase (Fig. 1).

One set of control, D-myo-Ins(1,4,5)P₃, and PC groups received no additional treatment (protocol 1A). To assess the possible role of PKC, PI3-kinase, and mitochondrial and sarcocemmal KATP channels in D-myo-Ins(1,4,5)P₃-induced cardioprotection, control, D-myo-Ins(1,4,5)P₃, and PC groups in protocol 1, B–E, were infused with chelerythrine chloride (final concentration of 5 μM), LY-294002 (15 μM), 5-hydroxydecanoate (5-HD; 200 μM), and HMR-1098 (5 μM), respectively. Because kinase activation and/or K_ATP channel opening could potentially occur and contribute to cardioprotection at any time from the administration of D-myo-Ins(1,4,5)P₃ throughout the period of sustained ischemia, all infusions were initiated 5 min before the administration of D-myo-Ins(1,4,5)P₃ and terminated after the start of coronary artery occlusion (i.e., infused for a total of 30 min via a second proximal side port; Fig. 1). Doses of the antagonists were chosen on the basis of previous studies (11, 13, 17, 23, 26, 28) and have been shown to inhibit kinase activities and/or attenuate infarct-size reduction in response to PC ischemia and/or other cardioprotective strategies.

For all hearts, hemodynamics (i.e., maximum LV systolic pressure, end-diastolic pressure, as well as peak positive and negative change of LV pressure over time (±dP/dt)) were recorded at 1-min intervals throughout the protocol on a computerized data acquisition system (Micro-Med, Louisville, KY), and coronary flow was measured by a timed collection of effluent before occlusion, at 10 min into coronary occlusion, and at 10 min and 2 h after reflow.

At the conclusion of the 2-h reperfusion period, the coronary branch was briefly reoxygenated, and fluorescent polymer beads (2–9 μm; Duke Scientific, Palo Alto, CA) were injected into the coronary circulation to delineate the extent of the occluded vascular bed or area at risk of infarction (AR). The heart was immediately removed from the apparatus, sliced into 5-7 transverse slices, illuminated under ultraviolet light, and digitally photographed. To distinguish necrotic from viable myocardium, the heart slices were then incubated in triphenyltetrazolium chloride for 15 min at 37°C, rephotographed, and stored in formalin (2, 6, 10, 24).

End points. The primary end point of protocol 1 was infarct size. For all hearts, right ventricular tissue was trimmed and each LV heart slice was weighed. AR and area of necrosis (AN) in each heart slice were quantified from the 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 percentage of the total LV weight, whereas AN was expressed as a percentage of the AR (AR/LV and AN/AR, respectively) (2, 6, 10, 24, 32).

Secondary end points were hemodynamics and coronary flow. LV pressures and LV dP/dt were tabulated for each heart at baseline (before randomization); immediately before coronary artery occlusion; at 5 and 30 min into coronary artery occlusion; and at 15 min, 30 min, 1 h, and 2 h after relief of ischemia. For each time point, LV developed pressure was calculated as the difference between maximum LV systolic pressure and end-diastolic pressure.

Protocol 2: Activity of PKC-ε and PI3-Kinase in D-myo-Ins(1,4,5)P₃-Treated Hearts

Study design. Previous studies have shown that PC-induced cardioprotection is associated with subcellular redistribution (and thus, presumably, activation) of PKC-ε (22, 23) and increased activity of PI3-kinase/Akt (11, 27) during the initial minutes of the sustained ischemic insult. Accordingly, our aim in protocol 2 was to determine, by standard Western blot analysis, whether hearts treated with exogenous D-myo-Ins(1,4,5)P₃ display a similar upregulation of these kinases at the onset of the prolonged coronary occlusion. An additional 12 hearts were randomized into control, PC-, and D-myo-Ins(1,4,5)P₃-treated groups as described above (n = 4 hearts/group). At the time corresponding to the start of sustained ischemia in protocol 1, the artery was ligated, fluorescent polymer beads were injected to delineate the ischemic territory, and the hearts were rapidly frozen in liquid nitrogen (Fig. 1). All hearts were stored at −80°C until processed.

