Inducible Nitric Oxide Synthase Mediates Delayed Myocardial Protection Induced by Activation of Adenosine A1 Receptors
Evidence From Gene-Knockout Mice

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Background—The mechanism of delayed preconditioning induced by activation of adenosine A1 receptors (A1ARs) is not fully understood. We determined the role of inducible nitric oxide synthase (iNOS) in mediating adenosine-induced late cardioprotection using pharmacological inhibitors and iNOS gene–knockout mice.

Methods and Results—Adult male mice were treated with saline or an A1AR agonist, 2-chloro-N\textsuperscript{6}-cyclopentyladenosine (CCPA). Twenty-four hours later, the hearts were perfused in Langendorff mode and subjected to 30 minutes of global ischemia followed by 30 minutes of reperfusion. 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX; 0.1 mg/kg IP) and \textit{S}-methylisothiourea (SMT; 3 mg/kg IP) were used to block A1ARs and iNOS, respectively. Infarct size (IS) was measured by triphenyltetrazolium chloride staining, and iNOS expression was measured by Western blots. Myocardial IS was reduced from 24.0\textpm3.2\% in the saline group to 12.2\textpm2.5\% in CCPA-treated mice (\textit{P}, 0.05). The infarct-reducing effect of CCPA was abrogated by DPCPX (29.3\textpm3.4\%) and SMT (32.3\textpm2.6\%) and was absent in mice with targeted ablation of iNOS (23.9\textpm1.6\%). CCPA produced improvement in postischemic end-diastolic pressure, developed pressure, and rate-pressure product, which was also blocked by DPCPX and SMT. Increased iNOS protein expression observed in CCPA-treated hearts was diminished by DPCPX.

Conclusions—Selective activation of A1ARs produces delayed cardioprotection against ischemia/reperfusion injury in the mouse. Increased iNOS expression concomitant with the lack of protective effect of A1AR activation in iNOS gene–knockout mice suggests a direct cause-and-effect relationship of iNOS in adenosine-induced late cardioprotection. (\textit{Circulation}. 2000;102:902-907.)

Key Words: adenosine \n receptors \n ischemia \n reperfusion \n nitric oxide synthase \n myocardial infarction

Brief episodes of ischemia increase the tolerance of myocardium to injury by a second ischemic insult 24 to 72 hours later.\textsuperscript{1} This phenomenon is known as the delayed phase or second window of preconditioning (SWOP). The delayed phase of preconditioning is preceded by an early phase, which is known to last 2 to 3 hours after the initial preconditioning insult.\textsuperscript{2} Several studies suggest that adenosine is one of the important mediators of the early and delayed preconditioning. Liu et al\textsuperscript{1} blocked the early preconditioning effect when endogenous adenosine was pharmacologically antagonized with nonselective adenosine receptor antagonist. Similarly, the nonselective adenosine receptor antagonist 8-(\textit{p}-sulfophenyl)-theophylline (SPT) abolished SWOP when given before and during ischemic preconditioning (IPC) by coronary artery occlusion in rabbits.\textsuperscript{4} In addition, the administration of the selective adenosine A1 receptor (A1AR) agonist 2-chloro-N\textsuperscript{6}-cyclopentyladenosine (CCPA) in place of IPC induced SWOP after 24 hours in rabbit hearts.\textsuperscript{4} The mechanism(s) underlying the delayed protection with adenosine is not fully understood. Because the half-life of adenosine is short,\textsuperscript{5} it is obvious that mere occupation of the A1ARs would not provide cardioprotection, but rather its “activation” starts the cascade of signal transduction events leading to limitation in infarct size 24 hours later. We originally hypothesized that CCPA-induced delayed preconditioning is mediated by opening of the K\textsubscript{ATP} channel.\textsuperscript{6} The mediator in opening of the K\textsubscript{ATP} channel after adenosine-induced protection is not known. Because NO has been proposed to open K\textsubscript{ATP} channels,\textsuperscript{7} we hypothesized that the A1AR may mediate this protective effect via an NO-sensitive mechanism. We carried out the present investigation in the isolated mouse and in genetically engineered inducible nitric oxide synthase (iNOS) gene–knockout mice to provide the direct cause-and-effect relationship of iNOS in adenosine-induced delayed cardioprotection.

