Delayed adenosine A₁ receptor preconditioning in rat myocardium is MAPK dependent but iNOS independent

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Lasley, Robert D., Byron J. Keith, Gentian Kristo, Yukihiro Yoshimura, and Robert M. Mentzer, Jr. Delayed adenosine A₁ receptor preconditioning in rat myocardium is MAPK dependent but iNOS independent. Am J Physiol Heart Circ Physiol 289: H785–H791, 2005. First published April 20, 2005; doi:10.1152/ajpheart.01008.2004.—Adenosine A₁ receptor delayed preconditioning (PC) against myocardial infarction has been well described; however, there have been limited investigations of the signaling mechanisms that mediate this phenomenon. In addition, there are multiple conflicting reports on the role of inducible nitric oxide synthase (iNOS) in mediating A₁ late-phase PC. The purpose of this study was to determine the roles of the p38 and extracellular signal-regulated kinase (ERK) mitogen-activated protein kinases (MAPKs) in in vivo delayed A₁ receptor PC and whether this protection at the myocyte level is due to upregulation of iNOS. Myocardial infarct size was measured in open-chest anesthetized rats 24 h after treatment with vehicle or the adenosine A₁ agonist 2-chloro-N⁶-cyclopentyladenosine (CCPA; 100 μg/kg ip). Additional rats receiving CCPA were pretreated with the p38 inhibitor SB-203580 (1 mg/kg ip) or the MAPK/ERK kinase (MEK) inhibitor PD-098059 (0.5 mg/kg ip). At 24 h after CCPA administration, a group of animals was given the iNOS inhibitor 1400 W 10 min before ischemia. Treatment with CCPA reduced infarct size from 48 ± 2 to 28 ± 2% of the area at risk, an effect that was blocked by both SB-203580 and PD-098059 but not 1400 W. Ventricular myocytes isolated 24 h after CCPA injection exhibited significantly reduced oxidative stress during H₂O₂ exposure compared with myocytes from vehicle-injected animals, and this effect was not blocked by the iNOS inhibitor 1400 W. Western blot analysis of whole heart and cardiac myocyte protein samples revealed no expression of iNOS 6 or 24 h after CCPA treatment. These results indicate that adenosine A₁ receptor delayed PC in rats is mediated by MAPK-dependent mechanisms, but this phenomenon is not associated with the early or late expression of iNOS.

agonist; reperfusion; infarct size; p38; extracellular signal-related kinase; cardiac myocytes; mitogen-activated protein kinase; inducible nitric oxide synthase

SIGNIFICANT EVIDENCE HAS ACCUMULATED that ischemic and pharmacological preconditioning (PC) against myocardial ischemia-reperfusion injury occurs in two distinct phases. The effects of acute PC may persist for ≥60 min, after which protection is lost. A second window of protection, also referred to as delayed or late-phase PC, appears 24 h after the PC stimulus and may persist for up to 72 h (5, 6, 29). The primary difference between acute and delayed PC appears to be the role of induced or increased expression of various proteins [such as inducible NO synthase (iNOS), manganese superoxide dismutase, and cyclooxygenase-2, to name a few] after the acute PC stimulus (5, 6, 9, 18, 32, 41).

Despite numerous reports implicating the upregulation of various cardioprotective proteins in delayed PC, there have been few reports investigating the upstream signaling pathways in this phenomenon. The stimulation of one or more mitogen-activated protein kinases (MAPKs) such as the p38 and p42/p44 extracellular signal-related kinase (ERK) MAPK families results in the translocation of these active kinases to the nucleus to initiate gene transcription (24). In the only in vivo study to date addressing this signaling mechanism, Fryer et al. (11) reported that opioid-induced late-phase PC was blocked by both p38 and ERK inhibitors. Although there are numerous reports of adenosine A₁ receptor delayed PC (2–4, 7–10, 21–23, 26, 38, 46, 47) and evidence that adenosine and A₁ receptor agonists induce the activation of both p38 and ERK (1, 13, 15, 25, 34, 35, 37), there have been no in vivo studies determining the role of these kinases in delayed PC.

