Adenosine-induced late preconditioning in mouse hearts: role of p38 MAP kinase and mitochondrial K$_{ATP}$ channels

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Zhao, Ting C., Denise S. Hines, and Rakesh C. Kukreja. Adenosine-induced late preconditioning in mouse hearts: role of p38 MAP kinase and mitochondrial K$_{ATP}$ channels. Am J Physiol Heart Circ Physiol 280: H1278–H1285, 2001.—We investigated the role of p38 mitogen-activated protein kinase (MAPK) phosphorylation and opening of the mitochondrial ATP-sensitive K$^+$ ([K$_{ATP}$]$_{mito}$) channel in the adenosine A$_1$ receptor (A$_1$AR)-induced delayed cardioprotective effect in the mouse heart. Adult male mice were treated with vehicle (5% DMSO) or the A$_1$AR agonist 2-chloro-$N^6$-cyclopentyladenosine (CCPA; 0.1 mg/kg ip). Twenty-four hours later, hearts were subjected to 30 min of global ischemia and 30 min of reperfusion in the Langendorff mode. Genistein or SB-203580 (1 mg/kg ip) given 30 min before CCPA treatment was used to block receptor tyrosine kinase or p38 MAPK phosphorylation, respectively. 5-Hydroxydecanoate (5-HD; 200 µM) was used to block ([K$_{ATP}$]$_{mito}$) channel. CCPA produced marked improvement in left ventricular function, which was partially blocked by SB-203580 and 5-HD and completely abolished with genistein. CCPA caused a reduction in infarct size (12.0 ± 2.0 vs. 30.3 ± 3.0% in vehicle), which was blocked by genistein (29.4 ± 2.3%), SB-203580 (28.3 ± 2.6%), and 5-HD (33.9 ± 2.4%). CCPA treatment also caused increased phosphorylation of p38 MAPK during ischemia, which was blocked by genistein, SB-203580, and 5-HD. The results suggest that A$_1$AR-triggered delayed cardioprotection is mediated by p38 MAPK phosphorylation. Blockade of cardioprotection with 5-HD concomitant with decrease in p38 MAPK phosphorylation suggests a potential role of ([K$_{ATP}$]$_{mito}$) channel opening in phosphorylation and ensuing the late preconditioning effect of A$_1$AR.

BRIEF ISCHEMIA before a second sustained ischemia decreases the size of myocardial infarction (26). This cardioprotective phenomenon, known as ischemic preconditioning, has been classified into two temporally distinct phases: an early phase, which wanes within 2–4 h, and a late phase, which manifests 24–72 h later (2). Intense efforts are being made to discover the novel triggers and effectors of preconditioning that could potentially be developed to mimic the effect of ischemic preconditioning. Adenosine is one such agent that has been proposed to be an important trigger of the early and delayed preconditioning (4, 34). However, the underlying intracellular signaling mechanisms of protection induced by this agent are not fully understood. We (37) recently demonstrated that the adenosine A$_1$ receptor (A$_1$AR) agonist 2-chloro-$N^6$-cyclopentyladenosine (CCPA)-induced late preconditioning was due to enhanced synthesis of nitric oxide (NO) in the CCPA-treated heart. This late ischemic protection was abolished by S-methylisothiourea, a selective pharmacological inhibitor of inducible NO synthase (iNOS), and was absent in the mouse with targeted ablation of the iNOS gene (37). Because NO has a role in the opening of the mitochondrial ATP-sensitive K$^+$ ([K$_{ATP}$]$_{mito}$) channel (32), recently, we and others (3–6) demonstrated that the late protection due to A$_1$AR stimulation or ischemic preconditioning was abolished by glibenclamide and 5-hydroxydecanoate (5-HD), the blockers of the K$_{ATP}$ channel. However, the cellular signaling pathways potentially modulating the K$_{ATP}$ channel and late preconditioning after A$_1$AR activation need further investigation.