End points. To assess the activation/translocation of PKC-ε (i.e., the redistribution of the isoform from the cytosol to membranes and mitochondria), LV samples harvested from the center of the risk region were processed for the preparation of cytosolic and particulate fractions as...
Table 1. Left ventricular developed pressure

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Baseline (mmHg)</th>
<th>Pre-CO (mmHg)</th>
<th>Occlusion, 30 min (mmHg)</th>
<th>Reperfusion 15 min (mmHg)</th>
<th>Reperfusion 1 h (mmHg)</th>
<th>Reperfusion 2 h (mmHg)</th>
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<td>33±4†</td>
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<td>43±4†</td>
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<td>53±8†</td>
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<td>32±4†</td>
<td>55±4†</td>
<td>48±4†</td>
<td>41±7†</td>
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Values are means ± SE (in %). CO, coronary artery occlusion; PC, preconditioned; d-myo-IP3, d-myo-Ins(1,4,5)P3-treatment. *P < 0.05 vs. control; †P < 0.05 vs. d-myo-IP3; ‡P < 0.05 vs. 5-HD.

RESULTS

Protocol 1: Effect of Pharmacological Antagonists on d-myo-Ins(1,4,5)P3-Induced Cardioprotection

Hemodynamics. Baseline values of LV developed pressure and coronary flow averaged 96 mmHg and 84 ml/min, with no significant differences among the 15 treatment groups.

Protocol 1A confirmed that, as expected (10, 24), d-myo-Ins(1,4,5)P3 had no effect on hemodynamics or coronary flow, whereas hearts that received brief PC ischemia were modestly but significantly “stunned” before the onset of sustained coronary occlusion (Tables 1 and 2). Recovery of LV developed pressure after relief of ischemia was depressed in all hearts, with no differences in PC- and D-myo-Ins(1,4,5)P3-treated hearts versus controls (Table 1); i.e., as reported previously, d-myo-Ins(1,4,5)P3 and PC had no beneficial effect on the acute recovery of function of viable but stunned myocardium in this model (24). Results obtained for peak LV dP/dt were similar to those observed for LV developed pressure (data not shown).

In protocol 1, B-E, LV developed pressures tended to be depressed in all inhibitor-treated groups at the onset of sustained coronary occlusion, averaging 79–97% of baseline values. In addition, as described in previous studies (13), infusion of chelerythrine was associated with significant increases in coronary flow that persisted into coronary occlusion (Table 2). However, the only antagonist to alter posts ischemic recovery of function was LY-294002: LV developed pressure after reperfusion were significantly lower in all LY-294002-treated hearts when compared with no-inhibitor controls (Table 1).

Infarct size. Risk regions did not differ significantly among the 15 study groups and ranged from 38–41% of the total LV weight for no-inhibitor-treated hearts, 39–45% for hearts that received chelerythrine, 41–46% for LY-294002-treated hearts, 41–46% for hearts infused with 5-HD, and 39–45% for cohorts treated with HMR-1098.

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PI3-kinase and mitochondrial KATP channels, but not PKC or similar in magnitude to that obtained with PC (mean AN/AR of 30% of the risk region. As anticipated (10, 24), the administration of enroled in Fig. 2. Area of necrosis, expressed as a percentage of risk region for hearts D-myo-Ins(1,4,5)P3 (Fig. 2). These data implicate the involvement of abrogated the reduction of infarct size initiated by D-myo-Ins(1,4,5)P3, whereas, in contrast, PKC and sarcolemmal KATP channels do not appear to play a role. These findings constitute, to our knowledge, the first insight into the cellular mechanisms contributing to the reduction in infarct size triggered by prophylactic administration of D-myo-Ins(1,4,5)P3 treatment.