Another unresolved aspect of A₁ receptor delayed PC is what specific proteins are upregulated in this phenomenon. The protein that has received the most attention in mediating delayed ischemic and pharmacological PC is iNOS. Although adenosine A₁ receptor agonists induce delayed PC in multiple species, there is conflicting evidence for the involvement of iNOS (4, 8, 30, 31, 47). Two different iNOS inhibitors failed to block A₁ delayed PC in rabbit myocardium (8). Two studies in murine myocardium utilizing iNOS-knockout mice have also yielded conflicting results on the role of iNOS in adenosine A₁ receptor late-phase PC (4, 47). In addition, although there is evidence that delayed ischemic PC in murine and canine myocardium is associated with iNOS upregulation in cardiac myocytes (19, 41), there have been no reports of direct testing of whether A₁ delayed PC protects cardiac myocytes via an iNOS-dependent mechanism.

The present study was designed to address these limitations in the literature. In vivo studies were conducted to determine whether A₁ receptor delayed PC against myocardial infarction in rats is dependent on p38 and/or ERK MAPKs. The role of iNOS in mediating this phenomenon was tested both in adult rat ventricular myocytes submitted to oxidative stress and in vivo rat hearts.

MATERIALS AND METHODS

All animals in this study received humane care according to the guidelines set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, 1996) and the Guide for the Care and Use of Laboratory Animals. Address for reprint requests and other correspondence: R. D. Lasley, Dept. of Surgery, Univ. of Kentucky College of Medicine, MN276, Chandler Medical Center, 800 Rose St., Lexington, KY 40536-0298 (E-mail: rlasley@uky.edu).

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with a mixture of room air and 100% O2. Tidal volume, respiratory
the rats were ventilated (model 683; Harvard Apparatus; Natick, MA)
and xylazine (1 mg/kg ip). Anesthesia was maintained with additional
(300–375 g body wt) were anesthetized with ketamine (75 mg/kg ip)
24 h before the experiment. Additional rats were treated 30 min before
delayed PC was induced by injecting rats with the A1 agonist
N2-chloro-
tal protocol for the in vivo infarct protocols. All rats were subjected to
administered vehicle (10% DMSO in saline; 1 ml ip; 1400W, and CGS-21680 were made as concentrated stock solutions in
DMSO and were diluted in saline immediately before use.
Determination of infarct size. After 2 h of reperfusion, the coronary
artery was reoccluded, and 1.5 ml of 5% Evans blue solution was
injected via the jugular vein. The ischemic area at risk (AAR) was
devoid of the Evans blue stain. The heart was excised, and the left
ventricle (LV) was removed from the remaining tissue and subse-
sequently cut into four slices of equal thickness in a plane parallel to the
atrioventricular groove. The slices were incubated in a 1.0% triphen-
ylterazolium chloride ( TTC) solution in phosphate-buffered saline
solution at 37°C for 15 min. Slices were then compressed to a uniform
thickness of 2 mm by placing them between two transparent Plexiglas
plates. The area of nonischemic LV (stained blue), the AAR (stained
red by TTC), and the infarcted area (TTC negative) were quantified
using graphic analysis software (SigmaScan Pro automated image-
analogue software; Jandel Scientific; SPSS; San Rafael, CA). AAR
was expressed as a percentage of the LV (AAR/LV), and infarct size
(IS) was expressed as a percentage of the AAR (IS/AAR).
Isolation of rat ventricular myocytes. Ventricular myocytes were isolated from adult male Sprague-Dawley rats (275–325 g body wt) as
previously described (21) with minor modifications. The excised heart
was perfused with HEPES medium that contained (in mM) 118 NaCl,
4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 10 HEPES, 11 glucose, and 2
pyruvate, pH 7.2 for 5 min in a nonrecirculating mode. Collagenase
(Liberase type 2; 0.14 mg/ml; Roche Biochemicals; Indianapolis, IN)
was then added to the medium and recirculated for 15–20 min. During
the final 10 min, calcium was gradually reintroduced to a final
concentration of 500 μM. The heart was removed from the cannula
and minced, and the myocytes were mechanically dispersed. Disso-
ciated myocytes were suspended in HEPES buffer (pH 7.40, 1 mM
CaCl2) until use. Myocytes were allowed to equilibrate for 60 min
before protocols were initiated. Experiments were conducted in
HEPES buffer within 6 h of isolation. Rats were injected with vehicle
or CCPA (100 μg/kg ip) 24 h before isolation of ventricular myocytes.
Isolated myocyte protocols. Myocytes were exposed to H2O2, and
the extent of oxidative stress was determined with the fluorescent dye
dichlorofluorescein diacetate (DCFDA) as previously described (21).
Myocytes were loaded with DCFDA (5 μM) for 15 min at room
temperature in the dark. The myocytes were washed with HEPES
medium to remove extracellular dye and incubated for an additional
30 min to allow for intracellular decacytation of DCFDA, which
results in the formation of the reduced and weakly fluorescent dichlo-
rofluorescein moiety. Upon oxidation by reactive oxygen and reactive
nitrogen species (ROS and RNS, respectively), reduced dichlorofluo-
rescein is converted to the highly fluorescent dichlorofluorescein
(DCF).
An aliquot of dye-loaded myocytes was then allowed to settle on
laminin-coated coverslips placed in a 300-μl temperature-controlled
recording chamber (model RC-24 chamber and model TC-324B
temperature controller; Warner Instruments; Hamden, CT) on the
stage of an Olympus IX-70 inverted microscope (Olympus America; Melville, NY). Cells were suffused with normal HEPES buffer (pH
7.4 at 37°C) at a flow rate of 1 ml/min. The first baseline DCF fluo-
rescence values were recorded after 5 and 20 min of suffusion before the
cells were exposed to H2O2 (150 μM), which induced oxidative stress
that was maintained for 10 min. All cell fluorescence values were
background corrected, and increases in DCF during H2O2 exposure
were expressed relative to each cell’s baseline fluorescence.
An excitation wavelength of 490 nm was used, and emission
fluorescence was collected at 520 ± 5 nm. To reduce excitation-
dependent oxidation of the dye, a neutral-density filter (ND12) was
placed in the path of the excitation source (a 75-W xenon arc lamp),
and sampling times were limited to 160 ms. Epifluorescence was
collected by a charge-coupled device camera attached to a side port of
the microscope, and fluorescence intensity analysis was done on a
Pentium processor with custom-made software.
A subset of CCPA-preconditioned myocytes was treated with the selective iNOS inhibitor 1400W (1 μM). Treatment was initiated for 15 min before the final baseline DCF measurement was made and was continued throughout the H2O2 exposure.

