Adenosine A$_1$ Receptor Induced Delayed Preconditioning in Rabbits

Induction of p38 Mitogen-Activated Protein Kinase Activation and Hsp27 Phosphorylation via a Tyrosine Kinase– and Protein Kinase C–Dependent Mechanism

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Abstract—Transient adenosine A$_1$ receptor (A$_1$R) activation in rabbits induces delayed preconditioning against myocardial infarction 24 to 72 hours later. The cellular mechanisms downstream of A$_1$R mediating this delayed cardioprotection have not been elucidated. This study examined the role of protein kinase C (PKC) and tyrosine kinases (TKs) in the signaling cascade mediating A$_1$R-induced late preconditioning in rabbits. The small heat shock protein Hsp27 has been shown to confer cytoskeletal protection when in the phosphorylated state. We therefore also evaluated the potential role of the p38 mitogen-activated protein kinase (p38 MAPK) and Hsp27 as distal mediators of A$_1$R-induced delayed preconditioning. Pharmacological preconditioning of rabbits with the selective A$_1$ agonist 2-chloro-N$_6$-cyclopentyladenosine (CCPA; 100 mg/kg) significantly reduced myocardial infarct size compared with control animals, after 30-minute regional ischemia/2-hour reperfusion in vivo 24 hours later (23.7±3.1 versus 43.0±4.1%; P<0.05). This delayed protection was abrogated by prior inhibition of either PKC with chelerythrine chloride (5 mg/kg) or of TKs with lavendustin A (1.3 mg/kg), suggesting that both PKC and TK are crucial for the development of delayed preconditioning after A$_1$ receptor activation in the rabbit. Myocardial tissue extracts obtained 24 hours after CCPA treatment were analyzed for p38 MAPK catalytic activity using an in vitro kinase assay. This showed an almost 7-fold increase in p38 MAPK activity in myocardial samples pretreated with CCPA compared with control hearts. Two-dimensional gel electrophoresis revealed an increase in the phosphorylated isoforms of Hsp27 in hearts pretreated with CCPA compared with control hearts. Prior inhibition of either PKC or TK prevented the CCPA-induced increase in p38 MAPK activity and phosphorylation of Hsp27. This study identifies new components of the signaling mechanism of A$_1$ R-induced delayed preconditioning. Our results suggest an important role for both PKC and TK as mediators of late preconditioning against infarction after A$_1$R activation and, although correlative, point to the p38 MAPK/Hsp27 pathway as a potential distal effector of this protection. (Circ Res. 2000;86:989-997.)

Key Words: adenosine • myocardial infarction • delayed preconditioning • protein kinase • heat shock protein

The delayed phase (second window) of ischemic preconditioning (PC) is the phenomenon whereby brief periods of sublethal ischemia induce a state of enhanced myocardial tolerance to subsequent prolonged ischemic injury, which becomes apparent 12 to 24 hours after the PC ischemic insult and persists up to 72 hours.1–7 This subacute form of myocardial adaptation many hours after ischemic PC is associated with a reduction in infarct size,1,2,4–6 postischemic myocardial dysfunction (stunning),5,8,9 and ischemia/reperfusion–induced ventricular arrhythmias.10 The remarkable potency and reproducibility of this phenomenon in all species studied has generated intense interest in exploiting delayed PC to develop therapeutic strategies that can enhance myocardial tolerance to ischemia/reperfusion injury in patients with coronary artery disease. However, the cellular mechanisms that underlie this subacute adaptive response have remained elusive.

We have previously reported an important role for endogenous adenosine, released from the myocardium during PC ischemia, as a trigger of delayed protection against infarction in rabbit myocardium. We11 have shown that the delayed anti-infarct effects of ischemic PC are abolished by pretreatment with adenosine receptor antagonists. Conversely, transient adenosine A$_1$ receptor (A$_1$R) activation with the selective agonist 2-chloro-N$_6$-cyclopentyladenosine (CCPA) induces a delayed and sustained protection against infarction...
in the rabbit\textsuperscript{11–13} and rat.\textsuperscript{14} These studies point to the crucial role of A\textsubscript{1}R in initiating the cellular events that result in delayed myocardial protection against infarction.

