Targeted deletion of A2A adenosine receptors attenuates the protective effects of myocardial postconditioning

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¹Division of Critical Care Medicine, St. Jude Children’s Research Hospital, Memphis and ²Department of Physiology, University of Tennessee Health Sciences Center, Memphis, Tennessee; ³Université Libre de Bruxelles, Brussels, Belgium; and ⁴Department of Physiology and Pharmacology, West Virginia University, Morgantown, West Virginia

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Morrison RR, Tan XL, Ledent C, Mustafa SJ, Hofmann PA. Targeted deletion of A2A adenosine receptors attenuates the protective effects of myocardial postconditioning. Am J Physiol Heart Circ Physiol 293: H2523–H2529, 2007. First published August 3, 2007; doi:10.1152/ajpheart.00612.2007.—Endogenous adenosine is an important ligand trigger for the cardioprotective effects of postconditioning (POC), yet it is unclear which adenosine receptor subtype is primarily responsible. To evaluate the role of A2A adenosine receptors in POC-induced protection, global ischemia-reperfusion was performed with and without POC in isolated wild-type (WT) and A2A adenosine receptor knockout (A2A,KO) mouse hearts. Injury was measured in terms of postischemic functional recovery and release of cardiac troponin I (cTnI). Activation of protective signaling with POC was assessed by Akt and extracellular signal-regulated kinase (ERK) 1/2 phosphorylation. In WT hearts, POC improved recovery of postischemic developed pressure in early (81.6 ± 6.4% of preischemic baseline vs. 37.5 ± 5.6% for non-POC WT at 1 min) and late (62.2 ± 4.2% of baseline vs. 45.5 ± 5.3% for non-POC WT at 30 min) reperfusion, reduced cTnI release by 37%, and doubled the phosphorylation of both Akt and ERK1/2. These beneficial effects of POC were blocked by treatment with the selective A2A adenosine receptor antagonist ZM-241385 during reperfusion. Postischemic functional recovery, cTnI release, and phosphorylation of Akt and ERK1/2 were not different between non-POC WT and A2A,KO hearts. In A2A,KO hearts, POC did not improve functional recovery, reduce cTnI release, nor increase phosphorylation of Akt or ERK1/2. Thus the protective effects of POC are attenuated by both selective A2A receptor antagonism and targeted deletion of the gene encoding A2A adenosine receptors. These observations support the conclusion that endogenous activation of A2A adenosine receptors is an essential trigger leading to the protective effects of POC in isolated murine hearts.

ischemia-reperfusion; cardiac troponin I; phosphorylated Akt; phosphorylated extracellular signal-regulated kinase 1/2

MYOCARDIAL INFARCTION FOLLOWING prolonged ischemia is substantially reduced by applying short interruptions in coronary flow at the onset of reperfusion. This cardioprotective phenomenon is termed postconditioning (POC). The first account of POC by Zhao et al. in 2003 (38) established that its infarct-sparing effect is on par with classical ischemic preconditioning (IPC) as described by Murry et al. in 1986 (19). While two decades of research has yielded considerable insight into the mechanisms and benefits of IPC, its clinical application remains limited since it must be initiated before an ischemic event. The potential to apply POC after an ischemic event represents a valuable therapeutic opportunity (15, 28), and therefore drives vigorous investigation into its triggers, mediators and effector mechanisms (reviewed in 8, 10, 33, 39).

Mounting evidence suggests POC and IPC share some of the same ligand triggers (8) and protective signaling pathways (10). Early studies of POC implicate endogenous adenosine as one such ligand trigger (8, 14, 15, 22, 33, 35, 39), fitting with the established notion of adenosinergic cardioprotection (12, 20, 32). Few studies have yet addressed which of the four known adenosine receptor subtypes (A1, A2A, A2B, or A3) are involved in protection by POC. Two recent reports by Kin et al. (14) and Philipp et al. (22) indicate that endogenous adenosine elicits the infarct-sparing effect of POC but not through activation of A1 receptors. However, these two studies failed to agree on whether POC-induced protection is triggered by activation of A2A, A2B, or A3 receptors, or some combination thereof. Further study is required to determine whether and to what extent each of these adenosine receptor subtypes contributes to the beneficial effects of POC.

