Acute adenosine preconditioning is mediated by p38 MAPK activation in discrete subcellular compartments

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Baller-Croft, Cherry, Gentian Kristo, Yukihiro Yoshimura, Easton Reid, Byron J. Keith, Robert M. Mentzer, Jr., and Robert D. Lasley. Acute adenosine preconditioning is mediated by p38 MAPK activation in discrete subcellular compartments. Am J Physiol Heart Circ Physiol 288: H1359–H1366, 2005. First published November 11, 2004; doi:10.1152/ajpheart.01006.2004.—Although acute adenosine preconditioning (PC) is well established, the signaling pathways mediating this cardioprotection remain unclear. Because adenosine receptor agonists activate p38 MAPK and this kinase has been implicated in ischemic and pharmacological PC, the purpose of this study was to determine the role of p38 MAPK in acute adenosine receptor PC. The role of p38 MAPK activation in discrete subcellular compartments during ischemia-reperfusion was also determined. The following groups were used in an in vivo rat ischemia-reperfusion model: 1) control (10% DMSO iv), 2) the A1/A2a adenosine receptor AMP-579 (50 μg/kg iv), 3) AMP-579 + the A1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 100 μg/kg iv), 4) AMP-579 + the p38 MAPK inhibitor SB-203580 (1 mg/kg iv), and 5) SB-203580 alone. p38 MAPK activation was measured by Western blot analysis in cytosolic, mitochondrial, membrane, and nuclear/myofibrillar fractions obtained from hearts at preischemic, ischemic, and reperfusion time points. A significant reduction in infarct size was observed with AMP-579 PC, an effect blocked by DPCPX or SB-203580 pretreatment. AMP-579 treatment was associated with a significant increase in p38 MAPK activation in the nuclear/myofibrillar fraction before ischemia, whereas no activation of this kinase occurred during ischemia or reperfusion. In contrast, p38 MAPK was activated in the mitochondrial fraction by ischemia and in the cytosolic, mitochondrial, and membrane fractions by reperfusion in the control group. SB-203580 blocked the AMP-579-induced increase in phosphorylation of the downstream p38 substrate activating transcription factor-2. These results suggest a role for p38 MAPK activation in discrete subcellular compartments in acute adenosine A1 receptor PC.

A1 receptor; in vivo rat model; compartmentation; ischemia-reperfusion

MYOCARDIAL PRECONDITIONING (PC) is the phenomenon whereby a brief period of ischemia-reperfusion or exposure to certain G protein-coupled receptor (GPCR) agonists protects the heart against damage induced by a subsequent period of sustained ischemia (7, 25). Adenosine A1 receptor agonists are one type of GPCR agonist. Although adenosine A1 receptor agonist PC is well established in multiple species (7, 24), the mechanism of this cardioprotection is unclear. PC may occur via protein kinase C activation of mitochondrial ATP-sensitive K+ (KATP) channels, but there is no definitive evidence that adenosine A1 receptor activation before ischemia is associated with either of these events (7, 18, 24).

Most recently, mechanistic studies on ischemic and pharmacological PC have focused on the role of MAPKs. There are three MAPK subtypes, which include ERK1/2, JNK1/2, and p38 MAPK (22). Numerous studies have provided evidence for an important role of p38 in acute PC against myocardial infarction (2, 20, 23, 26, 34, 40). Adenosine receptor agonists have been shown to activate p38 MAPK in several tissues, including myocardium and isolated ventricular myocytes (10, 17, 31, 36, 37). There is also evidence that delayed adenosine A1 PC is associated with the modulation of p38 MAPK activity (8, 45). However, the role of p38 in acute adenosine PC has not been investigated.

Although there are numerous reports implicating p38 MAPK in the modulation of myocardial ischemia-reperfusion injury, there are conflicting results on the specific role of this kinase. Several investigators have shown that activation of p38 MAPK by ischemic PC results in a further increase in p38 MAPK activation during subsequent sustained ischemia (2, 23, 26). In contrast, others have observed that prior activation of p38 MAPK by ischemic PC attenuates its activity during sustained ischemia (19, 33–34). Similarly, there are contradictory reports on the ability of the selective p38 MAPK inhibitor SB-203580 to block the cardioprotective effects of acute ischemic and pharmacological PC (2, 9, 18–20, 23, 26, 33–35, 40).