The protein tyrosine kinase-mitogen-activated protein kinase (MAPK)-regulated pathway plays an important role for the control of cell growth and differentiation (8). More recently, evidence has been obtained that supports a role for the involvement of tyrosine kinase in early classic preconditioning (22, 35) and late preconditioning (15, 28). Since a novel member of the MAPK family, p38 MAPK, was cloned (11), at least six isoforms of p38 MAP kinase have been described (19). p38 MAPK is activated by dual phosphorylation on a Thr-Gly-Tyr motif in response to endotoxin, cytokines, physical stress (such as hyperosmolarity), and chemical stress (such as hydrogen peroxide) (11). Most interestingly, p38 MAPK has been shown to be activated on exposure to adenosine in perfused rat hearts, which is consistent with MAPK involvement in adenosine-mediated ischemic preconditioning (12). Tyrosine kinase-induced phosphorylation of p38 MAP kinase was shown by preconditioning (22), and the level of phosphorylation of the activation site of p38 MAPK is spe...
cifically increased during ischemia (35). A recent study (10) suggested that transient activation of A1AR caused a significant rise in the activity of p38 MAPK 24 h later in the rabbit heart. In addition, the delayed cardioprotection due to A1AR stimulation is abrogated by either the protein kinase C inhibitor chelerythrine or the receptor tyrosine kinase inhibitor lavendustin A.

Considering the fact that late preconditioning induced by A1AR is mediated by opening of K_{ATP} channels and is also associated with p38 MAPK phosphorylation, the relationship of these two processes is currently unknown. Although the association of phosphorylation of p38 MAP kinase with A1AR-induced late preconditioning has been reported, it is not known whether such a protective effect is abrogated by the selective MAPK inhibitor SB-203580 in the intact heart (10). SB-203580 is a pyrindinylimidazole compound that inhibits the catalytic activity of p38 MAPK by competitive binding in the ATP pocket and has been found to be equipotent against p38-α and p38-β (21). In the present study, we sought to investigate the direct cause and effect relationship of p38 MAPK phosphorylation with A1AR-induced late preconditioning in the mouse heart. Our second goal was to show whether the abrogation of A1AR-induced late cardioprotection with the (K_{ATP})_mito channel inhibitor 5-HD is also associated with inhibition of p38 MAPK phosphorylation during ischemia. These investigations were carried out in our well-established model of the isolated perfused mouse heart as reported previously (36, 38).

METHODS

**Animals.** Adult outbred male mice (IRC Strain) of 30–40 g body wt were supplied by Harlan (Indianapolis, IN). All animal experiments were conducted under the Guidelines on Human Use and Care of Laboratory Animals for Biomedical Research published by the NIH and according to the experimental protocols approved by the Animal Welfare Committee of the Medical College of Virginia Commonwealth University.

**Chemicals.** CCPA, 5-HD, and SB-203580 were obtained from Research Biochemicals International (Natick, MA); gel electrophoresis supplies were obtained from Bio-Rad Laboratories (Hercules, CA); and genistein and all other chemicals were obtained from Sigma (St. Louis, MO).

**Heart perfusion.** All animals were anesthetized with a lethal intraperitoneal injection of pentobarbital sodium (100 mg/kg with 33 units heparin). Hearts were rapidly excised and arrested in ice-cold Krebs-Henseleit buffer. They were then cannulated via the ascending aorta for retrograde perfusion to aortic root, and the heart was cannulated for retrograde perfusion by the Langendorff method using Krebs-Henseleit buffer containing (in mM) 110 NaCl, 4.7 KCl, 1.2 MgSO_4, 7H_2O, 2.5 CaCl_2, 2H_2O, 11 glucose, 1.2 KH_2PO_4, 25 NaHCO_3, and 0.5 EDTA. The buffer, aerated with 95% O_2–5% CO_2 to give a pH of 7.4 at 37°C, was perfused at a constant pressure of 55 mmHg. A small incision was made at the base of the pulmonary artery to drain coronary effluent.

**Left ventricular function.** A left atrial incision was made to expose the mitral annulus, through which a tiny balloon was passed into the left ventricle (LV). A water-filled latex balloon, attached to the tip of polyethylene tubing, was then sufficiently inflated to provide a LV end-diastolic pressure (LVEDP) of <10 mmHg, which was measured by means of a disposable Gould pressure transducer. LV pressure and heart rate were recorded using a RF11 A recorder (Sensor Medics, Anaheim, CA). LV maximum changes in positive and negative pressure over time (dP/dt max) were recorded using a Heart Performance Analyzer (Micro-Med; Louisville, KY). Cardiac contractile function, the rate-pressure product (RPP), was expressed as being the product of LV developed pressure (LVDP) and heart rate; LVDP was calculated by subtracting LVEDP from LV systolic pressure.