**Western blot analysis.** To determine whether A1 receptor delayed-phase PC was associated with upregulation of iNOS, rats (n = 3) were injected with vehicle or CCPA (100 μg/kg ip) 6 and 24 h before isolation of ventricular myocytes. Myocytes were washed with ice-cold PBS and resuspended in two volumes of ice-cold buffer A that contained 250 mM sucrose, 20 mM HEPES, 10 mM KCl, and 1.5 mM MgCl2, pH 7.4 with aprotinin (2 μg/ml), leupeptin (2 μg/ml), pepstatin A (2 μg/ml), benzamidine (0.1 mM), and phenylmethylsulfonyl fluoride (0.1 mM). This suspension reached 20 strokes with a Teflon Dounce homogenizer on ice, which was followed by sonication (three times for 10 s each at 4°C) and centrifugation (750 g, for 10 min at 4°C) to sediment the nuclear fraction. The postnuclear supernatant was centrifuged at 105,000 g for 1 h to generate cytosolic fractions. Additional rats received injections of lipopolysaccharide (LPS, 10 mg/kg ip) either 6 or 16 h before isolation of the heart. Whole heart and liver cytosolic fractions were generated as described above after hearts were homogenized with a Polytron. Protein concentrations were determined by Bradford assay.

Proteins (50–120 μg) from the myocyte-, heart-, and liver-soluble fractions were subjected to 8% SDS-PAGE under reducing conditions. After electrophoresis, the proteins were electroblotted to polyvinylidene difluoride membranes. The membranes were blocked (for 1 h) with Tris-buffered saline (TBS) that contained 5% milk and 0.5% Tween 20. Incubation (overnight at 4°C) of anti-iNOS (1:1,000 dilution) affinity-purified IgG (Transduction Labs; Lexington, KY) in TBS (1% milk with 0.2% Tween 20) was followed by washes with TBS (0.2% milk with 0.2% Tween 20). Secondary antibody (1:7,500 dilution) conjugated with horseradish peroxidase was incubated (for 1 h) with TBS (1% milk with 0.2% Tween 20) followed by washing. The membrane was placed in enhanced chemiluminescent substrate (for 5 min) and exposed to Kodak X-OMAT film for ≤10 min.