The intracellular signaling pathways downstream of A\textsubscript{1}R mediating this delayed cardioprotection have not been elucidated. Protein kinase C (PKC) has been shown to play an important role in A\textsubscript{1}R-induced signal transduction in myocardial tissue.\textsuperscript{15–17} Furthermore, several studies indicate the involvement of PKC in mediating both the early phase (reviewed in Reference 18) and the second window\textsuperscript{19,20} of ischemic PC. On the other hand, increasing evidence has recently implicated involvement of tyrosine kinase (TK) activation in the signaling mechanism of ischemic PC.\textsuperscript{21–27} TK signaling is activated by a number of G protein–coupled receptors\textsuperscript{28–31} and, as suggested by the study of Maulik et al,\textsuperscript{21} may form an early step in the mechanism of ischemic PC. Conversely, studies in noncardiac tissue suggest that tyrosine phosphorylation may occur downstream of PKC,\textsuperscript{32,33} a view that has been supported by recent evidence in rabbit myocardium subjected to ischemic PC protocols.\textsuperscript{22,25} On the other hand, Vahlhaus et al\textsuperscript{27} recently reported that blockade of both TK and PKC is necessary to abolish ischemic PC in pigs, suggesting that these enzymes may act in parallel pathways to mediate PC. However, the involvement of these 2 families of protein kinases in A\textsubscript{1}R-induced delayed PC has not been evaluated. Therefore, in the first part of the present study, we examined the role of PKC and TK signaling in acquisition of delayed tolerance to myocardial ischemia 24 hours after A\textsubscript{1}R activation in the rabbit.

Another important issue regarding the delayed protection against infarction conferred by transient adenosine A\textsubscript{1}R activation is the nature of the distal effector or target protein(s) mediating this protection. One potential end-effector protein that has been the subject of recent interest is the constitutively phosphorylated cytoskeletal protein, Hsp27. Overexpression of mammalian Hsp27 has been shown to confer significant cellular resistance against heat shock, tumor necrosis factor, oxidative stress, and a number of cytotoxic drugs.\textsuperscript{34–37} Importantly, recent evidence suggests that overexpression of Hsp27 in adult cardiac myocytes confers enhanced resistance against injury mediated by simulated ischemia.\textsuperscript{38} These small heat shock proteins can function in different, seemingly unrelated cytoprotective processes, such as RNA stabilization,\textsuperscript{35} molecular chaperoning and preventing unfolded proteins from irreversible aggregation,\textsuperscript{39} regulation of apoptosis,\textsuperscript{40} and interaction with and stabilization of the cytoskeleton.\textsuperscript{41} This latter function of Hsp27 seems to be dependent on its state of phosphorylation. Thus, unphosphorylated Hsp27 behaves as an F-actin capping protein and inhibits actin polymerization, whereas the phosphorylated Hsp27 isoforms promote polymerization, which confers resistance against stress-induced microfilament disorganization (reviewed in Reference 41). In this regard, it has been shown that cells overexpressing Hsp27 contain an actin cytoskeleton that is more resistant to disruption due to oxidative stress than control cells or those overexpressing a nonphosphorylatable mutant of Hsp27.\textsuperscript{36,42} Hsp27 is phosphorylated by the mitogen-activated protein kinase-activated protein kinase-2 (MAPKAPK-2), a stress-sensitive kinase that is sequentially phosphorylated in a cascade of kinases involving p38 mitogen-activated protein kinase (p38 MAPK).\textsuperscript{42,43} The p38 MAPK/MAPKAPK-2 pathway in the myocardium is activated by a number of stressful stimuli (reviewed in Reference 44), and several groups have demonstrated its activation by ischemic PC protocols.\textsuperscript{45–49} Importantly, recent evidence suggests that p38 MAPK/MAPKAPK-2 pathway is also activated after exposure to adenosine in both cultured cardiomyocytes\textsuperscript{50} and isolated perfused rat hearts.\textsuperscript{51}

On the basis of the aforementioned, and considering the fact that cytoskeletal disruption is one of the determining events in the cascade of ischemia/reperfusion injury,\textsuperscript{52} we hypothesized that A\textsubscript{1}R-induced delayed cardioprotection may be mediated by activation of the p38 MAPK/MAPKAPK-2 pathway, resulting in enhanced cytoskeletal stabilization during the index ischemic insult. In the second part of the present study, we investigated this hypothesis by measuring p38 MAPK activity and Hsp27 expression, and phosphorylation in vivo.