There is also evidence that IPC and POC share postreceptor signaling pathways to induce myocardial protection (5, 24, 26, 27, 31, 36, 40, and reviewed in Ref. 9–11). Most notable among these is the reperfusion injury salvage kinase (RISK) pathway involving phosphorylation of Akt and/or extracellular signal-regulated kinase (ERK) 1/2, which ultimately leads to inhibition of mitochondrial permeability transition pore formation (10, 11). POC activates both Akt and ERK1/2 signaling in several models, including rabbit (5, 36), rat (2, 31, 40), and pig (26). Akt phosphorylation early in reperfusion is an important component of A2A-mediated cardioprotection (3, 4, 27, 34), and phosphorylation of ERK1/2 can occur through activation of all known adenosine receptor subtypes (7, 25). Although activation of the RISK pathway is common to both POC and adenosinergic cardioprotection during reperfusion, it remains unclear which adenosine receptor subtype is primarily responsible for initiating these signaling events in POC-induced protection.

Advent of gene-modified models with either targeted deletion or transgenic overexpression of adenosine receptor subtypes has improved the characterization of cardiac effects associated with the activation of each receptor (1), particularly with adjunctive use of selective pharmacological analogs (17, 29, 30). Exploiting this specificity, we sought to more fully evaluate the role of A2A adenosine receptors in POC by examining its effects on posts ischemic functional recovery, cardiac troponin I (cTnI) release, and protective signaling in...
isolated wild-type (WT) and A_{2A} adenosine receptor knockout (A_{2A}KO) mouse hearts. We hypothesized that the beneficial effects of POC would be attenuated in hearts from mice with targeted deletion of A_{2A} adenosine receptors.

**MATERIALS AND METHODS**

**Animals.** All animals were cared for in accordance with protocols approved by the Animal Care and Use Committees of St. Jude Children’s Research Hospital and the University of Tennessee Health Sciences Center. A_{2A}KO mice and their respective WT littermates were obtained from a sub colony of the original lines generated and previously characterized by Ledent et al. (16). Colonies of WT and A_{2A}KO mice were bred and maintained on site at St. Jude Children’s Research Hospital, and genotype was randomly confirmed by tail snip real-time PCR. All animals used in a given experiment originated from the same breeding series and were matched for age and weight. Both male and female mice were used, and gender representation was equal within all experimental groups. Standard laboratory food and water were available ad libitum. All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication no. 85-25, revised 1996).

A Langendorff isolated heart model. Isolated heart experiments were performed as previously described using a murine model that has been fully characterized (12, 18). In brief, mice were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg), a thoracotomy was performed, and hearts were excised in ice-cold heparinized (5 U/ml) perfusate. The aorta was cannulated with a 20-gauge, blunt-ended needle, and retrograde coronary perfusion was initiated at constant perfusate. The aorta was cannulated with a 20-gauge, blunt-ended needle, and retrograde coronary perfusion was initiated at constant pressure of 80 mmHg with modified Krebs-Henseleit buffer containing (in mM): 120 NaCl, 25 NaHCO3, 4.7 KCl, 1.2 KH2PO4, 2.5 CaCl2, 1.2 MgSO4, 15 glucose, 1 pyruvate, and 0.05 EDTA. The perfusate was equilibrated with 95% O_{2}-5% CO_{2} at 37°C, giving a pH of 7.4 and PO_{2} of ~550 mmHg. The left atrium was removed, and the left ventricle was vented with a small polyethylene apical drain. A fluid-filled balloon constructed of plastic film was inserted in the left ventricle across the mitral valve and connected to a pressure transducer. Balloon volume was modified through a stopcock attached to the pressure transducer using a 500-μl glass syringe to maintain a left ventricular diastolic pressure of 2–5 mmHg. Hearts were immersed in a water-jacketed perfusate bath maintained at 37°C. Coronary flow was continuously monitored via a Doppler flow probe (Transonic Systems, Ithaca, NY) located in the aortic perfusion line. Coronary flow, aortic pressure, and left ventricular pressure were all recorded on a PowerLab multichannel data acquisition system (ADInstruments, Castle Hill, Australia) connected to a MacIntosh G4 computer. The ventricular pressure signal was digitally processed to yield diastolic and systolic pressures, heart rate, positive dP/dt, and negative dP/dt. Left ventricular developed pressure was calculated as the difference between systolic and diastolic pressures.