Differences in the dosage and timing of SB-203580 administration as well as species-dependent variations have been cited to explain these discrepancies. Another explanation that has not been explored is the subcellular compartmentation of p38 MAPK. Although Baines et al. (3) reported that p38 MAPK is expressed in mitochondria, and others have shown that activation of mitochondrial KATP channels may be associated with cardioprotection (7, 18, 24), there have been no studies examining whether p38 MAPK activation in this fraction modulates myocardial ischemia-reperfusion injury. Mauilik et al. (21) showed that ischemic PC induces the translocation of p38 MAPK from the cytosol to the nucleus and to cytoplasmic cross-striations. However, this group did not examine the location of active p38 MAPK. Thus the purpose of the present study was to determine the role of p38 MAPK in acute adenosine A1 PC and to determine the role of p38 MAPK activation in discrete subcellular compartments.

MATERIALS AND METHODS

All animals in this study received humane care according to the guidelines set forth in “The Principles of Laboratory Animal Care” formulated by The National Society for Medical Research and the
National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 86-23, Revised 1996). In addition, animals were used in accordance with the guidelines of the University of Kentucky Institutional Animal Care and Use Committee.

**In vivo ischemia-reperfusion.** Adult male Sprague-Dawley rats (Charles River, Portage, MI) weighing 350–399 g were used. Rats were anesthetized with ketamine-xylazine (60 and 6–9 mg/kg, respectively) with supplemental doses of ketamine as needed. The right jugular vein was cannulated for fluid and drug administration and the right femoral artery for the measurement of blood pressure, heart rate, and blood gases. A tracheotomy was then performed, and the animal was connected to a small-animal ventilator (model 683; Harvard Apparatus, South Natick, MA). Room air ventilation (with positive end-expiratory pressure) was supplemented with 100% O2.

A median sternotomy was performed, and the pericardium was removed. A 6-0 prolene suture was then passed below the left coronary artery in the area immediately below the left atrial appendage. The ends of the suture were then fed through a short length of propylene tubing to form a snare. After 30-min recovery from the surgical procedures, experimental protocols were initiated. Regional myocardial ischemia was induced by pulling up on the snare and clamping it onto the epicardial surface with a small hemostat. Coronary artery occlusion was confirmed by epicardial cyanosis and a decrease in blood gas pressure. After 25-min regional ischemia, the occlusion was released and the heart was reperfused for 2 h.

**Determination of infarct size.** After 2-h reperfusion the ligature at the coronary occlusion site was permanently tied off, and Evans blue solution (1%) was injected into the venous line to demarcate the left ventricular area at risk (AAR). The animal was then killed with a pentobarbital overdose, the heart was excised, and the atria and great vessels were removed. The heart was sliced into three or four pieces (>2-mm thickness) from base to apex for staining with triphenyltetrazolium chloride (TTC) solution to measure infarct size as previously described (13). The AAR was devoid of Evans blue dye, whereas the infarcted tissue within the AAR was the TTC-negative stained region. The areas were then quantified by computerized planimetry. Infarct size was expressed as a percentage of the AAR.

**Drugs.** 15-[(1a,2b,3b,4a(S*)]-4-[7-[(2-(3-chloro-2-thienyl)-1-methylpropyl]amino]-3H-imidazo[4,5-b]pyridyl]-1-yl)cyclopentane carboxamide (AMP-579) was obtained from Aventis Pharmaceuticals (Bridgewater, NJ). SB-203580 was purchased from LC Laboratories (Woburn, MA), and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) was purchased from Sigma-Aldrich (St. Louis, MO). All of these agents were initially dissolved in DMSO and then diluted in saline solution to reduce the final DMSO concentration to 10%.