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cardioprotection. The goals of the present investigation were (1) to show that selective activation of A1/ARs with CCPA induces delayed cardioprotection; (2) to demonstrate that adenosine-induced late cardioprotection is abrogated by either S-methylisothiourea (SMT), an iNOS inhibitor absent in iNOS gene–knockout mice; and (3) to show that A1AR activation enhances the expression of iNOS in the mouse heart.

Methods

Animals
Adult male outbred mice (ICR strain; body weight, 25 to 40 g) were supplied by Harlan Sprague Dawley Co (Indianapolis, Ind). Adult male iNOS gene–knockout (−/−) B6,129 mice were purchased from the Jackson Laboratory (Bar Harbor, Me). Chimeric mice were generated by injecting C57BL/6j (B6) blastocysts with recombinant 129-derived embryonic stem cells and implanted into pseudopregnant females for development. Chimeric mice were then mated with B6 females, and the resulting B6,129 F1 heterozygous mutant mice (+/−) were interbred to generate F2 homozygous mutant (−/−) mice for the disruption of iNOS. Their progeny were genotyped by Southern analysis. On their arrival, the animals were allowed to readjust to the new housing environment for ≥3 days before any experiment. Standard rodent food and water were freely accessible. All animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by the National Institutes of Health (publication No. 85-23, revised 1996).

Drugs and Chemicals
CCPA and the A1AR antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were purchased from Research Biochemicals Inc. Lipopolysaccharide (LPS), SMT, and triphenyltetrazolium chloride (TTC) were purchased from Sigma Chemical Co. The iNOS primary antibody was purchased from Santa Cruz Biotechnology Inc, and an anti-rabbit Ig horseradish peroxidase–linked whole antibody was purchased from Amersham.

Langendorff-Perfused Isolated Heart Preparation
The methodology of the Langendorff-perfused mouse heart preparation was described previously in detail, except that the ventricular function was determined with an isovolumic balloon as follows. A left atrial incision was made to expose the mitral annulus, through which a water-filled latex balloon was passed into the left ventricle. The balloon was attached via polyethylene tubing to a Gould pressure transducer that was connected to a Sensormedics polygraph recorder (model RF511A) and a heart performance analyzer (HPA-100, Micro-Med). The balloon was inflated to adjust the left ventricular end-diastolic pressure (LVEDP) to ~10 mm Hg. Myocardial ischemic injury was measured with multiple, independent end points of tissue injury. These included infarct size, left ventricular developed pressure (LVDP), LVEDP, rate-pressure product (RPP), heart rate, and coronary flow. Heart rate, LVDP, LVEDP, and maximum positive or negative first derivative of left ventricular pressure (±dP/dtmax) (the index of the inotropic state) were monitored and recorded continuously. LVDP was calculated by subtracting LVEDP from the peak systolic pressure. RPP, an index of cardiac work, was calculated by multiplying LVDP by heart rate.

Drug Pretreatment and Experiment Protocol
Mice were randomized into 7 experimental groups that received the following treatments, as shown in Figure 1: (1) Saline (n=8): mice were treated with saline (IP) 24 hours before ischemia/reperfusion (I/R); (2) CCPA (n=7): mice were treated with CCPA (0.1 mg/kg IP) 24 hours before I/R; (3) DPCPX+CCPA (n=6): the A1AR antagonist DPCPX (0.1 mg/kg IP) was given 30 minutes before the treatment with CCPA (0.1 mg/kg IP), which was given 24 hours before I/R; (4) DPCPX (n=6): DPCPX (0.1 mg/kg IP) was given 24 hours before I/R; (5) CCPA+SMT (n=6): mice were treated with CCPA (0.1 mg/kg IP) 24 hours before I/R, and SMT (3 mg/kg IP) was administered 30 minutes before I/R; (6) SMT (n=6): SMT (3 mg/kg IP) was given 30 minutes before I/R; and (7) CCPA+iNOS-KO (n=8): iNOS-knockout mice were treated with CCPA (0.1 mg/kg IP) 24 hours before I/R. All hearts were then isolated and subjected to 30 minutes of stabilization, 30 minutes of global ischemia, and 30 minutes of reperfusion.