Protocol 2: Activity of PKC-ε and PI3-Kinase in D-myo-Ins(1,4,5)P3-Treated Hearts

Both brief PC ischemia and administration of D-myo-Ins(1,4,5)P3 were associated with the activation of PI3-kinase; although there were no differences in total Akt among groups (data not shown), immunoreactivity of phospho-Akt was increased by 1.9- and 1.3-fold in PC- and D-myo-Ins(1,4,5)P3-treated hearts versus controls (1.501 and 1.014 vs. 773 arbitrary units; P < 0.05, Fig. 3). In contrast, subcellular redistribution of PKC-ε was only seen with PC. The proportion of PKC-ε in the particulate fraction averaged 35% in the PC group versus 26% in controls (P < 0.05) but remained unchanged at 23% in hearts treated with D-myo-Ins(1,4,5)P3 (Fig. 4); i.e., D-myo-Ins(1,4,5)P3-treated hearts displayed activation of PI3-kinase/Akt but not activation/translocation of PKC-ε at the time corresponding to the onset of the prolonged ischemic insult.

DISCUSSION

In the current study, we provide evidence that activation of PI3-kinase/Akt signaling and opening of mitochondrial KATP channels participate in the infarct-sparing effect of D-myo-Ins(1,4,5)P3, whereas, in contrast, PKC and sarcolemmal KATP channels do not appear to play a role. These findings constitute, to our knowledge, the first insight into the cellular mechanisms contributing to the reduction in infarct size triggered by prophylactic administration of D-myo-Ins(1,4,5)P3.

Reduction of Infarct Size With D-myo-Ins(1,4,5)P3

Ins(1,4,5)P3 is a second messenger generated in parallel with diacylglycerol via activation of G protein-coupled receptors and subsequent phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate. In addition to its classic role in the regulation of calcium homeostasis (3, 4, 29), endogenous Ins(1,4,5)P3 signaling has been implicated to contribute to cardioprotection initiated by both brief PC ischemia and opioid receptor activation (1, 2). Evidence...
from our group has further shown that prophylactic administration of exogenous D-myo-Ins(1,4,5)P3 reduced infarct size in rabbit hearts, i.e., the sodium salt, delivered as a slow bolus 25 min before the onset of coronary artery occlusion, evoked a 35–40% reduction of infarct size that was comparable in magnitude with that obtained by PC (10, 24). Initial progress has been made in identifying the mechanisms by which D-myo-Ins(1,4,5)P3 triggers its infarct-sparing effect, with Ins(1,4,5)P3 receptor binding and communication via connexin 43-formed channels implicated in playing a role (24). However, there are, at present, no data regarding the identity of the distal mediators that may contribute to D-myo-Ins(1,4,5)P3-induced protection.

PKC, PI3-Kinase, and KATP Channels as Mediators of Cardioprotection

Considerable attention has focused on elucidating the cellular mechanisms that contribute to the infarct-sparing effects of ischemic PC, pharmacological PC (including, for example, preischemic administration of adenosine receptor agonists acetylcholine, bradykinin, nicorandil, diazoxide, etc.), and treatment with other agents reported to attenuate lethal myocardial ischemia-reperfusion injury. Among the numerous candidates under investigation, PKC, PI3-kinase, and mitochondrial and/or sarcolemmal KATP channels have emerged as pivotal mediators among multiple protective strategies (5–7, 13, 14, 17, 18, 20, 23, 25, 27, 28, 31). Indeed, for ischemic PC [arguably the most robust, reproducible, and extensively studied cardioprotective stimulus (reviewed in Refs. 16 and 31). With regard to KATP channels, current evidence appears to favor a major involvement of the mitochondrial, rather than sarcolemmal, site (16, 31). However, there is a reportedly complex interplay between the two channels in mediating protection, i.e., activation of mitochondrial KATP channels may play a role in the limitation of cell death, whereas opening of the surface channels may contribute to an attenuation of stunning (reviewed in Ref. 9). Our observation that 5-HD, but not HMR, blocked the benefits of PC in isolated rabbit hearts subjected to regional ischemia-reperfusion (a model in which PC reduces infarct size but has no apparent salutary effect on acute recovery of LV function) is consistent with these concepts.