**Experimental protocols.** The treatment protocols in the infarct size studies are shown in Fig. 1. Vehicle control rats (n = 5) received 10% DMSO 30 min before coronary occlusion. Adenosine A1 receptor antagonists were administered the adenosine A1/A2a receptor agonist AMP-579 (50 μg/kg iv; n = 9) 30 min before ischemia. Two other groups received the adenosine A1 receptor antagonist DPCPX (100 μg/kg iv; n = 4) or the p38 MAPK inhibitor SB-203580 (1 mg/kg iv; n = 6) 5 or 30 min, respectively, before AMP-579. The final group was administered SB-203580 (1 mg/kg iv; n = 3) 1 h before ischemia.

To determine p38 MAPK subcellular localization and activation, hearts from additional vehicle- and AMP-579-treated rats were collected at the following time points: 1) 10 min after vehicle or AMP-579 treatment (n = 3), 2) 15 min of ischemia (n = 5), and 3) 10 min of reperfusion (n = 4). In the latter two groups, the ischemic zone of the heart was demarcated with an epicardial 4-0 prolene suture around the perimeter. The bordering ischemic and nonischemic tissue located within a 2- to 3-mm distance of this suture was discarded on tissue collection. The extent of activating transcription factor-2 (ATF-2) phosphorylation after vehicle, AMP-579, or SB-203580 + AMP-579 treatment was measured at the preischemic time point (n = 3).

**Cardiac tissue subcellular fractionation.** At the aforementioned time points, the heart was rapidly excised and placed in ice-cold phosphate-buffered saline (pH 7.4). The ischemic and nonischemic zones of the heart were removed and placed in homogenization buffer containing (in mM) 250 sucrose, 20 HEPES, 1.5 MgSO4, 1 EDTA, 1 EGTA, 1 dithiothreitol, 0.5 β-glycerophosphate, and 1 sodium vanadate, with 0.1 mg/ml PMSF and 45 μg/ml aprotinin, pH 7.4. After homogenization of the ischemic and nonischemic zones with a Tissumizer (Tekmar; Cincinnati, OH), the samples were centrifuged at 750 g for 10 min to pellet the nuclear/myofilament fraction. The resulting supernatant was centrifuged at 10,000 g for 10 min to obtain a crude mitochondrial pellet, which was further purified by two washes in homogenization buffer. The supernatant from the first 10,000 g centrifugation step was centrifuged at 100,000 g for 30 min to obtain cytosolic and membrane fractions. The fractions were tested for purity with standard markers: histone deacetylase 1 for the nucleus, α-actinin for myofilaments, cytochrome-c oxidase for the mitochondria, and caveolin-3 for the membrane. Total protein in

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Fig. 1. Experimental protocols in the infarct size studies. All rats were subjected to 25 min of regional ischemia followed by 120-min reperfusion. The vehicle or adenosine A1/A2a receptor agonist AMP-579 was administered 30 min before ischemia. The A1 antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) was given 5 min before AMP-579 treatment. Additional rats receiving either vehicle or AMP-579 were given the p38 MAPK inhibitor SB-203580 (SB) 1 h before ischemia.