**Chemicals.** PD-098059 and SB-203580 were purchased from LC Laboratories (Woburn, MA). CCPA, CGS-21680, 1400W, and LPS were obtained from Sigma-Aldrich (St. Louis, MO). DCFDA was purchased from Molecular Probes (Eugene, OR).

**Data analysis.** Data are expressed as means ± SE. Among-group differences in infarct size were determined by one-way ANOVA. For the H2O2 protocols, 6–10 myocytes were studied from 4 or 5 myocyte isolations. Group differences were determined by a two-way ANOVA and subsequent Newman-Keuls post hoc analysis. A P value < 0.05 was considered statistically significant.

**RESULTS**

**In vivo results.** Seven animals were excluded from the study, three due to technical errors and four due to a mean blood pressure < 30 mmHg during reperfusion. A summary of the hemodynamic parameters during the ischemia-reperfusion protocols is shown in Table 1. There were no differences among the groups in heart rate or mean arterial pressure (MAP) before or during ischemia or during reperfusion. Coronary occlusion was associated with decreased MAP (vs. baseline values) in the vehicle control, 24-h CCPA, PD-098059 with CCPA, CCPA with 1400W, and high-dose CGS-21680 groups, which persisted throughout reperfusion in the former four groups.

Figure 2 summarizes the in vivo IS findings. There were no significant differences in the sizes of the ischemic zones, which were ~35% of the LV. Rats injected with vehicle 24 h before the open-chest protocol exhibited an IS of 48 ± 2% of the AAR (Fig. 2). IS after CCPA 24-h PC was 28 ± 2%, which is a 42% reduction. Treatments with the p38 and MEK inhibitors SB-203580 and PD-098059, respectively, before CCPA injection resulted in ISs of 40 ± 2 and 50 ± 4%, respectively. Preischemic administration of the iNOS inhibitor 1400W in rats injected with CCPA 24 h before the experiment was associated with 31 ± 1% infarction. Treatment with two doses of the adenosine A2a agonist CGS-21680 24 h before regional ischemia had no effect on IS (42 ± 5 and 41 ± 7% in the low- and high-dose groups, respectively).

**In vitro results.** In cells loaded with the reactive species-sensitive fluorescent indicator DCFDA, exposure to H2O2 (150 μM) resulted in a significant increase in DCF fluorescence. As shown in Fig. 3 in myocytes (n = 43) isolated from saline-

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**Table 1. Hemodynamics during in vivo regional ischemia protocol**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CCPA</th>
<th>SB-203580 + CCPA</th>
<th>PD-098059 + CCPA</th>
<th>CCPA + 1400W</th>
<th>CGS-21680</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Low</td>
<td>High</td>
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<tr>
<td>Heart rate, beats/min</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>249 ± 5</td>
<td>253 ± 10</td>
<td>266 ± 11</td>
<td>272 ± 23</td>
<td>252 ± 6</td>
<td>255 ± 9</td>
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<tr>
<td>Ischemia</td>
<td>225 ± 16</td>
<td>229 ± 11</td>
<td>276 ± 19*</td>
<td>266 ± 23</td>
<td>223 ± 11</td>
<td>238 ± 21</td>
</tr>
<tr>
<td>Reperfusion (2 h)</td>
<td>264 ± 16</td>
<td>279 ± 15</td>
<td>262 ± 13</td>
<td>234 ± 27</td>
<td>235 ± 13</td>
<td>229 ± 17</td>
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<tr>
<td>Mean arterial pressure, mmHg</td>
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<tr>
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<td>91 ± 2</td>
<td>88 ± 3</td>
<td>89 ± 1</td>
<td>93 ± 5</td>
<td>86 ± 1</td>
<td>88 ± 1</td>
</tr>
<tr>
<td>Ischemia</td>
<td>74 ± 3†</td>
<td>76 ± 2†</td>
<td>77 ± 8</td>
<td>62 ± 4†</td>
<td>58 ± 3†</td>
<td>72 ± 5</td>
</tr>
<tr>
<td>Reperfusion (2 h)</td>
<td>74 ± 6†</td>
<td>76 ± 2†</td>
<td>71 ± 6</td>
<td>60 ± 8†</td>
<td>59 ± 6†</td>
<td>72 ± 8</td>
</tr>
</tbody>
</table>

