Mechanism of decreased adenosine protection in reperfusion injury of aging rats

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Gao, Feng, Theodore A. Christopher, Bernard L. Lopez, Eitan Friedman, Guoping Cai, and Xin L. Ma. Mechanism of decreased adenosine protection in reperfusion injury of aging rats. Am J Physiol Heart Circ Physiol 279: H329–H338, 2000.—The purpose of this study was to determine whether the protective effects of adenosine on myocardial ischemia-reperfusion injury are altered with age, and if so, to clarify the mechanisms that underlie this change related to nitric oxide (NO) derived from the vascular endothelium. Isolated perfused rat hearts were exposed to 30 min of ischemia and 60 min of reperfusion. In the adult hearts, administration of adenosine (5 μmol/l) stimulated NO release (1.06 ± 0.19 nmol · min⁻¹ · g⁻¹, P < 0.01 vs. vehicle), increased coronary flow, improved cardiac functional recovery (left ventricular developed pressure 79 ± 3.8 vs. 57 ± 3.1 mmHg in vehicle, P < 0.001; maximal rate of left ventricular pressure development 2,385 ± 103 vs. 1,780 ± 96 in vehicle, P < 0.001), and reduced myocardial creatine kinase loss (95 ± 3.9 vs. 159 ± 4.6 U/100 mg protein, P < 0.01). In aged hearts, adenosine-stimulated NO release was markedly reduced (+0.42 ± 0.12 nmol · min⁻¹ · g⁻¹ vs. vehicle), and the cardioprotective effects of adenosine were also attenuated. Inhibition of NO production in the adult hearts significantly decreased the cardioprotective effects of adenosine, whereas supplementation of NO in the aged hearts significantly enhanced the cardioprotective effects of adenosine. The results show that the protective effects of adenosine on myocardial ischemia-reperfusion injury are markedly diminished in aged animals, and that the loss in NO release in response to adenosine may be at least partially responsible for this age-related alteration.

myocardium; nitric oxide

IN ELDERLY PATIENTS, survival after acute myocardial infarction is decreased (14). This is recognized to be attributed to the increased susceptibility of the aged heart to ischemic-reperfusion injury (3, 15). However, it is not known whether this age-associated decrease in survival rate is also related to and exacerbated by a loss in the protective actions of endogenous or exogenous cardioprotectants.

Adenosine is a purine nucleotide that has been demonstrated to regulate a variety of cardiovascular functions. In adult animals, substantial evidence exists indicating that adenosine exerts cardioprotective effects against myocardial ischemia and reperfusion injury (39). The mechanisms by which adenosine confers protection are primarily achieved by activation of specific surface receptors located on cardiomyocytes, endothelial cells, and vascular smooth muscle cells. Activation of A₁ adenosine receptors results in antiadrenergic effects, reduction in cardiac work, and restoration of high-energy phosphate stores (10, 11). Activation of A₂ adenosine receptors causes coronary vasodilatation (38), inhibition of neutrophil function and free radical generation (41), and inhibition of platelet aggregation (34). Although it was previously believed that A₂ receptor activation exerts its cardiac protection against reperfusion injury by reducing inflammation and leukocyte-mediated damage, a more recent study by Cargnoni et al. (5) provides clear evidence that A₂ receptor activation may also exert direct cardioprotection against reperfusion injury in a blood-cell-free environment. Several studies, including those from our laboratory (4, 12, 13, 15), demonstrate that the responsiveness of both A₁ and A₂ adenosine receptors to adenosine is significantly decreased in aged hearts. However, the impact of the age-associated decrease in adenosine receptor functions on the cardioprotective effects of adenosine in myocardial ischemia-reperfusion has not been previously studied.

Nitric oxide (NO), a molecule produced from L-arginine by a family of enzymes known as nitric oxide synthase (NOS), has been demonstrated to exert marked cardioprotective effects in myocardial ischemia-reperfusion injury (2). Proposed mechanisms for these antireperfusion injury effects are almost identical to those proposed for adenosine. These include vasodilatation, antineutrophil effects, and free radical scavenging effects (39). Strong evidence now exists indicating that NO plays a critical role in mediating the cardiovascular effects of adenosine. Blocking NO production with L-arginine analogs not only markedly decreases the vasodilatation effect of adenosine in the

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coronary circulation but also decreases the antiadrenergic effect of adenosine (19, 28, 29, 35, 38). These data indicate that the cardiovascular effects of adenosine may be mediated through the L-arginine-NO pathway. Moreover, it has been shown that adenosine-stimulated NO release is mediated by A2-receptor activation, which occurs primarily during reperfusion. However, it has not been determined whether adenosine-stimulated NO release during reperfusion contributes to the cardioprotective effects of adenosine in myocardial ischemia-reperfusion injury. Thus the aims of the present study were 1) to determine whether the cardioprotective effects of adenosine in posts ischemic myocardial injury are altered in aged animals, and if so, 2) to elucidate the role of adenosine-stimulated NO release during reperfusion in these age-related alterations in the cardioprotection of adenosine against myocardial ischemia and reperfusion injury.