**Ischemia-reperfusion and POC protocol.** All hearts were equilibrated for 30 min before ischemia-reperfusion. Beginning 10 min into equilibration, hearts were paced at 7 Hz with 2-ms square wave pulses at 20% above threshold through the remainder of equilibration. After equilibration, hearts underwent 20 min global normothermic ischemia followed by 30 min reperfusion. Global ischemia was produced by clamping the aortic inflow and simultaneously bubbling 95% O_{2}-5% CO_{2} through the organ bath to maintain a left ventricular diastolic pressure of 2–5 mmHg. Hearts were immersed in a water-jacketed perfusate bath maintained at 37°C. Coronary flow was continuously monitored via a Doppler flow probe (Transonic Systems, Ithaca, NY) located in the aortic perfusion line. Coronary flow, aortic pressure, and left ventricular pressure were all recorded on a PowerLab multichannel data acquisition system (ADInstruments, Castle Hill, Australia) connected to a MacIntosh G4 computer. The ventricular pressure signal was digitally processed to yield diastolic and systolic pressures, heart rate, positive dP/dt, and negative dP/dt. Left ventricular developed pressure was calculated as the difference between systolic and diastolic pressures.

**Western analysis of Akt and ERK1/2 activation.** Pilot studies demonstrated optimal detection of phosphorylated Akt and ERK1/2 (pAkt and pERK1/2) at 15 min reperfusion (data not shown). For this reason, a subset of hearts from each group was reperfused for 15 min, and ventricular tissue was subsequently homogenized in a lysis buffer containing 50 mM HEPES, 1 mM EDTA, 2 mM dithiothreitol (DTT), 10 mM o-phenylenediamine, 1 mM sodium orthovanadate, 1% protease cocktail inhibitor (Sigma Chemical, St. Louis, MO), and 1% Triton X-100. The lysate was incubated for 30 min on ice and centrifuged at 10,000 g for 10 min at 4°C. Aliquots of the resulting supernate were diluted 1:1 in Laemmli buffer containing 50 mM NaF and stored frozen until Western analysis. Activation of Akt and ERK1/2 was assessed by Western blot using antibodies specific for the phosphorylated forms of each kinase (pAkt antibody no. CS4058, pERK1/2 antibody no. CS9101; Cell Signaling Technology, Danvers, MA) with concomitant determination of total kinase [Akt antibody no. CS9272 (Cell Signaling Technology); ERK1/2 antibody no. SC94 (Santa Cruz Biotechnology, Santa Cruz, CA)]. All primary antibodies were applied at 1:1,000 dilution, and secondary antibody (goat anti-rabbit no. A0545; Sigma Chemical) was applied at 1:8,000. Immunoblots were visualized by enhanced chemiluminescence (Pierce, Rockford, IL) followed by autoradiography. Densitometry was performed on scanned images using NIH Image J software. Akt, pAkt, ERK1/2, and pERK1/2 were all normalized to an appropriate positive control within each gel, and the phosphorylated kinase was expressed as a ratio of the respective total kinase signal.

**Statistical analysis.** Baseline functional data for WT and A_{2A}KO groups were analyzed by unpaired Student’s t-test. Functional parameters during ischemia-reperfusion were analyzed by two-way repeated-measures ANOVA using Student-Newman-Keul’s post hoc test for multiple comparisons. One-way ANOVA was used to assess group differences in cTnI release and phosphorylated kinase. For all tests, a P value of <0.05 was considered significant.
RESULTS

Baseline function in isolated WT and A2A KO hearts. Table 1 summarizes functional parameters after 30 min equilibration in isolated murine hearts perfused at constant pressure under normothermic aerobic conditions. Average body weight, heart weight, and heart-to-body weight ratio were not different between WT and A2A KO groups. No functional differences were observed between WT and A2A KO hearts before ischemia-reperfusion. Baseline data (Table 1) are consistent with a previous characterization of hearts from this knockout model (17).