**Materials and Methods**

All work was conducted in accordance with the Guidelines on the Operation of Animals Act 1986 (scientific procedures), published by the Stationery Office, London, UK. Male New Zealand White rabbits weighing 2.1 to 2.8 kg were used in these experiments. On day 1, conscious animals were pharmacologically preconditioned and/or received protein kinase inhibitors. Animals were assigned to 6 experimental groups (Figure 1). Group I (control) received an IV bolus of sterile 0.9% saline (0.5 mL). Group II (CCPA) animals were pharmacologically preconditioned with a single IV bolus of CCPA (100 \(\mu\)g/kg). Animals in groups I and II were also treated with the vehicle used for protein kinase inhibitors (4% vol/vol ethanol in sterile water). To examine the role of PKC in mediating A\textsubscript{1}R-induced late PC, groups III (chelerythrine chloride [CHE]+saline) and IV (CHE+CCPA) received the same treatment as groups I and II, respectively; in addition, they were given an IV infusion of the selective PKC inhibitor CHE (5 mg/kg) 10 minutes before the saline/CCPA bolus. Rabbits in groups V

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** Experimental protocol. On day 1, animals were pharmacologically preconditioned with an IV bolus injection of CCPA or were treated with saline (groups I and II). Animals in these groups were also treated with protein kinase inhibitor vehicle (4% vol/vol ethanol in sterile water). Animals in groups III and IV received an infusion of 5 mg/kg CHE IV 10 minutes before the saline/CCPA bolus. Animals in groups V and VI received an IV infusion of 1.3 mg/kg LDA before the saline/CCPA bolus. On day 2, animals were anesthetized and subjected to 30 minutes of regional myocardial ischemia (isch) and 2 hours of reperfusion in vivo. Arrowheads indicate timing of myocardial sampling in animals that were not subjected to ischemia/reperfusion, for analysis of p38 MAPK activity, and Hsp27 expression, and phosphorylation.
(lavendustin A [LDA]+saline) and VI (LDA+CCPA) were given the same treatment as those in groups I and II. To evaluate the potential role of TK in the signaling pathway downstream of A1R, these animals were also treated with the selective TK inhibitor LDA (1.3 mg/kg) 10 minutes before the saline/CCPA bolus.

Ischemia/Reperfusion Protocol In Vivo

On day 2, 24 hours after various treatments, rabbits were anesthetized and underwent an infarction procedure in vivo, consisting of 30-minute regional myocardial ischemia and 2-hour reperfusion, as described previously.11–13 At the end of reperfusion, myocardial area at risk was determined with fluorescent microspheres, and infarct size was assessed by triphenyl tetrazolium chloride staining, as previously described.11–13 The areas of infarcted tissue (I) and myocardium at risk (R) were quantified by computerized planimetry, and infarct size was expressed as a percentage of the risk area (I/R).

Analysis of Myocardial p38 MAPK Activity

In a different group of animals, 24 hours after the various treatments outlined above, rabbits (n=3 per group) were euthanized by an overdose of pentobarbital sodium, and hearts were immediately excised and rinsed in ice-cold saline. Left ventricular myocardial samples were rapidly frozen by immersion in liquid nitrogen and stored at −80°C.

In these myocardial samples, p38 MAPK catalytic activity was determined by an in vitro kinase assay using recombinant activating transcription factor-2 (ATF2) as a substrate, according to the New England Biolabs instructions. After probing the immunoblots with the phospho-ATF2 antibody, filters were stripped and probed with anti-p38 MAPK. The ratio of phospho-ATF2 to p38 MAPK immunoreactivity was determined for each sample, and the results were expressed as fold activation over control.

Hsp27 Expression and Phosphorylation

One- and two-dimensional gel electrophoresis was performed on myocardial samples 24 hours after various pretreatments to assess expression and posttranslational phosphorylation of Hsp27. Sample preparation and 1-dimensional SDS-PAGE were carried out as described previously.53,54 Two-dimensional gels were carried out to determine the phosphorylation state of Hsp27 using a Bio-Rad mini-protean II 2-dimensional cell according to the Bio-Rad protocol.

Statistical Analysis

The data are presented as mean±SEM. The significance of the differences in mean values of I, R, and I/R between the experimental groups was evaluated by 1-way ANOVA followed by the Fisher protected least significant difference. Differences between hemodynamic measurements at different time points were assessed by 2-way ANOVA with repeated measures. The null hypothesis was rejected at P<0.05.

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Results

A total of 66 rabbits were used in these experiments. Forty-eight animals were used for the infarct studies. Of these, 6 were excluded, 4 because of intractable ventricular fibrillation during the infarct protocol (2 in the control group, 1 in the CHE+CCPA group, and 1 in the LDA+CCPA), 1 because of failure of triphenyl tetrazolium chloride stain (CHE+saline group), and 1 because the risk zone was <0.4 cm3 (CHE+CCPA group). Data on infarct size are therefore presented for 42 animals that successfully completed the infarct protocol. Eighteen animals were used for analysis of p38 MAPK activity and Hsp27 expression/phosphorylation (n=3 per group).