**Control:**
- 30 min
- DMSO
- Ischemia
- Reperfusion

**AMP579:**
- 30 min
- AMP579

**DPCPX + AMP579:**
- 35 min
- 30 min
- DPCPX
- AMP579

**SB + AMP579:**
- 1 hr
- 30 min
- SB
- AMP579

**SB:**
- 1 hour
- 30 min
- SB
- DMSO
each fraction was determined with a Lowry protein assay (Bio-Rad, Hercules, CA).

Western blot analysis. Western blot analysis was performed as described previously (4). Briefly, protein samples (15 μg for phospho-p38 MAPK or 30 μg for phospho-ATF-2) were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose (Bio-Rad). Ponceau S staining was used to verify equal protein loads. The membrane was then blocked overnight at 4°C in a Tris-buffered saline Tween solution (TBST) containing 0.2% I-Block (Tropix, Bedford, MA). The phospho-p38 MAPK (Santa Cruz) or the phospho-ATF-2 antibody (Santa Cruz) in 5% BSA was then incubated with the membrane for 1 h. After three TBST washes, the secondary antibody was then added to the membrane. The bound antibodies were visualized by enhanced chemiluminescence (Amersham; Piscataway, NJ). The phospho-p38 MAPK membrane was then stripped to reprobe with p38 antibody (Santa Cruz). Briefly, the membranes were washed twice in TBST and incubated overnight at 4°C with blocking buffer (0.2% I-Block). Primary and secondary antibodies were incubated with membrane as described above. Scion Image software (Frederick, MD) was used for densitometric analysis of the protein bands. All of the results were normalized to a positive control included on each membrane, which consisted of a whole cell lysate of adult rat ventricular myocytes exposed to 100 μM H2O2 (15 min, 37°C). Myocytes were isolated from one heart by collagenase perfusion as previously described (11).

Statistical analysis. All data are expressed as means ± SE. A one-way ANOVA followed by Tukey’s post hoc test was used to determine significant differences among the groups in infarct size and hemodynamics. For the Western blot data, statistical significance between the nonischemic and ischemic zones of the same heart was determined with a paired Student’s t-test, and significant difference between ischemic zones from different experimental groups was assessed by an unpaired Student’s t-test. Statistical significance was defined as P < 0.05.

RESULTS

The hemodynamic data are summarized in Table 1. The baseline heart rates and blood pressures were lower than in pentobarbital-anesthetized rats (9) because of the cardiodepressant effects of ketamine-xylazine anesthesia (41). However, these baseline parameters were not different among the groups. Administration of the adenosine A1/A2a receptor agonist AMP-579 produced a marked depression in heart rate and mean arterial pressure, with the former effect inhibited by the A1 adenosine receptor antagonist DPCPX. The p38 MAPK inhibitor SB-203580 had no effect on heart rate or mean arterial pressure when given alone or before AMP-579 injection. The AAR, which was not different among the groups, was 35–42% of the left ventricle (data not shown). Treatment with AMP-579 significantly reduced infarct size from 50.9 ± 4.0% in the vehicle group to 28.7 ± 3.4% (Fig. 2). This AMP-579-mediated cardioprotection was blocked by pretreatment with the A1 adenosine receptor antagonist DPCPX. The p38 MAPK inhibitor SB-203580 also prevented AMP-579 cardioprotection but had no effect on infarct size when given alone (Fig. 2).

Because SB-203580 prevented AMP-579-mediated cardioprotection, the effect of AMP-579 on p38 MAPK activation in myocardial subcellular fractions was investigated. Figure 3 illustrates the purity of these fractions. The nuclear/myofilament fraction contained the nuclear marker histone deacetylase 1 and the myofilament marker α-actinin (Fig. 3A). The α-actinin concentration was lower in the AMP-579 group than in the control group. The caveolin-3 concentration was higher in the AMP-579 group than in the control group. The cytochrome C oxidase concentration was lower in the AMP-579 group than in the control group. The HDAC-1 concentration was lower in the AMP-579 group than in the control group.

Table 1. Systemic hemodynamics in acute AMP-579 preconditioning protocol

<table>
<thead>
<tr>
<th>Vehicle/Drug</th>
<th>5 min</th>
<th>Vehicle/Drug</th>
<th>30 min</th>
<th>2-h Refusion</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>231 ± 7</td>
<td>236 ± 13</td>
<td>237 ± 18</td>
<td>232 ± 23</td>
</tr>
<tr>
<td>AMP-579</td>
<td>235 ± 11</td>
<td>165 ± 11 †</td>
<td>205 ± 11</td>
<td>220 ± 15</td>
</tr>
<tr>
<td>AMP-579 + DPCPX</td>
<td>226 ± 8</td>
<td>230 ± 11 †</td>
<td>241 ± 16</td>
<td>210 ± 13</td>
</tr>
<tr>
<td>AMP-579 + SB-203580</td>
<td>232 ± 13</td>
<td>166 ± 14 †</td>
<td>194 ± 13</td>
<td>205 ± 13</td>
</tr>
<tr>
<td>SB-203580</td>
<td>241 ± 13</td>
<td>230 ± 11</td>
<td>200 ± 30</td>
<td>191 ± 27</td>
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</tbody>
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Heart Rate, beats/min

<table>
<thead>
<tr>
<th>Mean Arterial Pressure, mmHg</th>
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<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>AMP-579</td>
</tr>
<tr>
<td>AMP-579 + DPCPX</td>
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<tr>
<td>AMP-579 + SB-203580</td>
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<td>SB-203580</td>
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Values are means ± SE. DPCPX, 8-cyclopentyl-1,3-dipropylxanthine. *P < 0.05 vs. control; †P < 0.05 vs. AMP-579; ‡P < 0.05 vs. baseline.