Measurements of Myocardial Infarct Size
At the end of I/R, hearts were removed from the Langendorff perfusion apparatus and immediately weighed, frozen, and stored in a freezer. The frozen hearts were then cut from apex to base into 6 or 7 transverse slices ≤0.8 mm thick. The slices were placed into a small dish and then incubated in 10% TTC at room temperature for 30 minutes. After staining, TTC buffer was replaced, and the slices were fixed in 10% formaldehyde for 40 minutes before measurement of infarcted tissue by computer morphometry with Bioquant 98 software. The risk area was the sum of total ventricular area minus cavities. The infarct size was calculated and presented as percentage of risk area.

Western Blot Analysis
Hearts were weighed and homogenized with 6 bursts of 15 seconds each at 4°C with a Polytron PT 20 in 1 mL RIPA buffer (1×PBS, 1% Nonidate P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μmol/L PMSF, 30 μL/mL aprotinin, and 100 μmol/L sodium orthovanadate). Subsequently, the samples were centrifuged at 14 000 rpm for 10 minutes. The pellet was discarded, and the protein in the
supernatant was determined. Standard SDS gel electrophoresis was performed with 20 μg of protein loaded in each well of a 12% polyacrylamide gel. After electrophoresis, the protein was transferred to a nitrocellulose membrane for 2 hours at 100 V. The membrane was blocked with 5% non-fat dry milk in 1× Tris-buffered saline containing 0.1% Tween 20 (TBST 0.1%) for 1 hour. The membrane was incubated with the primary iNOS antibody (dilution, 1:1000) for 1 hour at room temperature. After a washing with TBST, the membrane was incubated with an anti-rabbit horseradish peroxidase–linked antibody (dilution, 1:500) for 1 hour. The membranes were developed with enhanced chemiluminescence (Amer sham) and exposed to x-ray film for the appropriate time.

Statistical Analysis
The results are expressed as mean±SEM. Differences between groups for a given parameter were analyzed by 1-way ANOVA followed by Student-Newman-Keuls post hoc test for pairwise comparisons. A value for a given parameter were analyzed by 1-way ANOVA followed by Student-Newman-Keuls post hoc test for pairwise comparisons. A value of P<0.05 was considered statistically significant.

Results
Exclusions
In total, 54 mice were originally entered into the I/R experiments. Five of these animals (ie, 9% of the total) were excluded from further data analysis because of aortic damage or cannulation time delay (>3 minutes).

Preischemic Ventricular Functional Parameters
The Table shows preischemic baseline functional parameters of the hearts in the experimental groups. The mean values of LVDP, LVEDP, RPP, heart rate, coronary flow, and +dP/dt max were not significantly different between the groups. Also, there was no significant difference in the body weights and heart weights among all the groups (not shown).

Postischemic Myocardial Infarct Size
Myocardial infarct size, an index of irreversible myocardial injury, was 24.2±3.2% of the risk zone in the saline-treated mouse hearts after I/R (Figure 2). Treatment with CCPA 24 hours before I/R reduced the infarct size (12.2±2.5%, P<0.05 versus saline). The selective A1AR antagonist DPCPX blocked the protective effect of CCPA, as indicated by an increase in the infarct size to 29.3±4.5% (P<0.05 versus CCPA). The infarct sizes between the saline-treated and DPCPX-treated CCPA groups were not significantly different (P>0.05). The infarct size in mice treated with DPCPX was 25.1±4.5%, which was also not different from that in the saline-treated mice (P>0.05). SMT, a selective iNOS inhibitor, administered before I/R blocked the protective effect of CCPA, as indicated by an increase in the infarct size to 32.3±3.4% (P<0.05 versus CCPA). The infarct size in SMT-treated control mice was 30.8±4.7%, which was also not different from that in the CCPA+SMT and saline-treated mice (P>0.05). CCPA treatment in the iNOS-knockout mice failed to reduce infarct size after I/R. Target disruption of the iNOS gene had no effect on infarct size after I/R injury, as reported previously.9 The area at risk for the globally ischemic hearts was not different between the groups (not shown).