Systemic Hemodynamic Changes During Ischemia/Reperfusion

Table 1 summarizes the changes in heart rate, systolic blood pressure (SBP), and rate-pressure product (RPP) during the infarction protocol in the 6 experimental groups. There were no differences in baseline hemodynamic performance between any of the groups. There was a small decline in SBP and RPP during 30-minute ischemia with no recovery during reperfusion. These hemodynamic changes with time were very similar among all the groups.

Myocardial Risk and Infarct Size

Table 2 presents the body weights and the volumes of risk and infarct zones in the 6 experimental groups. In these groups, the mean volume of myocardial tissue at risk during coronary artery occlusion was in the range 0.9 to 1.2 cm3, representing ∼40% to 45% of total left ventricular tissue volume. There were no significant differences in ischemic risk zone among the experimental groups. The absolute infarct size was also similar between the groups. Infarct size expressed as a percentage of area at risk (I/R) in the 6 experimental groups is presented in Figure 2. Pretreatment with CCPA 24 hours before myocardial infarction resulted in a significant 45% reduction in I/R compared with saline-treated controls (23.7±3.1 versus 43.0±4.1%, respectively; P<0.05). Thus, in accordance with previous studies in this model,11–13,55 transient activation of A1R induced a delayed PC effect against infarction at 24 hours. The effect of inhibition of PKC was evaluated by administering CHE before CCPA or saline injections. Prior treatment with CHE 5 mg/kg completely abolished the infarct-limiting effect of delayed pharmacological PC with CCPA, whereas it did not significantly affect infarct size in saline-treated animals (I/R; 37.3±4.1 and 41.1±4.7%, respectively; P=NS versus control). Similarly, inhibition of TK using the selective antagonist LDA (1.3 mg/kg), administered before the CCPA bolus, abrogated the limitation of infarction in these animals, whereas LDA on its own had no effect on infarct size (I/R; 38.2±4.9 and 42.8±4.8%, respectively; P=NS versus control). These results point to an important role for these 2 groups of protein kinases in the signaling mechanism downstream of A1R and in mediating its delayed cardioprotective effects.

p38 MAPK Activity

Activation of p38 MAPK in myocardial samples obtained from rabbits pretreated 24 hours earlier was determined by measurement of its catalytic activity using the in vitro kinase assay with recombinant ATF2 as substrate. As seen in Figure 3, pretreatment 24 hours earlier with the A1R agonist CCPA resulted in an almost 7-fold increase in the activity of p38 MAPK (689±63%, P<0.01). This increased activity was abolished by prior inhibition of either PKC or TK. Pretreatment with CHE or LDA alone did not significantly affect p38 MAPK activity at 24 hours. None of the above treatments affected total expression of p38 MAPK protein as seen when filters were reprobed with anti–p38 MAPK antibody.

Hsp27 Expression and Phosphorylation

One-dimensional PAGE was used to assess expression of Hsp27 protein in myocardial samples obtained 24 hours after
Ser82). This increases the total negative charge of the protein, with a resultant decrease in isoelectric point and an acidic shift in the position of the Hsp27 isoforms corresponding to increased phosphorylation of the protein and probably representing the di- and triphosphorylated isoforms of Hsp27. This phosphorylation pattern was completely inhibited by prior inhibition of either PKC or TK, so that phosphorylation of Hsp27 can occur on up to 3 sites (Ser15, Ser78, and Ser82). This increases the total negative charge of the protein, with a resultant decrease in isoelectric point and an increased leftward mobility of Hsp27 into the acidic (anode) region of the isoelectric focusing gel. The 4 major phosphorylation isoforms of Hsp27 are the nonphosphorylated and the mono-, di-, and triphosphorylated. As seen in Figure 4, in the control hearts Hsp27 was detected primarily in the nonphosphorylated form (the most positively charged), with minor contribution from the monophosphorylated isoforms. Prior treatment with CCPA (100 μg/kg) 24 hours earlier resulted in an acidic shift in the position of the Hsp27 isoforms corresponding to increased phosphorylation of the protein and probably representing the di- and triphosphorylated isoforms of Hsp27. This phosphorylation pattern was completely inhibited by prior inhibition of either PKC or TK, so that 2-dimensional gels of myocardial samples from these animals were similar to that from controls (Figure 4). Prior treatment with CHE or LDA alone had no effect on Hsp27 phosphorylation (data not shown). These results indicate that adenosine A1R activation induces phosphorylation of Hsp27 at a time point that corresponds to the delayed infarct-limiting effects of this treatment and that Hsp27 phosphorylation is mediated by a PKC- and TK-dependent signaling pathway.