Fig. 2. AMP-579 reduces infarct size. Infarct size in open-chest rats was measured as a percentage of the area at risk (AAR). *P < 0.05 vs. control.

Fig. 3. Distribution of subcellular markers. Preischemic fractions were analyzed for the presence of subcellular markers by Western blot analysis. A: either 15 or 30 μg of nuclear/myofilament fraction was probed for α-actinin (left) or histone deacetylase 1 (HDAC-1, right), respectively. B: determination of cytochrome-c oxidase in preischemic cytosolic (Cyto) and mitochondrial (Mito) fractions. C: caveolin-3 in cytosolic and membrane (Memb) fractions.
tinin immunoreactivity was greater than the histone deacetylase 1 immunoreactivity, even though the latter blot contained twice as much protein as the former (Fig. 3A). The mitochondrial fraction was enriched in cytochrome-c oxidase, whereas this enzyme was not present in the cytosolic fraction (Fig. 3B). The membrane marker caveolin-3 was enriched in the membrane fraction but absent from the cytosolic fraction (Fig. 3C).

p38 MAPK activation 10 min after vehicle or AMP-579 treatment is shown in Fig. 4, A and B. Administration of AMP-579 significantly activated p38 MAPK in the nuclear/myofilament fraction, whereas a reduction in the activation of this kinase occurred in the membrane fraction (Fig. 4, A and B). AMP-579 had no effect on p38 MAPK phosphorylation in cytosolic or mitochondrial fractions. To verify that preischemic changes in p38 activation were not due to alterations in protein, the amount of total p38 MAPK in the fractions was determined (Fig. 4, C and D). Because no p38 immunoreactivity was detected (data not shown), total p38 MAPK protein was ascertained with a p38α-selective antibody. No difference in total p38α protein between control and AMP-579-treated animals was detected in any of the fractions (Fig. 4, C and D). There were, however, differences in p38α subcellular expression. The cytosolic fraction contained the greatest amount of total p38α protein, 50% and 70% more than present in the nuclear/myofilament and membrane fractions, respectively. The cytosolic p38α protein was also 3.5-fold greater than in the mitochondrial fraction.

Activation of p38 MAPK during ischemia is shown in Fig. 5A. In the control group, ischemic zone p38 MAPK activation in the mitochondrial fraction was 22% greater than in the nonischemic zone, but there were no changes in the other fractions (Fig. 5A). The only statistically significant effects during ischemia in the AMP-579 group occurred in the membrane fraction. In this fraction, a 25% reduction in p38 MAPK phosphorylation occurred in the ischemic zone compared with the nonischemic zone and a 30% decrease compared with control.

Although there were limited changes in phospho-p38 during ischemia, there were redistributions of p38α protein during ischemia (Fig. 5B). Cytosolic and membrane p38α in AMP-
579-treated rats was significantly reduced in the ischemic zone compared with the corresponding nonischemic zone. In contrast, AMP-579 PC was associated with a significantly greater amount of p38α in ischemic zone mitochondrial fraction compared with the control group. The only significant effect in control rats was a decrease in ischemic zone nuclear/myofilament p38α.

The activation of p38 MAPK during reperfusion was next investigated (Fig. 6A). Reperfusion in control rats produced a significant activation of p38 MAPK in the cytosolic, mitochondrial, and membrane fractions (Fig. 6A). In contrast, no difference in p38 MAPK phosphorylation was observed in the ischemic zones compared with the nonischemic zones in the cytosolic, mitochondrial, and nuclear/myofilament fractions of AMP-579-treated rats. p38 MAPK activation was significantly reduced in the membrane fraction of the AMP-579 group. There were no differences between the groups in terms of total p38α distribution during reperfusion, but there were differences between ischemic and nonischemic zones (Fig. 6B). Reperfusion caused a significant reduction in ischemic zone p38α immunoreactivity in the cytosolic and membrane fractions in both groups, whereas a significant elevation of the ischemic zone p38α protein was observed in the mitochondrial fractions.