Functional effects of ischemia-reperfusion and POC in WT hearts. Figure 1 shows functional responses to ischemia-reperfusion and POC in isolated WT hearts (A–C) and isolated A2A KO hearts (D–F). Upon interruption of coronary flow at time 0 min hearts entered ischemia and rapidly lost developed pressure (Fig. 1, A and D). Diastolic pressure rose steadily between 10 and 20 min after onset of ischemia (Fig. 1, C and F). Upon reperfusion, all hearts recovered coronary flow (Fig. 1, B and E) and developed pressure (Fig. 1, A and D) and demonstrated immediate but transient reduction in diastolic pressure (Fig. 1, C and F). In early reperfusion, POC resulted in higher developed pressure compared with ischemia-reperfused but nonpostconditioned [control (CTL)] WT hearts (81.6 ± 6.4 vs. 37.5 ± 5.6% of baseline, respectively, at 1 min reperfusion; Fig. 1A). POC elicited higher coronary flow during the first 5 min of reperfusion (Fig. 1B) and

Table 1. Baseline functional data in wild-type and A2A adenosine receptor knockout mouse hearts

<table>
<thead>
<tr>
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<th>Wild Type (n = 65)</th>
<th>A2A Knockout (n = 38)</th>
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<tbody>
<tr>
<td>Age, wk</td>
<td>12.1 ± 0.2</td>
<td>12.4 ± 0.3</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>25.7 ± 0.5</td>
<td>26.8 ± 0.7</td>
</tr>
<tr>
<td>Heart wt, mg</td>
<td>111 ± 2.0</td>
<td>112 ± 3.4</td>
</tr>
<tr>
<td>Heart-to-body wt ratio, %</td>
<td>0.43 ± 0.01</td>
<td>0.42 ± 0.01</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>371 ± 4.3</td>
<td>364 ± 5.7</td>
</tr>
<tr>
<td>Coronary flow, ml·min⁻¹·g⁻¹</td>
<td>17.8 ± 0.3</td>
<td>17.6 ± 0.4</td>
</tr>
<tr>
<td>Left ventricular developed pressure, mmHg</td>
<td>139 ± 2.7</td>
<td>136 ± 3.6</td>
</tr>
<tr>
<td>Positive dP/dt, mmHg/s</td>
<td>5,704 ± 98</td>
<td>5,992 ± 100</td>
</tr>
<tr>
<td>Negative dP/dt, mmHg/s</td>
<td>3,488 ± 69</td>
<td>3,622 ± 91</td>
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All values are means ± SE; n, no. of hearts. Functional parameters were measured after 30 min of normothermic aerobic perfusion using a Langendorff apparatus. Heart rate was recorded before pacing.
lower initial diastolic pressure (18.1 ± 1.7 vs. 32.5 ± 2.9 mmHg for CTL at 1 min reperfusion; Fig. 1C). These immediate differences were not sustained through reperfusion; however, POC resulted in greater final recovery of developed pressure (62.2 ± 4.2 vs. 45.5 ± 5.3% of baseline for CTL at the end of reperfusion; Fig. 1A). Coronary flow was attenuated by treatment with the A2A receptor antagonist ZM-241385 through all but the early phase of reperfusion (Fig. 1B). Importantly, both the early and late beneficial effects of POC on recovery of developed pressure were completely blocked by ZM-241385 (Fig. 1A). In the absence of POC, ZM-241385 treatment did not effect final recovery of developed pressure in WT hearts in spite of reduced coronary flow during most of reperfusion (48.6 ± 2.0, n = 6 ZM-241385-treated vs. 45.5 ± 5.3% of baseline for untreated CTL).