Role of PKC, PI3-Kinase, and KATP Channels in Infarct-Sparing Effect of D-myo-Ins(1,4,5)P3

There are, at present, no published data on the effects of exogenous administration of D-myo-Ins(1,4,5)P3 on the activities of PKC, PI3-kinase/Akt, and opening of KATP channels. Nonetheless, there is limited and poorly defined evidence suggesting that inositol polyphosphates [including endogenous Ins(1,4,5)P3] and exogenous inositol phosphate analogs may interact with these protective mediators. For example, chelerythrine has been implicated to both inhibit Ins(1,4,5)P3 metabolism in neonatal rat cardiomyocytes (15) and increase flavoprotein oxidation (an index of mitochondrial KATP channel activity) via production of endogenous Ins(1,4,5)P3 in guinea pig ventricular myocytes (12). In addi-
tion, exogenous inositol pentakisphosphate and endogenous inositol tetrakisphosphate reportedly inhibit PI3-kinase/Akt signaling in cancer cell lines and intestinal epithelial cells, respectively (8, 21), whereas others (30) have concluded that structurally modified Ins(1,4,5)P3 analogs, but not Ins(1,4,5)P3 per se, inhibit in vitro PI3-kinase activity immunoprecipitated from a leukemic T cell line.

We found that the pan-PKC inhibitor chelerythrine, at a concentration (5 µM) shown to abrogate infarct-size reduction with PC in our model, had no effect on the infarct-sparing effect of d-my-o-Ins(1,4,5)P3. Although these data could be interpreted as evidence that PKC does not play a role in the reduction of infarct size seen with d-my-o-Ins(1,4,5)P3, an alternative explanation is that the administration of d-my-o-Ins(1,4,5)P3 evoked an augmented activation/translocation of cardioprotective PKC isoforms that was not blocked by the 5-µM dose of the inhibitor. Two pieces of evidence argue against the latter possibility. First, results obtained in protocol 2 demonstrate that, although PC ischemia was associated with an expected, significant subcellular redistribution of PKC-ε [the specific isoform implicated to contribute to cardioprotection in this model (22, 23)], the proportion of PKC-ε in the particulate fraction did not differ in d-my-o-Ins(1,4,5)P3-treated hearts versus controls. Second, in supplemental post hoc experiments, we pursued the issue of dose and administered 10 µM of chelerythrine to an additional 4 hearts treated with d-my-o-Ins(1,4,5)P3. Even with a twofold increase in the concentration of the inhibitor, persistent protection with d-my-o-Ins(1,4,5)P3 was observed (i.e., mean infarct size was 28 ± 3% of the risk region). Although we cannot definitively exclude the possibility that brief and transient activation/translocation of PKC-ε may have occurred at earlier time points after d-my-o-Ins(1,4,5)P3 treatment or that PKC isoforms implicated in other cardioprotective strategies (possibly δ or γ) may have played a role, these data strongly suggest that PKC does not contribute to the reduction of infarct size seen with d-my-o-Ins(1,4,5)P3.

In apparent contrast to the aforementioned studies (8, 21) reporting inhibition of PI3-kinase/Akt signaling by inositol polyphosphates in isolated cells, our results revealed that the administration of d-my-o-Ins(1,4,5)P3 was associated with a significant ~30% increase in the immunoreactivity of phospho-Akt manifest at the onset of sustained coronary occlusion. Moreover, we found that the infarct-sparing effect of d-my-o-Ins(1,4,5)P3 was blocked by infusion of the PI3-kinase inhibitor LY-294002. Although these data support the concept that PI3-kinase/Akt plays a role in d-my-o-Ins(1,4,5)P3-induced cardioprotection, we nonetheless sought, in a small number of post hoc studies, to obtain corroborative evidence using a second antagonist: wortmannin. Indeed, infarct size in d-my-o-Ins(1,4,5)P3-treated hearts that received a 30-min infusion of wortmannin (100 nM; n = 3) averaged 62% of the risk region, similar to the results seen in protocol 1 with LY-294002 plus d-my-o-Ins(1,4,5)P3.