Figure 7 provides the signaling evidence that SB-203580 blocked AMP-579-mediated p38 MAPK activation. Because AMP-579 increased p38 MAPK phosphorylation only in the preischemic nuclear/myofilament fraction, this time point and fraction were used. The ability of SB-203580 to block p38 MAPK-mediated phosphorylation of the downstream substrate ATF-2 was assessed. Administration of AMP-579 caused a significant increase in the amount of phosphorylated ATF-2 in the preischemic nuclear/myofilament fraction, an effect blocked by SB-203580 (Fig. 7).

DISCUSSION

The results of the present study provide the first in vivo evidence that acute adenosine A1 receptor PC involves mediation via p38 MAPK. This cardioprotection appears to be associated with modulation of p38 MAPK activation and distribution in discrete subcellular compartments. Ischemia-reperfusion activated p38 MAPK in the cytosolic, mitochondrial, and membrane fractions. By contrast, p38 MAPK activation only occurred before ischemia in the nuclear/myofilament fraction in AMP-treated rats.

The role of p38 MAPK in ischemic PC has been widely examined (19, 23, 28, 33, 40), but very few studies on the involvement of this kinase in acute pharmacological PC exist (18, 20, 26, 34). Marais et al. (20) reported that isoproterenol PC in isolated rat hearts activated p38 MAPK but reduced the activation of this kinase during subsequent sustained ischemia. Blockade of p38 MAPK caused a significant increase in the amount of phosphorylated ATF-2 in the preischemic nuclear/myofilament fraction, an effect blocked by SB-203580 (Fig. 7).
role of p38 in acute A1 PC. Nakano et al. (26) found that acute A1 PC activated the p38 MAPK substrate MAPKAPK-2, but the role of this kinase in adenosine cardioprotection was not studied. The p38 inhibitor SB-203580 was shown to block acute adenosine protection in human atrial appendages, but the activation of p38 MAPK was not measured (18).

The elucidation of the role of p38 MAPK in ischemia-reperfusion has been limited by the lack of in vivo studies. To date, there has been only one in vivo study examining the role of p38 MAPK in acute pharmacological PC. Fryer et al. (9) reported that opioid-mediated acute cardioprotection in an in vivo rat model was neither associated with p38 MAPK activation nor blocked by the p38 inhibitor SB-203580. The results of the present study, however, indicate that adenosine A1 receptor acute PC is associated with preischemic activation of p38 MAPK in an in vivo rat model. The p38 MAPK inhibitor SB-203580 blocked both the cardioprotective effect of the adenosine agonist AMP-579 and the associated increase in preischemic p38 activity. These findings indicate that acute A1 PC is mediated via a p38-dependent mechanism.

The second major finding of the present study is the observation that the distribution and activation of this kinase are compartmentalized within the myocardium. The majority of ischemia-reperfusion studies have used whole heart or cardiomyocyte lysates (6, 19, 26, 27, 35), although a few investigators have assessed p38 MAPK activation in cytosolic and nuclear fractions (9, 28). The importance of subcellular p38 is demonstrated in a study by Ping et al. (28), who observed that single-cycle ischemic PC in conscious rabbits increased myocardial p38 activity in the cytosolic but not the nuclear fraction. This group also showed that p38 MAPK is located in mouse heart mitochondrial fractions (3), but changes in mitochondrial p38 activity with PC or ischemia-reperfusion have not been assessed. The results from the present study indicate that in normal myocardium the mitochondrial fraction contained only 10% of the total myocardial p38 protein compared with 40% in the cytosol, but the amounts of active mitochondrial and cytosolic p38 MAPK were similar. In fact, p38α protein was present in all of the fractions that we isolated. Attempts to identify the expression of p38β with several different antibodies proved negative, consistent with the lack of p38β expression in neonatal rat myocytes and human myocardium (15, 30).