### TABLE 1. Hemodynamic Variables During Myocardial Ischemia/Reperfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>5 Minutes</th>
<th>29 Minutes</th>
<th>60 Minutes</th>
<th>120 Minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, bpm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>241 ± 7</td>
<td>239 ± 8</td>
<td>240 ± 9</td>
<td>230 ± 8</td>
<td>225 ± 7</td>
</tr>
<tr>
<td>CCPA</td>
<td>236 ± 6</td>
<td>240 ± 7</td>
<td>247 ± 5</td>
<td>237 ± 7</td>
<td>234 ± 6</td>
</tr>
<tr>
<td>CHE + saline</td>
<td>240 ± 6</td>
<td>247 ± 9</td>
<td>245 ± 9</td>
<td>237 ± 9</td>
<td>243 ± 9</td>
</tr>
<tr>
<td>CHE + CCPA</td>
<td>248 ± 12</td>
<td>248 ± 12</td>
<td>238 ± 15</td>
<td>235 ± 9</td>
<td>228 ± 9</td>
</tr>
<tr>
<td>LDA + saline</td>
<td>238 ± 3</td>
<td>236 ± 4</td>
<td>233 ± 7</td>
<td>231 ± 4</td>
<td>232 ± 5</td>
</tr>
<tr>
<td>LDA + CCPA</td>
<td>248 ± 7</td>
<td>240 ± 8</td>
<td>246 ± 6</td>
<td>235 ± 6</td>
<td>238 ± 6</td>
</tr>
</tbody>
</table>

| SBP, mm Hg |          |           |            |            |             |
| Control    | 93 ± 4   | 85 ± 3    | 79 ± 4     | 76 ± 2     | 73 ± 2      |
| CCPA       | 90 ± 3   | 82 ± 3    | 72 ± 2     | 70 ± 1     | 71 ± 2      |
| CHE + saline | 92 ± 3  | 87 ± 2    | 77 ± 4     | 70 ± 3     | 70 ± 2      |
| CHE + CCPA | 93 ± 6   | 79 ± 6    | 76 ± 4     | 75 ± 4     | 71 ± 4      |
| LDA + saline | 90 ± 3 | 81 ± 3    | 82 ± 4     | 72 ± 3     | 73 ± 3      |
| LDA + CCPA | 89 ± 2   | 80 ± 3    | 72 ± 3     | 68 ± 2     | 66 ± 1      |

| RPP, mm Hg/min × 10³ |          |           |            |            |             |
| Control         | 22.6 ± 1.6 | 20.3 ± 1.2 | 19.1 ± 1.5 | 17.5 ± 0.9 | 16.5 ± 0.6 |
| CCPA            | 21.0 ± 0.5 | 19.6 ± 0.7 | 18.9 ± 0.5 | 16.6 ± 0.3 | 16.5 ± 0.6 |
| CHE + saline   | 22.3 ± 1.2 | 21.5 ± 1.3 | 19.1 ± 1.2 | 16.7 ± 1.1 | 16.9 ± 0.9 |
| CHE + CCPA     | 22.3 ± 1.6 | 19.7 ± 2.0 | 18.3 ± 1.6 | 16.9 ± 1.2 | 16.2 ± 0.9 |
| LDA + saline   | 21.4 ± 0.5 | 19.8 ± 0.9 | 19.2 ± 1.2 | 16.4 ± 1.2 | 16.2 ± 0.7 |
| LDA + CCPA     | 21.1 ± 0.9 | 20.4 ± 1.2 | 18.7 ± 1.0 | 16.3 ± 0.7 | 16.8 ± 0.6 |

Values are mean ± SEM.