It is interesting to note that, although d-my-o-Ins(1,4,5)P3 triggered a reduction of infarct size that was similar in magnitude to that achieved with PC, the 1.3-fold increase in phospho-Akt seen in d-my-o-Ins(1,4,5)P3-treated hearts was less robust than the 1.9-fold increase observed in hearts that received brief antecedent ischemia. We cannot provide a definitive explanation for this apparent lack of a direct correlation between the magnitude of Akt activation/phosphorylation and degree of cardioprotection with the two interventions. There are, however, at least three possibilities. First, the 30% increase in phospho-Akt may reflect a threshold required to render myocardites resistant to ischemia, with additional activation/phosphorylation yielding no further benefit. Second, phospho-Akt was assessed at only one time point; thus it is possible that a more substantive increase in phospho-Akt may have been manifest either earlier after treatment or beyond the start of coronary occlusion. Finally, although the administration of LY-294002 fully abrogated d-my-o-Ins(1,4,5)P3-induced cardioprotection, it is nonetheless possible that the infarct-sparing effect of d-my-o-Ins(1,4,5)P3 involves the activation of other as-yet unidentified mediators in synergy with PI3-kinase.

We observed in protocol 1 that infarct-size reduction with d-my-o-Ins(1,4,5)P3 was abolished by 5-HD, whereas a persistent infarct-sparing effect of d-my-o-Ins(1,4,5)P3 was seen despite HMR-1098 infusion. Although interpretation of these data must be tempered by the possibility that, as with all pharmacological antagonists, the agents may not be selective for the proposed targets (9, 31), the results implicate the involvement of mitochondrial, but not sarcolemmal, KATP channels in the protection afforded by d-my-o-Ins(1,4,5)P3.

We cannot, from the design of protocol 1, discern whether mitochondrial KATP channels are a distal component of the signaling pathway(s) culminating in d-my-o-Ins(1,4,5)P3-induced protection or whether, as postulated with ischemic PC, opening of mitochondrial KATP channels also serves a proximal, trigger role (9, 16, 19, 31). We did, however, conduct two sets of post hoc experiments in an effort to provide initial insight into this issue. First, AN/AR was delineated in six additional hearts in which administration of 5-HD was delayed: i.e., infusion was begun 5 min after d-my-o-Ins(1,4,5)P3 treatment. Interestingly, an intermediate infarct size of 49 ± 8% was obtained, thereby raising the possibility that the opening of mitochondrial KATP channels may commence within the initial minutes after d-my-o-Ins(1,4,5)P3 treatment. Second, an additional eight hearts [4 controls and 4 treated with d-my-o-Ins(1,4,5)P3] were infused with 5-HD as described in protocol 1, and tissue was sampled at the onset of sustained coronary occlusion for assessment of total- and phospho-Akt with the use of the same methods employed in protocol 2. We observed a 33% increase in the immunoreactivity of phospho-Akt in 5-HD plus d-my-o-Ins(1,4,5)P3-treated hearts versus 5-HD controls, similar to the 1.3-fold increase in phospho-Akt seen with the administration of d-my-o-Ins(1,4,5)P3 in protocol 2. This persistent activation of PI3-kinase/Akt in the presence of 5-HD may be interpreted as evidence that opening of mitochondrial KATP channels occurs downstream of Akt phosphorylation.

However, a second interpretation is that opening of mitochondrial KATP channels and activation of PI3-kinase/Akt may be in parallel and, possibly, proximal to signaling events, both of which are required for d-my-o-Ins(1,4,5)P3-induced cardioprotection. Although we cannot, on the basis of these pilot data, discriminate between these two explanations, the latter concept would appear to be consistent with our post hoc necrosis data, implying a potential trigger role of mitochondrial KATP channels in the infarct-size reduction achieved with d-my-o-Ins(1,4,5)P3.

In summary, our results reveal that PI3-kinase and mitochondrial KATP channels contribute to the limitation of infarct size afforded by d-my-o-Ins(1,4,5)P3. Further studies are, how-
ever, required to establish the precise temporal profile and relative sequence of PI3-kinase/Akt activation and opening of mitochondrial K$_{ATP}$ channels after administration of d-Ins(1,4,5)P$_3$ and to obtain a comprehensive characterization of the cellular signaling components (including identification of downstream targets) responsible for evoking d-Ins(1,4,5)P$_3$-induced protection. Moreover, the current protocols do not address the intriguing concept that, as with other cardioprotective strategies in which upregulation of PI3-kinase plays a role, a reperfusion component (i.e., the upregulation of kinases on relief of sustained ischemia) may be involved (11). Nonetheless, these data provide the first insight into the mechanisms by which d-Ins(1,4,5)P$_3$ renders the rabbit heart resistant to infarction.

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