**Experimental protocols.** Mice were randomized to receive different treatments by intraperitoneal injection of an agonist, antagonist, or vehicle 24 h before euthanization. The mice were then assigned to one of the following nine groups. 1) CCPA (n = 7): pretreatment with CCPA (0.1 mg/kg ip). 2) Vehicle (n = 9): pretreatment with 5% DMSO (0.1 ml/kg ip). 3) Vehicle + CCPA (n = 9): same as CCPA group except that DMSO was injected 30 min before CCPA. 4) Genistein (n = 7): pretreatment with genistein alone (1 mg/kg ip). 5) Genistein + CCPA (n = 9): same as CCPA group except that genistein (1 mg/kg ip) was injected 30 min before CCPA. 6) SB-203580 (n = 6): pretreatment with SB-203580 (1 mg/kg ip). 7) SB-203580 + CCPA (n = 9): same as the SB-203580 group except that SB-203580 was injected 30 min before CCPA. 8) CCPA + 5-HD (n = 6): same as CCPA group except that 5-HD (200 μM) was given in 0.9% saline through the drug line 5 min before ischemia. 9) Vehicle + 5-HD (n = 5): same as CCPA + 5-HD group except that the animals were treated with the vehicle in lieu of CCPA.

After 30 min of equilibration, the hearts were subjected to 30 min of global ischemia and 30 min of reperfusion. The protocol is shown in Fig. 1.

**Measurements of myocardial infarct size.** At the end of the ischemia–reperfusion, the hearts were removed from the Langendorff perfusion apparatus and immediately weighed, frozen, and stored in a freezer for 24 h. The frozen hearts were then cut from apex to base into six to seven transverse slices of ~0.8 mm in thickness and incubated in 10% triphenyltetrazolium chloride for 30 min. After staining, the slices were fixed in 10% formaldehyde for 40 min before measurement of the infarct area by computer morphometry (Bioquant system, R&M Biometrics). The infarct size was calculated and presented as the percentage of risk area, defined as the sum of total ventricular area minus cavities.

A subset of animals was treated with various drugs solely for the purpose of measuring the p38 MAPK phosphorylation during ischemic period. The mice were assigned into one of the following groups. 1) Vehicle (DMSO): mice pretreated with 5% DMSO (ip) 24 h before ischemia. 2) CCPA: mice pretreated with CCPA 24 h before ischemia (0.1 mg/kg ip). 3) Genistein + CCPA: mice pretreated with genistein (1 mg/kg ip) 30 min before CCPA treatment, as in the CCPA group. 4) SB-203580 + CCPA: mice pretreated with SB-203580 (1 mg/kg ip) 30 min before CCPA. 5) CCPA + 5-HD: 5-HD (200 μM) given into perfusate 5 min before sustained ischemia in CCPA-treated mice.

The hearts were then subjected to global ischemia for 5, 10, 15, 20, or 30 min, respectively.

**Tissue preparation.** After the indicated period of global ischemia, as described in the above groups, hearts were immediately frozen in liquid N_2. The tissue was ground and suspended in 1 ml of lysis buffer containing 50 mM Tris–HCl (pH 7.4), 0.1 mM sodium orthovanadate, 50 mM sodium fluoride, 150 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 5 mM EGTA, 2 μg/ml leupeptin, 2 μg/ml...
aprotinin, and 5 μg/ml pepstatin A. Mixtures were homogenized with a Brinkman polytron and microcentrifuged at 14,000 rpm for 10 min. The protein content of the supernatant was determined using the detergent compatible-protein assay (Bio-Rad).