### TABLE 2. Body Weight and Myocardial Area at Risk (R) and Infarct Size (I)

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Animals</th>
<th>Body Weight, kg</th>
<th>R, cm³</th>
<th>I, cm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>2.49 ± 0.09</td>
<td>1.03 ± 0.09</td>
<td>0.42 ± 0.05</td>
</tr>
<tr>
<td>CCPA</td>
<td>8</td>
<td>2.49 ± 0.05</td>
<td>1.12 ± 0.12</td>
<td>0.28 ± 0.06</td>
</tr>
<tr>
<td>CHE + saline</td>
<td>7</td>
<td>2.59 ± 0.07</td>
<td>1.17 ± 0.07</td>
<td>0.49 ± 0.07</td>
</tr>
<tr>
<td>CHE + CCPA</td>
<td>6</td>
<td>2.47 ± 0.11</td>
<td>0.99 ± 0.08</td>
<td>0.36 ± 0.05</td>
</tr>
<tr>
<td>LDA + saline</td>
<td>7</td>
<td>2.32 ± 0.06</td>
<td>0.93 ± 0.08</td>
<td>0.39 ± 0.06</td>
</tr>
<tr>
<td>LDA + CCPA</td>
<td>6</td>
<td>2.37 ± 0.06</td>
<td>1.19 ± 0.08</td>
<td>0.49 ± 0.07</td>
</tr>
</tbody>
</table>

Values are mean ± SEM.
An expanded Materials and Methods section is available online at http://www.circresaha.org.

Discussion

The present study provides new insight into the cellular mechanisms responsible for conferring increased myocardial tolerance to lethal ischemic injury 24 hours after transient activation of A1 R. We have shown that the significant protection against myocardial infarction observed in the rabbit, 24 hours after treatment with the selective A1 R agonist CCPA, is completely abolished by prior inhibition of either PKC or TK with their potent and selective inhibitors, CHE and LDA, respectively. These data point to the crucial role of these 2 families of protein kinases in mediating A1 R-induced delayed PC. In the second part of the present study, we demonstrated a significant increase in myocardial p38 MAPK activity 24 hours after CCPA treatment. Pharmacological PC with CCPA was also associated with increased phosphorylation of the cytoprotective protein Hsp27 at 24 hours. Both the increase in p38 MAPK activity and Hsp27 phosphorylation were PKC and TK dependent and were inhibited by prior treatment with either CHE or LDA. The activation of p38 MAPK therefore appears to occur distally to PKC and TK. Taken together, our results for the first time suggest an important role for the p38 MAPK/Hsp27 pathway in the signaling mechanism underlying A1 R-induced delayed PC and suggest that this pathway is downstream of, and dependent on, PKC and TK activation.

Role of Protein Kinases in Delayed PC

Previous studies have implicated PKC in mediating delayed cardioprotection after ischemic PC.19,20,60 In the present study, we have shown that PKC also mediates A1 R-induced late protection against infarction. We used CHE, a potent inhibitor of PKC (IC50 ≈ 0.7 μmol/L) with very high selectivity for PKC compared with protein kinase A (PKA; 250:1) or protein TK (150:1),61 at a dose (5 mg/kg) that has been shown to abolish delayed ischemic PC against both infarction19 and stunning20 in rabbit myocardium. Furthermore, Ping et al60 have reported that a PC protocol of six 4-minute coronary occlusions/4-minute reperusions in conscious rabbits induces translocation of PKC isoforms ε and η from the cytosolic to the particulate fraction, which is prevented by prior treatment with CHE at the same dose as that used in the present study. Similarly, Wilson et al62 reported an association between development of delayed cardioprotection against ventricular arrhythmias 24 hours after rapid cardiac pacing in dogs and sustained translocation of PKCe to the membrane fraction. Gray et al63 have implicated PKCe in hypoxic PC of cardiac myocytes using PKCe-selective inhibitor peptide. On the other hand, Kawamura et al64 have

![Figure 2. Myocardial infarct size. Infarct size is expressed as a percentage of the region at risk of infarction. ○, Individual animals; ●, mean±SEM. *P<0.01 vs control (1-way ANOVA).](image)

![Figure 3. Myocardial p38 MAPK activity. In myocardial samples obtained 24 hours after the various PC protocols, p38 MAPK catalytic activity was determined using an in vitro kinase assay to phosphorylate ATF2, and the reaction mixture was separated by SDS-PAGE. The same filter was probed sequentially with anti-phospho-ATF2 and anti-p38 MAPK. Ratio of activation was calculated as phospho-ATF2:p38 MAPK immunoreactivity and was normalized to 1 for the control group. A, Representative Western immunoblots from different experimental groups. B, Ratio of phospho-ATF2:p38 MAPK immunoreactivity. Data are mean±SEM of 3 animals per group. *P<0.05 (1-way ANOVA).](image)