Postischemic Ventricular Function
Overall postischemic ventricular contractile function was also improved after CCPA treatment. The recovery of postischemic LVDP was 51.8±7.2 mm Hg in the saline-treated group and increased to 65.7±5.9 mm Hg, although the differences were not significant (P>0.05, Figure 3A). Pretreatment with DPCPX blocked the protective effect of CCPA, as indicated by decreased LVDP (44.6±5.2 mm Hg, P<0.05 versus CCPA). No significant differences in LVDP were observed between the DPCPX- and DPCPX+CCPA-treated groups. CCPA-induced improvement in LVDP was abrogated by SMT, although the difference was not significant. A marginal improvement in LVDP was observed after treatment with SMT, although this was not significantly different from the saline-treated control. Furthermore, CCPA failed to demonstrate recovery in LVDP in the iNOS gene–knockout mice after I/R.

The postischemic LVEDP was reduced from 15.0±4.7 mm Hg in the saline group to 2.7±2.4 mm Hg in the CCPA-treated group (P<0.05, Figure 3B). Both DPCPX and SMT abrogated CCPA-induced improvement in LVEDP, without having a significant effect in the saline-treated animals. In addition, CCPA-induced improvement in LVEDP
was absent in the iNOS-knockout mice, as indicated by significantly higher pressure (13.6±3.9 mm Hg, versus 2.7±2.4 mm Hg in CCPA-treated mice). An identical trend in the changes in RPP was observed (Figure 3C). Postischemic recovery of heart rate and coronary flow was similar in all the groups (Figure 4A and 4B).

**iNOS Protein Expression**

Pretreatment with CCPA caused enhanced expression in the myocardial iNOS (lanes 3 and 4) compared with the saline-treated hearts (lanes 1 and 2, Figure 5A). The iNOS expression was decreased in the hearts pretreated with DPCPX, demonstrating that activation of A1 ARs led to the increased expression of iNOS (lanes 5 and 6). The positive control (lane 7) shows iNOS induction with LPS (5 mg/kg), which was also given 24 hours before the mice were killed. Figure 5B shows the results of densitometry analysis of iNOS expression. It quantitatively confirms CCPA-induced iNOS upregulation (P<0.05 versus saline), which is diminished by DPCPX pretreatment.

**Discussion**

**Salient Findings**

Identification of pharmacological intervention(s) capable of mimicking the protective actions of delayed preconditioning would help in developing clinically applicable strategies for treating patients at risk for myocardial infarction or other acute coronary events. Previous studies have shown that A1 AR activation induces delayed protection in the rabbit heart.4,6 This protection was attributed to the activation of protein kinase C (PKC)10 and opening of the KATP channel.6,11 In the present study, we have shown a novel mechanism of A1 AR-induced delayed cardioprotection in the isolated perfused mouse heart. Stimulation of A1 ARs with CCPA resulted in delayed cardioprotection, as indicated by a significant reduction in infarct size and improvement in postischemic ventricular function. This delayed protective effect was abrogated by selective inhibition of A1 ARs, confirming the role of this receptor in delayed cardioprotection. The A1 AR-mediated protection was also abolished by SMT, a competitive NOS inhibitor with selectivity toward the inducible isofrom and without significant effects on constitutive endothelial NOS.12 Furthermore, targeted disruption of iNOS in the gene-knockout mice eliminated the delayed protection by CCPA. Our studies also show that the administration of CCPA led to a mild increase in the expression of iNOS that was diminished by DPCPX, further confirming the direct effect of A1 AR activation in iNOS expression. Taken together, these data suggest that iNOS plays an important role in A1 AR-induced delayed ischemic protection in the isolated perfused mouse heart.