Western blot. Western blot analysis was performed as described previously with some modification (13). The proteins (40 μg), separated by SDS-PAGE (10% polyacrylamide), were transferred to a nitrocellulose membrane (0.2 μM) by tank transfer (Bio-Rad) for 2 h at 200 mA. The membranes were first probed with phospho-specific p38 MAPK (Thr 180/Tyr 182) antibody (New England Biolabs; Beverly, MA), after which they were stripped in 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7) for 30 min at 56°C, blocked, and reprobed with nonspecific p38 MAPK antibody (for total p38 MAPK; Santa Cruz Biotechnology; Santa Cruz, CA). Briefly, membranes were either blocked with 5% BSA and 0.1% Tween 20 in Tris-buffered saline for 2 h by overnight incubation at 4°C with phospho-specific p38 MAPK antibody (1:1,000 dilution) or blocked with 5% nonfat dry milk in Tris-buffered saline overnight at 4°C. The membranes were then incubated for 1 h with p38 MAPK antibody (1:1,000 dilution). The membranes were washed with Tris-buffered saline containing 0.1% Tween 20 and incubated with an anti-rabbit horseradish peroxidase-linked antibody (Amersham Pharmacia Biotech; Piscataway, NJ) diluted 1:500 for 1 h at room temperature followed by detection with chemiluminescence (Amersham ECL Western blotting detection reagent).

Statistical analysis. The results are expressed as means ± SE. Differences among the groups were analyzed by one-way analysis of variance (ANOVA). Statistical differences were considered significant if the P value was <0.05.

RESULTS

Infarct size. Myocardial infarct size was 30.3 ± 3.0% of the risk zone in the vehicle-treated hearts. Treatment with CCPA reduced infarct size to 12.2 ± 2.5% (P < 0.05; Fig. 2). The infarct size in the vehicle + CCPA group was 17.0 ± 2.9%, which was not different compared with the CCPA group. Genistein and SB-203580 completely abolished the protective effect of CCPA, as indicated by increase in the infarct size to 29.4 ± 2.3 and 28.3 ± 2.6%, respectively (P < 0.05 vs. 12.2 ± 2.5% in control). The infarct sizes in the genistein- and SB-203580-treated mice were 30.0 ± 1.6 and 28.3 ± 2.6%, respectively, which were indistinguishable from the vehicle group. Infusion of 5-HD before ischemia-reperfusion in CCPA-treated mice resulted in significant increases in the infarct size to 33.9 ± 2.4. Vehicle + 5-HD did not cause a significant increase in infarct size.
change in the infarct size compared with that of the vehicle group. The area at risk for the globally ischemic hearts was not different between groups (data not shown).

LV function. Myocardial functional parameters, such as LV systolic pressure, LVEDP, dP/dt max, heart rate, and coronary flow during preischemia, were not significantly different among the groups (Table 1). Also, average body and heart weights were similar among all the groups (data not shown). In the ischemic-reperfused heart, LVEDP was significantly lower in the CCPA-treated group as compared with the vehicle-treated control (P < 0.05; Fig. 3A). The CCPA-induced improvement in LVEDP was abrogated by genistein (P < 0.05 vs. CCPA). Genistein itself had no significant effect on LVEDP in the control group (non-CCPA-treated mice). SB-203580 did not abolish the improvement in LVEDP observed in the vehicle-treated CCPA group. In fact, the LVEDP was found to be significantly lower in the SB-203580-treated groups (with or without treatment with CCPA). Similarly, 5-HD failed to block the improvement in postischemic LVEDP when compared with the vehicle-CCPA group (P < 0.05). Also, 5-HD did not have significant effect on the postischemic levels of LVEDP when compared with the vehicle or CCPA-treated mice.

Recovery of RPP was 63.9 ± 6.9% in the vehicle control group, increased to 96 ± 7% with CCPA (P < 0.05; Fig. 3B), and was abolished by genistein (52.1 ± 9.5%), SB-203580 (70.2 ± 7.5%), and 5-HD (67.5% ± 2.9), although these differences were nonsignificant compared with CCPA. A similar trend in the changes in LVDP was observed (data not shown). No significant changes in +dP/dt max or –dP/dt max was observed among the groups (data not shown).

Also, the heart rate and coronary flow were also not different between the groups (Fig. 4, A and B).