![Figure 4. Myocardial Hsp27 phosphorylation. In myocardial samples obtained 24 hours after the various PC protocols, Hsp27 phosphorylation was determined by 2-dimensional PAGE. Figure shows representative 2-dimensional immunoblots from 3 animals per experimental group.](image)
suggested that in addition to PKCe, the δ isoform is also translocated to the membrane fraction after ischemic PC in isolated rat hearts and is involved in the development of protection against postischemic left ventricular dysfunction. Mitchell et al have reported similar results in the rat heart. These apparent inconsistencies may be due to the different species and models used in the above studies. Although some evidence suggests that A1R activation results in transient translocation of PKCδ in rat ventricular myocytes, it is currently not known which other PKC isoforms may be activated in the myocardium downstream of A1R and mediate its delayed PC effect against infarction.

Imagawa et al first demonstrated the involvement of TK in ischemic PC-induced second window of protection against infarction, using the TK inhibitor genistein. However, genistein, originally considered to be selective for TK, seems to have other nonselective effects such as inhibition of serine/threonine kinases (eg, PKC and PKA) at higher concentrations. Importantly, genistein has also been reported to inhibit A1R in noncardiac cells, although Imagawa et al showed that it did not abolish the hemodynamic effects of A1R receptor activation in rabbits. We therefore chose to use a more selective TK inhibitor, LDA, in the present study. LDA is a potent inhibitor of protein TK (IC50, 0.5 μmol/L) and the epidermal growth factor receptor kinase (IC50, 29 nmol/L), with much weaker actions at inhibiting PKC or PKA (IC50 >200 μmol/L). In the present study, we used LDA at a dose of 1.3 mg/kg, which, assuming distribution in total body water, would yield an approximate plasma concentration of 5 μmol/L, 10-fold higher than the IC50 for inhibition of protein TK but well below that for other protein kinases. This dose of LDA completely abrogated the protection induced by pretreatment with CCPA 24 hours earlier, whereas LDA alone had no effect on infarct size (Figure 2). A similar dose of LDA (1 mg/kg) has been shown to abolish the late PC effect against stunning in a conscious rabbit model. Moreover, Ping et al have recently demonstrated that a PC protocol of six 4-minute coronary occlusions/4-minute reperusions in conscious rabbits induces selective activation of 2 members of the Src family of protein TKs (Src and Lck) in the myocardium, with no effect on epidermal growth factor receptor kinases, and that this activation is abolished by pretreatment with LDA, at a dose similar to that used in the present study. In that study, LDA did not affect the translocation of PKCe after ischemic PC, thereby pointing to the selectivity of LDA. It is therefore very unlikely that LDA, at the dose used in our study, inhibited any protein kinases other than protein TK.

Taken together, the results of the present study indicate a crucial role for both PKC and TK in mediating delayed PC against infarction after A1R activation in rabbits. We did not examine the relative positions of PKC and TK in the signaling pathway downstream of A1R, although the fact that inhibition of either group of enzymes completely abolished CCPA-induced late PC would suggest that these kinases function in the same, rather than parallel, signaling pathways. Recent evidence suggests that after ischemic PC in rabbits, TK activation occurs downstream of and is dependent on PKC activation. It is also noteworthy that recent evidence in rats and pigs suggests that these 2 groups of enzymes may act in parallel to mediate early ischemic PC. Further studies are necessary to address the relative positions of these enzymes in the signaling cascade downstream of A1R.

In the present study we have also shown, in myocardium not subjected to ischemia/reperfusion, that 24 hours after treatment with CCPA, p38 MAPK activity was significantly increased compared with saline-treated controls. Previous studies have demonstrated activation of myocardial p38 MAPK after a number of stresses, including ischemia/reperfusion (reviewed in Reference 44), and also after activation of G protein–coupled receptors, including A1R. However, all of these studies have examined the acute profile of p38 MAPK activation. For example, Haq et al showed, in the isolated perfused rat heart, that infusion of adenosine resulted in rapid activation of p38 MAPK that was maximal at 5 minutes and declined thereafter. To the best of our knowledge, the present study is the first to show that transient A1R activation also induces a second phase of enhanced p38 MAPK activity at 24 hours in rabbit myocardium. Moreover, we have shown that this delayed p38 MAPK activation is downstream of, and dependent on, PKC and TK activation, given that pretreatment with either CHE or LDA, at doses that abrogated delayed protection at 24 hours, completely abolished the enhanced p38 MAPK activity. This is consistent with other reports of a role for PKC and TK in activation of p38 MAPK in both noncardiac tissue and myocytes. Taken together, these results point to a potential role for p38 MAPK in mediating delayed A1R-induced PC in the rabbit. In support of this concept, preliminary results by Carroll and Yellon have shown, in an adult human-derived cardiac myoblast cell line, that pretreatment with the selective p38 MAPK inhibitor SB203580 completely abolishes delayed protection 24 hours after ischemic or adenosine-induced PC. Similarly, Zhao et al have recently reported preliminary data suggesting that A1R-induced PC against infarction in mice is associated with delayed activation of p38 MAPK via a TK-sensitive mechanism.