Values are means ± SE. Baseline values were taken before the coronary artery occlusion; ischemia values were taken at 15 min of occlusion. CCPA, 2-chloro-N6-cyclopentyladenosine. *P < 0.05 vs. control group; †P < 0.05 vs. baseline.
injected rats, DCF fluorescence increased 10.7 ± 2.3- and 33.4 ± 4.3-fold compared with baseline values after 5 and 10 min of exposure to H₂O₂. In contrast, in ventricular myocytes (n = 44) from CCPA-injected rats, DCF fluorescence increased only 5.6 ± 0.7-fold after 5 min of H₂O₂ exposure and 15.4 ± 1.2-fold after 10 min of H₂O₂ exposure (both values, P < 0.05 vs. myocytes from vehicle-injected rats). Results in Fig. 2 also indicate that CCPA 24-h PC of cardiac myocytes was not blocked by the iNOS inhibitor 1400W, as myocytes in this group (n = 29) exhibited identical increases in DCF fluorescence as those in the absence of this inhibitor.

The Western blot results were consistent with the above pharmacological observations. Figure 4 shows that iNOS immunoreactivity was not observed in myocyte cytosolic fractions (lanes 2–4) isolated from vehicle-injected rats or in normal liver cytosol (lane 8). Cytosolic fractions (lanes 5 and 6) obtained from myocytes isolated from CCPA-injected rats showed no expression of iNOS, similar to what was observed in the vehicle-injected animals. Protein from LPS-injected rats was analyzed as a positive control. Robust expression of iNOS was observed in whole heart (lane 1) and liver (lane 7) cytosolic fractions 6 h after LPS treatment, although to a much greater extent in the liver.

In the course of generating the LPS-positive controls, we observed that there was much greater expression of whole heart iNOS at 6 h (lane 7) after LPS treatment than after 16 h (lane 8; Fig. 5). To exclude the possibility that iNOS was induced only in the early phase of delayed A₁ agonist PC, protein was obtained from myocytes 6 h after CCPA treatment. Cardiac myocytes from rats isolated 6 h after CCPA treatment (lanes 3 and 4) exhibited no induction of iNOS, similar to results observed after 24 h. Examination of endothelial NOS (eNOS) expression revealed no difference between CCPA- and saline-treated animals (data not shown).

**DISCUSSION**

The results of this study indicate that A₁ receptor delayed PC against infarction in rat myocardium appears to be mediated by the activation of both p38 and ERK MAPKs. Delayed A₁ PC was evident at the cardiac myocyte level, as ventricular myocytes isolated from A₁ agonist-injected rats exhibited increased resistance to oxidative stress. These beneficial effects, however, were not blocked by the iNOS inhibitor 1400W, nor were they associated with the inducement of iNOS in either the whole heart or cardiac myocytes.

Ischemic and pharmacological PC have been shown to exert both acute and delayed protection against myocardial cell death in multiple preparations and in every species tested to date including human cardiac tissue (5–7, 22, 26, 29). The observations that acute PC wanes after several hours but then reappears 24 h later has led to the hypothesis that this phenomenon is due to the upregulation or inducement of one or more cardioprotective proteins such as antioxidant enzymes, iNOS, and cyclooxygenase-2 (5, 6, 9, 18, 32, 41). One mechanism for increasing gene expression is the activation of MAPKs such as the p38 and p42/p44 ERK. The activation of MAPKs by extracellular stimuli and G protein-receptor agonists results in the phosphorylation of numerous substrates including various transcription factors that regulate gene expression (24).