### Table 1. Baseline functional parameters

<table>
<thead>
<tr>
<th></th>
<th>LVSP, mmHg</th>
<th>LVEDP, mmHg</th>
<th>+dP/dt max, mmHg/s</th>
<th>–dP/dt max, mmHg/s</th>
<th>HR, beats/min</th>
<th>CF, ml/min</th>
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<tr>
<td>CCPA</td>
<td>67.5 ± 5.9</td>
<td>3.7 ± 1.6</td>
<td>29.5 ± 2.0 × 10²</td>
<td>23.1 ± 2.1 × 10²</td>
<td>385 ± 18.7</td>
<td>3.0 ± 0.57</td>
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<tr>
<td>Vehicle</td>
<td>82.3 ± 3.6</td>
<td>5.6 ± 3.3</td>
<td>25.1 ± 1.1 × 10²</td>
<td>24.0 ± 1.4 × 10²</td>
<td>410.3 ± 26.9</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>Vehicle + CCPA</td>
<td>78.3 ± 8.1</td>
<td>1.7 ± 1.3</td>
<td>23.5 ± 2.5 × 10²</td>
<td>23.3 ± 3.0 × 10²</td>
<td>392.0 ± 21.3</td>
<td>2.97 ± 0.3</td>
</tr>
<tr>
<td>Genistein</td>
<td>94.6 ± 6.1</td>
<td>1.5 ± 1.1</td>
<td>28.5 ± 2.3 × 10²</td>
<td>28.9 ± 2.1 × 10²</td>
<td>364.4 ± 9.0</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>Genistein + CCPA</td>
<td>93.6 ± 4.2</td>
<td>4.3 ± 2.2</td>
<td>29.6 ± 1.7 × 10²</td>
<td>28.8 ± 1.6 × 10²</td>
<td>401.0 ± 14.1</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>SB-203580</td>
<td>76.7 ± 6.3</td>
<td>6.0 ± 1.6</td>
<td>23.9 ± 2.3 × 10²</td>
<td>21.6 ± 2.1 × 10²</td>
<td>355.7 ± 22.2</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>SB-203580 + 5-HD</td>
<td>80.6 ± 6.6</td>
<td>7.0 ± 2.2</td>
<td>26.5 ± 2.0 × 10²</td>
<td>19.5 ± 2.2 × 10²</td>
<td>403 ± 27</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>Vehicle + 5-HD</td>
<td>82.3 ± 6.1</td>
<td>2.7 ± 1.4</td>
<td>21.7 ± 2.1 × 10²</td>
<td>19.4 ± 2.2 × 10²</td>
<td>339.1 ± 30.2</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>Vehicle + 5-HD</td>
<td>78.2 ± 9.2</td>
<td>6.5 ± 2.5</td>
<td>21.1 ± 1.7 × 10²</td>
<td>18.1 ± 2.4 × 10²</td>
<td>329.4 ± 34.1</td>
<td>2.3 ± 1.0</td>
</tr>
</tbody>
</table>

Values are means ± SE. LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; +dP/dt max, maximum positive and negative change in pressure over time; HR, heart rate; CF, coronary flow; CCPA, 2-chloro-5-N6-cyclopentyladenosine; 5-HD, 5-hydroxydecanoate.
Western blot analysis of p38 MAPK phosphorylation. The total p38 MAPK protein expression was not different between groups, i.e., the levels detected remained fairly constant throughout the ischemia (Fig. 5, right). However, the phospho-specific p38 MAPK protein increased in hearts pretreated with CCPA during 10, 15, and 20 min of ischemia (Fig. 5, left). The increase in CCPA-induced phospho-p38 MAPK protein was inhibited in mice that were pretreated with genistein as well as SB-203580. Interestingly, similar abolition of phospho-p38 MAPK expression was observed in the CCPA-pretreated heart with infusion of 5-HD 5 min before ischemia. DMSO had no effect on the phosphorylation state of p38 MAPK protein expression.

DISCUSSION

Salient findings. The main goal of this investigation was to demonstrate the cause and effect relationship of p38 MAPK phosphorylation in A1AR receptor-induced delayed cardioprotection in the mouse heart. In addition, we wanted to know whether the opening of the (K\textsubscript{ATP}) mito channel is correlated with p38 MAPK phosphorylation. Our results show that selective activation of A1AR with CCPA reduced postischemic infarct size, improved recovery of LVEDP and RPP, and caused p38 MAPK phosphorylation in the ischemic heart 24 h after the receptor stimulation. The receptor tyrosine kinase inhibitor genistein as well as the selective p38
MAPK inhibitor SB-203580, when given before CCPA treatment, abolished the delayed cardioprotective effect as well as inhibited p38 MAPK phosphorylation during ischemia. Furthermore, selective blockade of the (KATP)mito channel with 5-HD not only abrogated the A1AR-induced late cardioprotection but also inhibited p38 MAPK phosphorylation. Pharmacological inhibitors (genistein, SB-203580, or 5-HD) were without significant effect on infarct size in mice that were not treated with CCPA. Taken together, our data suggest that p38 MAPK phosphorylation and opening of the (KATP)mito channel play essential role in A1AR-induced delayed cardioprotective effect in the isolated perfused mouse heart.