In contrast to the effect of ischemic PC on cytosolic p38 (28), we observed no effect of AMP-579 on cytosolic p38 in normal myocardium. The only subcellular fraction that exhibited p38 MAPK activation before ischemia-reperfusion was the low-speed spin nuclear/myofilament fraction. Because we did not separate these two fractions, it is not possible to discern whether this increase occurred in one or both of these fractions. Treatment with AMP-579 also increased the phosphorylation of the downstream p38 MAPK substrate ATF-2 in the nuclear/myofilament fraction before ischemia, and this effect was blocked by pretreatment with SB-203580.

In contrast to the activation of p38 MAPK in the nuclear/myofilament fraction, AMP-579 treatment was associated with a decrease in membrane p38 phosphorylation. Our observation of significant membrane p38 expression and activation is consistent with other reports that myocardial membranes do contain p38 MAPK (29, 39). It is not clear why AMP-579 decreased p38 phosphorylation in the membrane fraction before ischemia, but this was not due to translocation of p38α out of this compartment. Because no detergent was used in the subcellular fractionation, the membrane fraction contained caveolar membranes. These flask-shaped invaginations of the sarcolemma may play an important role in signal transduction because numerous signaling molecules, including p38 MAPK, are located in caveolae (1, 16). Because the caveolar fraction contains <10% of the total protein in cardiomyocytes (14), the effects of ischemia-reperfusion and adenosine PC on membrane p38 MAPK distribution and activation may have been underestimated.

Although the activation of p38 MAPK during ischemic or pharmacological PC is fairly well established (19–20, 23, 26, 28, 33, 40), whether this kinase is activated during subsequent sustained ischemia remains controversial. Some investigators have found that PC activates p38 MAPK during prolonged ischemia (19, 33, 35), whereas other studies have shown that a reduction in p38 MAPK activation occurs during the sustained ischemia following PC (20, 23, 38, 42). The results from the current study are in agreement with the latter findings, as no ischemia-mediated activation of p38 MAPK was observed in the AMP-579 group. In fact, the stimulation of membrane-associated p38 MAPK in the ischemic zone was significantly reduced in the AMP-579-treated rats. By contrast, p38 MAPK activation was significantly elevated by ischemia in the mitochondrial fraction of the control group, whereas no activation of this kinase was observed in the other fractions. Fryer et al. (9) also reported that p38 MAPK was not activated in the cytosolic or nuclear fractions by ischemia in an in vivo rat model, but this group did not determine p38 MAPK activity in mitochondrial and membrane fractions. Although the ischemia-mediated activation of p38 MAPK is supported by numerous studies in multiple species and ischemia-reperfusion models (19, 33, 38, 40), there are no reports of p38 MAPK activation in discrete subcellular compartments by ischemia. Therefore, the current in vivo study is the first to show that ischemia activates p38 MAPK in the mitochondrial compartment in the heart.

In addition to ischemia-mediated activation of p38 MAPK, significant changes in total protein between the nonischemic and ischemic zones were observed during ischemia. A movement of p38α protein from the cytosolic and membrane fractions to the mitochondria appeared to occur in the ischemic zones of AMP-579-treated rat hearts, a pattern that was not observed in the control rat hearts. These alterations in p38α protein in the ischemic zone were not due to cell death because both decreases and increases in total protein were observed.