There have been several reports to date implicating the p38 pathway in acute and delayed ischemic and pharmacological PC (11, 12, 27). There is also evidence that adenosine receptor activation can activate p38 MAPK in several tissues including heart (13, 15, 25, 35, 37). We (1) recently reported that acute adenosine receptor PC is associated with increased p38 activation before ischemia. Despite these findings, there have been few reports of the role of p38 in adenosine A₁ receptor delayed PC. In the only studies to date, the p38 inhibitor SB-203580 has been reported to block A₁ agonist late-phase PC in isolated mouse heart (46) and in human atrial tissue (7, 26). The results of an additional study (10) indicated that rabbit myocardium exhibited a significant increase in p38 activity 24 h after A₁ agonist treatment, but the effects of p38 inhibition were not assessed. Fryer et al. (11) reported that the p38 inhibitor SB-203580 blocked opioid receptor late-phase PC in vivo rat myocardium. The results of the present study suggest that p38

![Fig. 3. Dichlorofluorescin (DCF) fluorescence in isolated ventricular myocytes exposed to H₂O₂ (150 μM). Myocytes were isolated from vehicle or CCPA-injected rats 24 h after treatment. DCF fluorescence for each cell was normalized to baseline values. The iNOS inhibitor 1400W was applied to the myocytes for 15 min before H₂O₂ exposure. *P < 0.05 vs. vehicle-injected myocytes. PC, preconditioned.](image)

![Fig. 4. Western blot analysis of myocyte cytosolic fractions 24 h after treatment. Myocyte cytosolic proteins (50 μg) from vehicle- (lanes 2–4) and CCPA-injected (lanes 5 and 6) rats were loaded onto the gel. Heart (lane 1) and liver (lane 7) cytosolic fractions (50 μg) from rats treated with lipopolysaccharide (LPS; 6 h prior) were used as iNOS-positive controls. Lane 8 is the vehicle-treated liver cytosolic fraction. MW, Molecular weight.](image)

![Fig. 5. Western blot analysis of iNOS after 6 h of treatment. Lanes 1 and 2 are liver protein samples (20 μg) obtained 6 h after LPS (lane 1) or saline (lane 2) treatment, respectively. Lanes 3 and 4 are myocyte cytosol (50 μg) obtained 6 h after CCPA injection. Lanes 5 and 6 are CCPA and saline 24 h samples, respectively. Lanes 7 and 8 show supernatant from whole heart (50 μg) after 6 or 16 h of LPS treatment, respectively.](image)
plays a key role in mediating in vivo adenosine A1 receptor delayed PC in rat myocardium.

In contrast to the several reports of p38 involvement in late-phase PC, there has been only one study published to date on the role of ERK in this phenomenon. Fryer et al. (11) reported that the same MEK inhibitor that we used in the present study, PD-098059, blocked δ-opioid agonist delayed PC in in vivo rat myocardium. Adenosine receptor activation, including the A1 receptor, activates ERK in several tissues including neonatal rat cardiac myocytes (13, 15, 35, 37). We recently reported that acute adenosine receptor PC in in vivo rat myocardium is associated with significant activation of both p42 and p44 isoforms (34). Our findings are the first report that the MEK inhibitor PD-098059 blocks A1 receptor delayed PC, which suggests a key role for the downstream ERK pathway in mediating this phenomenon.

Although there have been numerous reports of adenosine A1 delayed PC, there has been only one study determining the effects of A2a receptor agonists. Takano et al. (7) reported that the A2a receptor agonist CGS-21680 did not induce delayed PC against myocardial infarction in rabbits. We observed similar results in the present study with two different doses of CGS-21680. On the surface, these results may be expected; however, there are reports that A2a receptor stimulation, in several tissues including the heart, activates MAPK ERK (13, 20, 37, 43). Studies of A2a receptor modulation of ERK in intact myocardium, however, have been limited to reduction of infarct size via reperfusion A2a agonist treatments (20, 43). Thus cardiac A2a receptors may not couple to the ERK pathway in normal myocardium.

Because activated p38 and ERK translocate to the nucleus to phosphorylate transcription factors, the evidence that p38 and ERK MAPKs are components of delayed PC is consistent with the hypothesis that this phenomenon is due to the upregulation of protective proteins. One of the proteins that has received the most attention is iNOS. Although there are multiple reports showing that both ischemic and pharmacological delayed PC are associated with the upregulation of iNOS (14, 18, 19, 32, 40–42, 44, 45, 47), there are conflicting reports on whether this protein plays a role in adenosine A1 receptor late-phase PC (4, 8, 30, 31, 47). Takano et al. (38) first reported that adenosine A1 delayed PC occurred via an NOS-dependent pathway in rabbit myocardium, although it was not determined whether iNOS was the mediator. In a subsequent study, also in rabbit myocardium, two different iNOS inhibitors, both of which blocked delayed ischemic PC, failed to inhibit A1 receptor delayed PC (8).