Functional effects of ischemia-reperfusion and POC in A2A KO hearts. Functional responses to ischemia-reperfusion in isolated A2A KO and WT hearts are shown in Fig. 2. The pattern and extent of postischemic recovery of developed pressure was not significantly different between A2A KO and WT hearts (Fig. 2A). Coronary flow was lower in A2A KO hearts compared with WT hearts at 30, 35, and 40 min of reperfusion (Fig. 2B). During ischemia, diastolic pressure rose earlier and with greater maximal ischemic contracture in A2A KO compared with WT hearts (50.0 ± 2.4 vs. 35.5 ± 3.4 mmHg, respectively, at the end of ischemia), however, there were no differences in diastolic pressure between groups during reperfusion (Fig. 2C). POC did not improve the recovery pattern or magnitude of postischemic developed pressure (Fig. 1D), coronary flow (Fig. 1E), or diastolic pressure (Fig. 1F) in A2A KO hearts.

Postischemic release of cTnI. Figure 3 shows coronary release of cTnI during reperfusion in isolated WT and A2A KO mouse hearts. Preischemic release of cTnI was not different between groups (data not shown). In WT hearts, POC reduced cTnI release by 37% (2,389 ± 326 vs. 3,807 ± 482 ng/g for CTL), and this effect was blocked by selective A2A receptor antagonism with ZM-241385 (2,389 ± 326 vs. 3,673 ± 211 ng/g for POC and PO CZM groups, respectively). In the absence of POC, ZM-241385 treatment during reperfusion did not effect cTnI release in WT hearts (3,873 ± 331, n = 6 ZM-241385-treated vs. 3,807 ± 482 ng/g for untreated CTL). No significant difference in coronary release of cTnI was observed between WT-CTL and A2A KO-CTL hearts (3,807 ± 482 vs. 2,947 ± 312 ng/g, respectively, P = 0.15). In A2A KO hearts, POC trended toward an increase in the release of cTnI (2,947 ± 312 vs. 4,418 ± 609 ng/g for CTL and POC groups, respectively, P = 0.052).

Phosphorylation of Akt and ERK1/2. Protective signaling events associated with ischemia-reperfusion and POC in isolated WT and A2A KO hearts are depicted in Fig. 4. In WT hearts, POC more than doubled the phosphorylation of Akt (7.4 ± 1.3 vs. 3.6 ± 0.5 for CTL; Fig. 4A), and this effect was blocked by A2A receptor antagonism with ZM-241385 (3.4 ± 0.8 vs. 7.4 ± 1.3 for POC). In A2A KO hearts, POC did not alter phosphorylation of Akt (5.0 ± 0.8 vs. 6.2 ± 1.5 for CTL, Fig. 4A). In the absence of POC, Akt phosphorylation appeared greater in A2A KO hearts compared with WT hearts; however, this trend was not statistically significant (6.2 ± 1.5 A2A KO-CTL vs. 3.6 ± 0.5 WT-CTL, P = 0.09). In WT hearts, ischemia-reperfusion demonstrated a pattern of phosphorylation of ERK1/2 (Fig. 4B) similar to that observed for Akt (Fig. 4A), but no such correlation was noted in A2A KO hearts. In WT hearts, POC nearly doubled ERK1/2 phosphorylation (7.5 ± 0.9 vs. 4.5 ± 0.9 for CTL), and again this effect was blocked by A2A receptor antagonism with ZM-241385 (3.8 ± 0.4 vs. 7.5 ± 0.9 for POC). In A2A KO hearts, POC did not significantly effect ERK1/2 phosphorylation (3.6 ± 0.5 for CTL vs. 5.6 ± 1.6 for POC, P = 0.26).

DISCUSSION

The purpose of this study was to clarify the role of A2A adenosine receptors in POC by examining its effects on postischemic functional recovery, myocardial release of cTnI, and
protective signaling in isolated WT and A2AKO mouse hearts. POC improved functional recovery, decreased coronary release of cTnI, and increased phosphorylation of Akt and ERK1/2 in isolated WT hearts. Each of these endpoints of protection was reversed by a selective A2A adenosine receptor antagonist delivered during reperfusion. Furthermore, POC failed to improve functional recovery, did not reduce cTnI release, and did not significantly alter phosphorylation of either Akt or ERK1/2 in isolated A2AKO hearts. Thus the protective effects of POC are abrogated by both acute A2A receptor antagonism and targeted deletion of the gene encoding the A2A adenosine receptor subtype. Together, these data suggest that endogenous activation of A2A adenosine receptors is an essential trigger leading to the beneficial effects of POC in isolated murine hearts.