Tyrosine kinase signaling in preconditioning. The tyrosine kinases are important for the control of cell growth and differentiation and are rapidly phosphorylated on tyrosine residues in response to various stimuli, leading to the activation of the MAPK signaling pathway (8). In the isolated perfused rat heart, p38 MAPK has been shown to be activated during global ischemia and sustained throughout reperfusion (9, 20). Involvement of p38 MAPK signaling pathway in the early phase of preconditioning has been extensively investigated, although the results are controversial. Maulik et al. (22) first suggested the involvement of tyrosine kinase-phospholipase D as a potential signaling pathway for ischemic preconditioning. Weinbrenner et al. (35) also showed decreased phosphorylation of p38 MAP kinase during ischemia in nonpreconditioned hearts, but phosphorylation was enhanced several-fold after 10 and 20 min of ischemia in the preconditioned hearts. Furthermore, when protection from ischemic preconditioning was blocked by adenosine receptor antagonist, the increased phosphorylation of p38 MAPK during ischemia also disappeared. In addition, anisomycin, an activator of the p38 MAPK pathway, was found to be as protective as preconditioning (35). Sakamoto et al. (31) also demonstrated partial blockade of the cardioprotective effects of CCPA with SB-203580 in the isolated perfused rat heart. On the other hand, Barancik et al. (1) suggested a negative role of the p38 MAPK pathway during ischemic activation of p38 MAPKs. Ma et al. (20) showed that administration of SB-203580 decreased myocardial apoptosis and improved posts ischemic cardiac function in the isolated perfused heart. In the present study, we observed significant blockade of A1AR-induced late preconditioning with SB-203580 as well as genistein, which also correlated with decreased expression of phosphorylated p38 MAPK during ischemia in the mouse heart. Although Dana et al. (10) also demonstrated the role of p38 MAPK in A1AR-induced protection, these studies arrived at this conclusion by showing association of p38 MAPK phosphorylation 24 h later and its blockade by lavendustin, a relatively selective inhibitor of receptor tyrosine kinase. These studies were carried out in a rabbit model of myocardial infarction, and LV function was not measured. However, the present study demonstrates the protective effect p38 MAPK phosphorylation in a mouse model with multiple end points, i.e., infarct size as well as LV function. This model could be useful in further evaluation of genes involved in A1AR-induced late preconditioning.

In the present study, we observed an increase in phosphorylation of p38 MAPK only during ischemia in the hearts treated with CCPA, whereas this was absent in the nontreated hearts. Maulik et al. (23) showed that pretreatment with genistein attenuated the increase in both MAPK and MAPK-activated protein kinase (MAPKAPK)-2 activities, which occurred in the preconditioned hearts. Another study (20) showed increased p38 MAPK activity in non preconditioned hearts subjected to ischemia-reperfusion, whereas Weinbrenner et al. (35) observed p38 MAPK phosphorylation only in preconditioned hearts.

From the present study, it appears that p38 MAPK is the mediator of the delayed cardioprotective effect of CCPA. It was recently proposed that MAPKs exhibited a biphasic activation on exposure to certain stimuli (21). Haq et al. (12) showed that infusion of adenosine resulted in rapid activation of p38 MAPK that was maximal at 5 min and declined thereafter. We did not measure the initial time course of p38 phosphorylation after CCPA treatment in the present study. However, from the results presented in this paper and those of others (10), it appears that p38 MAPK phosphorylation after 24 h of CCPA treatment is important in mediating the late phase of preconditioning.