Role of Hsp27 in Delayed PC

One substrate for p38 MAPK is the protein kinase MAPKAPK-2, which itself phosphorylates Hsp27. Phosphorylation of Hsp27 has been shown to increase its cytoprotective activity, an action that involves changes in the oligomeric structure of Hsp27 and stabilization of the actin cytoskeleton (reviewed in Reference 41). In the present study we did not find enhanced expression of Hsp27 at 24 hours after CCPA treatment. This is consistent with a preliminary report by Heads et al, who found no change in Hsp27 expression 24 hours after CCPA or ischemic PC in rabbit myocardium, but showed in subcellular fractionation studies that these PC protocols resulted in redistribution of Hsp27 from the membrane to the soluble fraction. In the present study, we have extended these findings and have shown that, whereas Hsp27 from control hearts is mainly nonphosphorylated, 24 hours after pharmacological PC with CCPA, Hsp27 is primarily in a phosphorylated form, and that this pattern of phosphorylation is mediated by a signaling mechanism dependent on both PKC and TK activation. Although our results are correlative
and do not allow us to establish a direct causal relationship between Hsp27 phosphorylation and delayed A1R-induced infarction, their temporal relationship and the fact that both are blocked by pretreatment with either CHE or LDA are strongly suggestive.

**Other Mediators of A1R-Induced Delayed PC**

Other cytoprotective proteins have been implicated as potential end effectors mediating delayed cardioprotection after A1R activation in the rabbit heart. For example, we and others have demonstrated that A1R-induced delayed PC is dependent on opening of the ATP-sensitive K⁺ channels (KATP) during the index ischemic insult.⁵⁵,⁷⁴ These studies showed that the delayed infarct-limiting effect of CCPA was abrogated by 5-hydroxydecanoate, a proposed selective blocker of the mitochondrial rather than the sarcolemmal KATP channels.⁷⁵ Hsp27 and Hsp40 may maintain opening of mitochondrial KATP channels with diazoxide. It may therefore be of interest to speculate that A1R-induced delayed PC, by activation of the p38 MAPK/Hsp27 pathway 24 hours after A1R activation in the rabbit heart. For example, we and others have demonstrated that A1R-induced delayed PC is dependent on opening of the mitochondrial KATP channels,⁷⁵ therefore suggesting that opening of the mitochondrial KATP channels may mediate the delayed cardioprotective effects of A1R agonists.⁵⁵ Recent evidence has shown that the integrity of the actin cytoskeleton may have a regulatory function in gating of cardiac KATP channels.⁷⁶–⁷⁸ More importantly, Baines et al.⁷⁹ have recently demonstrated that pharmacological disruption of the cytoskeleton by cytochalasin D completely abolishes the protection conferred to cardiomyocytes by direct opening of the mitochondrial KATP channels with diazoxide. It may therefore be of interest to speculate that A1R-induced delayed PC, by activation of the p38 MAPK/Hsp27 pathway, may result in preservation of actin microfilaments during the sustained ischemic insult. This in turn may maintain opening of mitochondrial KATP channels with ultimate reduction in ischemia/reperfusion-induced myocardial injury, although the mechanisms by which opening of these channels confers protection to the ischemic myocardi um are by no means certain.

In conclusion, we have shown that the delayed myocardial protection 24 hours after transient A1R activation with CCPA in rabbits is mediated by a signaling mechanism involving both PKC and TK. This signaling cascade in turn results in activation of actin microfilaments during the sustained ischemic insult. This in turn may maintain opening of mitochondrial KATP channels with ultimate reduction in ischemia/reperfusion-induced myocardial injury, although the mechanisms by which opening of these channels confers protection to the ischemic myocardi um are by no means certain.

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Adenosine A_1 Receptor Induced Delayed Preconditioning in Rabbits: Induction of p38 Mitogen-Activated Protein Kinase Activation and Hsp27 Phosphorylation via a Tyrosine Kinase– and Protein Kinase C–Dependent Mechanism

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