Cardioprotection through POC. Although the phenomenon of POC is only recently described (38), our understanding of its triggers, mediators, signaling mechanisms, and endpoints of protection is progressing rapidly (8, 10, 33, 39). The benefits of POC are observed across a variety of species, including human (15, 28), dog (38), pig (26), rabbit (5, 22, 35, 36), rat (2, 13, 14, 21, 31, 40), and mouse (13, 14). The hallmark of cardioprotection through POC is reduction of infarct size following ischemia-reperfusion, but its beneficial effects also include reduced acute tissue oncosis (2, 14, 21, 28, 36) and attenuation of postischemic myocardial (2, 13, 14, 40) and endothelial (38, 39) dysfunction. In the present study, we demonstrate that POC increases developed pressure and lowers diastolic pressure in the 1st min of reperfusion (Fig. 1, A and C). These early POC-induced functional benefits are associated with higher coronary flow through the first 5 min of reperfusion (Fig. 1B). Consistent with findings by Kaljusto et al. (13), we also note that POC developed pressure after 30 min without sustained effects on diastolic pressure or coronary flow.

Ischemia-reperfusion, POC, and A2A adenosine receptors. Several reports indicate that POC affects protection through endogenous adenosine (8, 9, 14, 15, 22, 33, 35, 39), but only two such studies have attempted to address which adenosine receptor subtypes participate in this response (14, 22). Because both reports show that selective A1 receptor antagonism fails to attenuate infarct size reduction by POC, it appears that POC-induced cardioprotection does not occur through A1 receptor activation. With an in vivo rat model, Kin et al. (14) demonstrated that the infarct-sparing effect of POC is reversed by treatment with either a selective A2A or A3 receptor antagonist just before and during reperfusion. This suggests that both A2A and A3 receptors contribute to protection by POC (14). In contrast, using an in situ rabbit model, Philipp et al. (22) demonstrated that the infarct-sparing effect of POC was not blocked by treatment with a selective A2A receptor antagonist but was abolished with a selective A2B receptor antagonist. This suggests that POC is mediated through activation of A2B but not A2A receptors (22). Thus there is an apparent conflict as to whether POC-induced protection involves A2A receptors. However, this disparity may be because of species variability or differences in the potency and/or selectivity of pharmacological agents.
logical ligands employed. Circumventing these issues requires either development of adenosine analogs with exclusive selectivity or the use of gene-modified models with targeted deletion of adenosine receptor subtypes (34). The present study combined a traditional pharmacological approach with the selectivity of an A2A KO model to clarify the role of A2A activation in POC-induced cardioprotection. We observed that both early and late functional benefits of POC in isolated WT hearts were eliminated by treatment with the selective A2A adenosine receptor antagonist ZM-241385 in reperfusion (Fig. 1, A and C). Furthermore, POC did not improve recovery of developed pressure, coronary flow, or diastolic pressure in A2AKO hearts (Fig. 1, D–F). These data support the hypothesis that endogenous activation of A2A adenosine receptors is a trigger of POC-induced cardioprotection.

It is important to note that this study also characterizes the functional recovery from global ischemia-reperfusion in non-POC A2AKO murine hearts using an isolated, blood-free system. In reperfusion, there were no significant differences in either developed or diastolic pressure between WT and A2AKO hearts in spite of modestly reduced coronary flow in hearts from A2AKO mice (Fig. 2). In the absence of inflammatory cells, our observation that functional recovery was neither improved nor impaired in isolated A2AKO hearts is consistent with lymphocyte inhibition as the primary protective effect of A2A activation in myocardial ischemia-reperfusion (37). Interestingly, the rise in diastolic pressure during ischemia occurred earlier and to a greater extent in A2AKO hearts (Fig. 2C) without adversely affecting diastolic or developed pressure (Fig. 2A) in reperfusion. Although not directly tested, this earlier onset of contracture could indicate decreased high energy phosphate availability and/or increased ATP utilization during ischemia in A2AKO hearts.