Role of (KATP)mito channel. In the present study, we also observed blockade of the delayed preconditioning effect of CCPA with 5-HD. Several previous studies from this laboratory and others (5, 6, 13, 25, 29) have shown that late preconditioning induced by sublethal ischemia, heat shock, and pharmacological agents (such as CCPA and monophosphoryl lipid A) are mediated by opening of the KATP channel in the rabbit heart. It has been suggested that (KATP)mito channels are the effectors of preconditioning, because diazoxide, a selective opener of this channel, induced an early as well as a delayed cardioprotective effect (27, 33), which was abolished by 5-HD. An interesting aspect of this study is that 5-HD, when given before ischemia, not only abolished the late cardioprotective effect of CCPA but also inhibited p38 MAPK phosphorylation during ischemia in this group. These data suggest that p38 MAPK phosphorylation is an essential step in mediating the late cardioprotective effect due to opening of the (KATP)mito channel. The precise interrelationship of p38 MAPK phosphorylation with the opening of the channel is not clear from the present study. A recent study (28a) demonstrated that pharmacological preconditioning induced by selective opening of the (KATP)mito channel with diazoxide is blocked by the free radical scavenger mercaptopyrrolionyl glycine. It is possible that activation of cardiac (KATP)mito channels during ischemia in the CCPA-treated hearts may have a role in the generation of reactive oxygen species, which could potentially phosphorylate p38 MAPK. Further investigations are needed to understand whether...
CCPA-induced activation of the \( \text{K}_{\text{ATP}} \) \text{mito} channel during the late phase is blocked by antioxidants.

**Role of NO and heat shock protein**. There are other possible consequences of MAPK phosphorylation also. For example, the 27-kDa heat shock protein (HSP27) is phosphorylated by MAPKAPK-2 and -3, which are the kinases downstream of p38 MAPK (16, 24, 30). HSP27 is known to exert its cardioprotective effect by stabilizing the microfilaments (18). Dana et al. (10) suggested an important role for both protein kinase C and receptor tyrosine kinase as mediators of late preconditioning against infarction after A1AR activation. It was proposed that the p38/HSP27 pathway was a potential distal effector of protection. In the present study, we did not measure the phosphorylated state of HSP27. However, it is quite possible that the inhibition of p38 MAPK phosphorylation with SB-203580 may have prevented the phosphorylation of HSP27 during ischemia, thereby promoting disruption of more actin filaments, resulting in enhanced fragility. Conversely, it has been shown that anisomycin, which activates p38 MAPK with subsequent phosphorylation of HSP27, could have prevented actin filament disruption (14). Additionally, p38 MAPK phosphorylation may have activated iNOS, resulting in enhanced NO release. We (37) recently reported that A1AR activation-induced delayed protection was mediated by an iNOS-sensitive mechanism. p38 MAPK is known to regulate iNOS gene expression in endotoxin-stimulated primary glial cultures (7). The inhibition of p38 MAPK partially reduced interleukin stimulation of iNOS protein and iNOS mRNA in cardiac myocytes, suggesting that interleukin induction of iNOS synthesis depends on p38 MAPK signaling pathways (17).

In conclusion, we have shown that selective activation of A1AR with CCPA induced late cardioprotective effect 24 h after the receptor stimulation in the mouse heart. This protective effect was blocked by the receptor tyrosine kinase inhibitor genistein and the p38 MAPK inhibitor SB-203580. In addition, A1AR receptor stimulation caused increased phosphorylation of p38 MAPK during ischemia 24 h later, which was also blocked by genistein as well as SB-203580. These data suggest a direct cause and effect relationship of receptor tyrosine kinase or, more specifically, p38MAPK phosphorylation in mediating A1AR-induced delayed cardioprotection. Furthermore, the blockade of A1AR-induced delayed cardioprotection with 5-HD also suggests an obligatory role of \( \text{K}_{\text{ATP}} \) \text{mito} channels. It is possible that reduced oxygen species generated as a result of \( \text{K}_{\text{ATP}} \) \text{mito} channel opening may have a role in p38 MAPK phosphorylation. The net protective effect may be due to combination of factors including generation of NO/reduced oxygen species, opening of the \( \text{K}_{\text{ATP}} \) \text{mito} channel, and phosphorylation of HSP27. Further studies are necessary to elucidate the precise relationship of these mediators and effectors in late preconditioning induced by “pharmacological” activation of A1AR.

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