**Adenosine Receptors and Signaling Pathway**

Adenosine is a ubiquitous biological compound formed as a consequence of the breakdown of ATP during ischemic preconditioning. Extracellular adenosine acts on specific adenosine receptors on the cell surface. Three types of adenosine receptors have been identified: A1, A2, and A3. The A1 AR, which is located on the cardiac myocytes,5 is involved in the cardioprotective effect of IPC. Intracoronary infusion of adenosine in isolated rabbit hearts or intravenous administration of the A1 selective agonist N6-[phenyl-2R-isopropyl]-
adenosine before the sustained period of ischemia and reperfusion resulted in significant short-term reduction of infarct size. In addition, overexpression of myocardial A1ARs protects the heart from ischemic damage in transgenic mice. However, the continuous activation of A1ARs with a high-dose long-term infusion of CCPA led to the downregulation of the signaling mechanism and loss of protection. Infarct size reduction with IPC is a receptor-mediated phenomenon initiated by stimulation of several G protein–coupled receptors, including adenosine A1 and A2 receptor subtypes. Stimulation of ≥1 of these receptors activates the signaling pathway, which involves activation of phospholipase C and/or D,15,16 production of diacylglycerol, and the resultant activation and subcellular distribution of ≥1 of the 12 known isoforms of PKC. Baxter et al19 suggested that a PKC signaling pathway may link the activation of adenosine receptor to the delayed cytoprotection that is observed many hours later. Their data demonstrated that chelerythrine, a specific PKC inhibitor, blocked delayed cardiac protection induced by ischemia in rabbit heart. PKC can induce downstream activation of MAP kinases. In addition, adenosine has been shown to activate p38 MAPK in neonatal cardiac myocytes. Recently, a preliminary study from our laboratory showed that A1AR stimulation enhances phosphorylation of p38 MAP kinase in the isolated perfused mouse heart (unpublished data).

NO and Intracellular Signaling

In the present investigation, we observed the presence of iNOS expression in the saline-treated mice, which was further enhanced in the CCPA-treated animals. LPS treatment also induced iNOS in these mice. Previous reports have shown the expression of iNOS in the unstimulated cardiac tissue. Pretreatment with DPCPX prevented the CCPA-induced increase in iNOS expression, suggesting that the selective activation of A1ARs indeed triggered the signaling pathway. Guo et al22 also demonstrated a mild upregulation of iNOS protein after IPC in the mouse heart. Because excess NO has been shown to be deleterious during I/R injury, these authors argued that a mild increase in iNOS expression may have been the reason for the protective role of iNOS in the preconditioned heart. Conversely, although we may assume that iNOS expression would lead to increased NO production, it has previously been shown that under pathophysiological conditions, such as ischemia, in which L-arginine and tetrahydrobiopterin may be depleted, iNOS can also generate superoxide from the reductase domain. Therefore, future studies with direct measurement of NO and superoxide are necessary to determine the exact mechanism by which iNOS exerts its effect in adenosine-induced protection.

NO has been suggested to modulate KATP channels by increasing the second messenger cGMP. The cGMP-dependent protein kinases may be capable of phosphorylating KATP channels and priming the channel to offer cardioprotection. Miyoshi et al7 provided direct evidence that NO enhances KATP channel activity in vascular smooth muscle cells. Recently, Sasaki et al30 confirmed this observation by demonstrating direct activation of the mitochondrial KATP channel with exogenously generated NO in myocytes. Opening of the KATP channel as a potential mechanism in early and late IPC has been suggested by several studies.

The intracellular signaling pathways by which A1AR activation causes iNOS expression are largely unresolved but may involve a series of events resulting in the transmission of the signal from the plasma membrane through the cytoplasm to the nucleus, possibly via activation of nuclear factor-κB. This may result in the stimulation of nuclear factor-κB–specific DNA–protein binding, initiating the expression of iNOS and, finally, the release of NO. NO may potentially open mitochondrial or sarcoplasmic KATP channels, resulting in delayed myocardial protection via preservation of mitochondrial ATP and reduction of intracellular Ca2+ overload.

Conclusions

Although previous studies have shown that activation of A1ARs induces delayed cardiac protection in the rabbit, the present study demonstrates a similar delayed anti-ischemic effect in the isolated perfused mouse heart. The delayed protective effect of A1AR activation is mediated by NO generated from mild upregulation of iNOS. To the best of our knowledge, this is the first study providing a direct link between A1AR activation and induction of iNOS that subsequently leads to delayed cardioprotection. We have proved it by demonstrating the abrogation of the delayed protective effect of adenosine by pharmacological inhibition and targeted ablation of the iNOS gene in the mouse. We conclude that adenosine can be used in modulating the iNOS activity for the generation of therapeutic levels of NO, which may provide long-lasting ischemic protection in the heart.

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