**POC and myocardial oncosis in isolated WT and A2AKO hearts.** Determinants of acute myocardial oncosis include creatine kinase (CK; 2, 14), lactate dehydrogenase (LDH; 2, 18, 21), and cTnI (2, 6), and levels of each parallel postsischemic injury both in vivo (6, 14) and in isolated Langendorff heart models (2, 18, 21). Bopassa et al. (2) have shown that POC equally reduces LDH, CK, and cTnI release in isolated Langendorff rat hearts with strong correlation to improved posts ischemic functional recovery. Eckle et al. (6) have further established cTnI as an excellent marker of injury by documenting its linear relationship to infarct size in an in vivo mouse model of regional coronary ischemia-reperfusion. We demonstrate here that POC decreases cTnI release in the coronary effluent of isolated WT mouse hearts (Fig. 3) concomitant with improved functional recovery at the end of reperfusion (Fig. 1A). The attenuated cTnI release in POC WT hearts was blocked by treatment with ZM-241385. This is consistent with a cardioprotective effect of endogenous A2A activation in reperfusion (3, 4, 12, 14, 20, 34, 37). Release of cTnI in non-POC A2AKO hearts was not different from that of non-POC WT hearts (Fig. 3). Again, this correlates with the lack of significant differences in posts ischemic function in ischemic-reperfused isolated WT and A2AKO hearts (Fig. 2).

**POC and phosphorylation of Akt and ERK1/2.** Reports indicate that both Akt (2, 26, 31, 40) and ERK1/2 (5, 26, 36) phosphorylation are required elements for POC-induced cardioprotection. The spectrum of upstream activators of these signals awaits full characterization (11). In the present study, we document that POC in isolated WT hearts doubles Akt and ERK1/2 phosphorylation and that this effect is blocked by selective A2A adenosine receptor antagonism with ZM-241385 (Fig. 4). Taken with the observation that neither Akt nor ERK1/2 phosphorylation is significantly altered by POC in A2AKO hearts, our data support the possibility that endogenous A2A activation acts as an important proximal trigger of POC-induced signaling. This notion is consistent with the observation that the selective A2A agonist CGS-21680 activates ERK1/2 (7) and with findings by Boucher et al. (4) in which CGS-21680 treatment just before and during the first several minutes of reperfusion leads to myocardial protection associated with Akt activation.

**Study limitations.** One of the limitations to the present study is that the expression of other adenosine receptor subtypes in the A2AKO model was not measured. This raises the possibility that changes in A1, A2B, or A3 receptor expression may have altered responses to POC. However, our results obtained with a selective A2A receptor antagonist suggest blocking the A2A receptor pathway is sufficient to attenuate the benefits of POC. Another potential limitation to the present study is that the effects of POC were examined under conditions of constant pressure perfusion. In isolated rat hearts, Penna et al. (21) have demonstrated POC was more effective with perfusion at constant flow rather than constant pressure. Although we have not evaluated different perfusion modes in isolated mouse hearts, our endpoints of interest were sufficiently improved to document the effects of POC. Finally, although we have assessed the role of A2A receptors in POC, we have not excluded the possibility that other adenosine receptor subtypes may modify POC-induced protection.

**Conclusions and future directions.** We demonstrate that the beneficial effects of POC in isolated WT mouse hearts include attenuated posts ischemic myocardial dysfunction, reduced myocardial release of cTnl, and increased activation of salvage kinase signaling through phosphorylation of Akt and ERK1/2. Each of these protective responses was reversed by selective pharmacological blockade of A2A adenosine receptors in reperfusion. Moreover, in A2AKO hearts, POC did not improve functional recovery, reduce cTnI release, or increase phosphorylation of either Akt or ERK1/2. Thus these observations support the conclusion that endogenous activation of A2A adenosine receptors is an essential trigger leading to the beneficial effects of POC in isolated murine hearts. Further work combining the A2AKO model with selective analogs of A1, A2B, and/or A3 receptors may clarify whether other adenosine receptor subtypes may modify POC-induced protection. Fully characterizing the adenosine receptor-mediated triggers of POC may provide a foundation for novel pharmacological therapies aimed at mimicking its protective effects in the clinical management of heart disease.

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**REFERENCES**


34. Yang XM, 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 quanlyl cyclase activation. Basic Res Cardiol 100: 57–63, 2005.