p38 MAPK was further activated by reperfusion in the cytosolic, mitochondrial, and membrane fractions. Although the activation of p38 MAPK by reperfusion is supported by other studies (40, 42), the finding that reperfusion-mediated activation of p38 occurs in discrete compartments in an in vivo model has not been previously shown. Fryer et al. (9) reported no reperfusion-mediated activation of p38 MAPK in the cytosolic fraction in the in vivo rat, but the effect of reperfusion on mitochondrial and membrane p38 MAPK activation was not examined in that study. Activation of p38 MAPK in the membrane fraction during reperfusion is consistent with the findings of Rafiee et al. (29), who found that a considerable activation of this kinase occurred in the particulate fraction of rabbits exposed to chronic hypoxia. These authors (29) also reported a translocation of p38 from the cytosolic to particulate...
fractions, consistent with our findings of significant changes in the subcellular distribution of this protein in the ischemic zone of the cytosolic, mitochondrial, and membrane fractions compared with the corresponding nonischemic zone. An apparent translocation of p38α protein occurred from the cytosolic and membrane fractions to the mitochondria. These alterations in p38α expression were not due to cell death because the AMP-579 group exhibited the same changes as the control group despite the lower amount of cell death in the AMP-579 group.

The effect of reperfusion on p38 MAPK activation in the AMP-579 group was quite different from that in the control group. No p38 MAPK activation was observed during reperfusion in any of the subcellular fractions in the AMP-579-treated rat heart. Furthermore, the ischemia-mediated reduction in membrane p38 MAPK activity in the AMP-579 group was even more pronounced with reperfusion. These results are consistent with Marais et al. (20), who reported that isoproterenol PC was associated with diminished p38 MAPK activation during reperfusion.

Despite numerous studies on the role of p38 MAPK in ischemic and pharmacological PC, the specific mechanism and downstream targets of p38 MAPK in A1 adenosine receptor PC are unclear. Both heat shock protein (HSP27) and mitochondrial K_ATP channels have been proposed to be potential downstream targets of p38 MAPK-mediated cardioprotection (7, 8, 18, 32, 33, 45). One of the downstream p38 MAPK substrates, MAPKAPK-2, phosphorylates HSP27, leading to its activation and subsequent translocation to myofilaments, where it acts to stabilize the actin cytoskeleton (22, 32). Sakamoto et al. (32) showed that ischemic PC causes translocation of HSP27 to the sarcomere, an effect that was blocked with SB-203580. Although the present study did not examine HSP27 translocation, the activation of p38 MAPK by AMP-579 before ischemia occurred in the nuclear/myofilament fraction, suggesting a potential involvement of HSP27-mediated stabilization of the cytoskeleton in the cardioprotective mechanism of p38 MAPK in adenosine A1 PC. Although p38 MAPK also activates several transcription factors in the nucleus (22), upregulation of gene expression may be more relevant in delayed rather than acute PC.

In addition to HSP27, several investigators have used inhibitors and activators of the mitochondrial K_ATP channels to implicate this channel as the end-effector of p38 MAPK-mediated cardioprotection in ischemic and delayed adenosine PC (6, 7, 18, 44, 45). However, data from the present in vivo study do not support this hypothesis because adenosine A1 receptor-mediated PC was not associated with an increase in p38 MAPK activity in the mitochondrial fraction at any time point. In contrast, AMP-579 blocked the activation of mitochondrial p38 MAPK during ischemia and reperfusion.

A limitation of our study was the use of the adenosine agonist AMP-579, which has high affinity for both A1 and A2a receptors, as evident by the decreases in both heart rate and blood pressure in these animals. However, our findings are consistent with A1 receptor PC because AMP-579 was administered as a bolus 30 min before ischemia and its protective effect was blocked by the adenosine A1 receptor antagonist DPCPX. Furthermore, activation of the A1 receptor during reperfusion does not reduce myocardial infarct size (5). Although AMP-579 can stimulate A2a receptors, activation of this adenosine receptor subtype before ischemia does not exert a PC effect (7, 24). Reperfusion treatment with AMP-579 has been reported to decrease myocardial infarct size via an A2a receptor-dependent effect (12). This effect was also blocked by a MEK inhibitor, although ERK activation was not measured. Thus it is possible that AMP-579 may also activate ERK.

Although p38 MAPK may be involved in delayed adenosine A1 receptor PC (8, 45), the role of this kinase in acute A1 PC has not been previously examined. This study is the first to report that in vivo acute adenosine A1 PC is mediated by p38 MAPK activation. Furthermore, this extensive study is the first to examine p38 MAPK activation and total protein in all subcellular fractions at preischemic, ischemic, and reperfusion time points in an in vivo rat model.

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