There are similar conflicting reports for murine myocardium. One report indicated that the A1 receptor agonist CCPA increased iNOS protein expression in wild-type mice, and delayed A1 PC in isolated perfused mouse hearts was blocked by an iNOS inhibitor (47). In addition, CCPA did not induce late-phase PC in iNOS-knockout mice (47). In contrast, Bell and colleagues (4) reported that, although A1 agonist treatment did increase iNOS upregulation in wild-type mice, this did not appear to be necessary for delayed PC, because delayed A1 PC could be still be induced in iNOS-knockout mice. These authors in fact observed that eNOS was upregulated 24 h after CCPA treatment (4). Nayem et al. (30) reported that isolated cardiac myocytes from A1 receptor-overexpressing mice did require iNOS for protection against simulated ischemia-induced cell death but that adenosine A1 delayed PC could be induced in myocytes from iNOS-knockout mice (31). However, both of these conclusions were based solely on results obtained with iNOS inhibitors, and iNOS expression was not examined.

The results of the present study indicate that adenosine A1 receptor delayed PC in rat myocardium is not associated with the expression of iNOS in ventricular myocytes. These results are consistent with the inability of the selective iNOS inhibitor 1400W to inhibit the anti-infarct effects of 24 h of PC with CCPA or to block the increased resistance to oxidative stress in cardiomyocytes isolated from CCPA-injected rats. Our findings are consistent with those of Igarashi et al. (16), who reported that iNOS induction in neonatal rat cardiomyocytes was associated with exacerbation of injury during oxidative stress. In addition, adenosine A1 receptor agonist pretreatment has been shown to decrease iNOS upregulation and mortality in mice after LPS treatment (28). Treatment with LPS was associated with robust myocardial iNOS expression 6 h after treatment, which was significantly diminished after 16 h. This same transient pattern of iNOS expression has been reported after LPS treatment in mice (28) and during monophosphoryl lipid A-induced PC in rats (40) and pigs (44). Given these time-dependent observations, we tested for myocyte iNOS expression 6 h after CCPA treatment, which also turned up negative. We were also not able to detect any iNOS expression in the whole heart. These findings suggest that iNOS is neither a trigger nor a mediator of adenosine A1 receptor delayed PC in rat myocardium.

Although numerous investigators have reported the upregulation of iNOS in late-phase ischemic and pharmacological PC (14, 18, 19, 32, 40–42, 44, 45, 47), there have been few attempts to determine the cellular or subcellular source of this protein. The importance of this issue is supported by the findings of Poon et al. (33), who reported that the differential expression of iNOS in myocytes and neutrophils during sepsis determined whether this protein exerted beneficial or detrimental effects. Late-phase ischemic PC in mouse myocardium is associated with increased iNOS expression with some immunohistochemical evidence that this occurred in myocytes (41). However, there was substantial constitutive iNOS expression in both cytosolic and membrane fractions, and iNOS expression increased in the nonischemic zones. In contrast, we observed essentially no iNOS in normal rat myocardium or myocytes. Ventricular myocytes isolated from canine myocardium 24 h after ischemic PC did exhibit functional evidence of increased iNOS expression, but immunohistochemical analysis of ventricular myocardium revealed only sparse myocyte iNOS with the greatest iNOS expression in the perivascular space (19). Rui et al. (36) observed that delayed PC (using anoxia-reoxygenation) could be induced in neonatal cardiomyocytes isolated from both wild-type and iNOS-deficient mice, and delayed PC in the former was not associated with upregulation of iNOS. To our knowledge the present findings are the first to directly examine whether delayed pharmacological PC is associated with the inducement or upregulation of a specific protein in isolated ventricular myocytes. Our functional and biochemical findings indicate no role for cardiomyocyte iNOS in mediating adenosine A1 receptor delayed PC in rat myocardium.
In summary, in vivo adenosine A₁ receptor delayed PC against myocardial infarction is mediated by both p38 and ERK-dependent pathways. This protection extends to the cardiomyocyte level, as isolated ventricular myocytes from A₁ agonist-injected rats exhibited significantly reduced oxidative stress during H₂O₂ exposure. However, A₁ receptor delayed PC in rat myocardium is not associated with the inducement of iNOS.

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