MATERIALS AND METHODS

Materials. N^6-p-sulphophenyladenosine [SPA, a selective A1-receptor agonist (37)] and 5′-([&-cyclopentyl)-carboxamidoadenosine [CPA, a selective A2-receptor agonist (36)] were purchased from Research Biochemicals (Natick, MA). All other chemicals were purchased from Sigma Chemical (St. Louis, MO). The experiments were performed in adherence to National Institutes of Health Guidelines on the use of laboratory animals and were approved by the Thomas Jefferson University Committee on Animal Care.

Heart preparation and experimental protocol. Male Fischer-344 rats [6 mo old, adult; and 24 mo old, aged (4, 8)] were anesthetized with pentobarbital sodium (50 mg/kg ip) and heparinized with heparin sodium (1,000 U/kg iv via dorsal penile vein). A midsternal thoracotomy was performed 5 min after the heparin sodium injection, and the hearts were excised and placed in ice-cold Krebs-Henseleit (KH) buffer solution consisting of (in mM) 118 NaCl, 4.75 KCl, 1.19 KH2PO4, 2H2O, 25 NaHCO3, 1.19 MgSO4·7H2O, 2.54 CaCl2·2H2O, 25 NaHCO3, 0.5 EDTA, and 11 glucose (31). Within 30 s, the ascending aorta was cannulated and retrograde perfusion of the heart with KH buffer solution was initiated in a nonrecirculating Langendorff heart perfusion apparatus (Radnoti Glass Technology, Monrovia, CA) at a constant pressure of 60 mmHg. KH buffer solution was oxygenated with 95% O2-5% CO2, which equilibrated to receive either vehicle (0.9% NaCl) or adenosine (5 μmol/l, a concentration that exerted significant cardioprotection without causing extreme bradycardia in adult hearts).

Drugs were infused into the heart via a sidearm in the perfusion line located just proximal to the heart cannula. The rate of infusion was adjusted based on the CF rate so that the desirable final concentration was obtained. Sham ischemic-reperfusion hearts were perfused with KH buffer solution for 2 h.

At the end of each experiment, the heart was removed from the perfusion apparatus and ~100 mg myocardial tissue was taken and subsequently homogenized in cold 0.25 M sucrose (1:10, wt/vol) containing 1 mM EDTA and 0.1 mM mercaptoethanol using a PRO 200 homogenizer (PRO Scientific, Monroeville, PA). Homogenates were centrifuged at 36,000 g at 4°C for 30 min. The supernatant was decanted and creatine kinase (CK) activity was analyzed using a Beckman DU 640 spectrophotometer, as reported previously (25). Protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL). The CK loss was calculated by subtracting CK activity of ischemic-reperfused hearts from CK activity of sham ischemic hearts and was expressed in international units per 100 mg of protein.

NOx measurement. Coronary effluent was collected for three 5-min periods. Samples were collected for 5 min immediately before ischemia (control), and at 0–5 and 55–60 min of reperfusion. NO concentrations (NOx) were measured using the previously reported vanadium reduction method (24). In brief, 50 μl of effluent solution were injected into a water-jacketed, oxygen-free purge vessel containing 5 ml of 0.1 M vanadate (III) chloride (Aldrich, Milwaukee, WI) in 2 N HCl. Acidic vanadate (III) chloride at temperatures above 80°C quantitatively reduces both nitrite and nitrate to NO, which is quantified by a chemiluminescence detector (270B Nitric Oxide Analyzer, Sievers, Boulder, CO) after reaction with ozone. Signals from the detector were collected and analyzed using a PC-based data recording and processing system (Duo-18, World Precision Instruments, Sarasota, FL). Standard curves were generated using the area under the curve after each injection of 50 μl of 0, 12.5, 25, 50, 75, and 100 μM sodium nitrate. The calculations to determine the NO content of the coronary effluent were done by the slope of the regression analysis using the linear formula $y = a + bx$. The amount of NO released was expressed in nanomoles per minute per gram of heart tissue.

Statistical analysis. All values in the text, tables, and figures are presented as means ± SE of n independent experiments. Hemodynamic and NO data were analyzed using super ANOVA repeated measurement, and CK data were analyzed using ANOVA followed by the Bonferroni correction for post hoc t-tests (StatView, SAS Institute, Cary, NC). Probabilities of $P \leq 0.05$ were considered to be statistically significant.

RESULTS

Initially, 114 male Fischer-344 rats were entered into the study, and 107 hearts (66 hearts from adult rats and 41 hearts from aged rats) were included in the final data analysis. Seven hearts were excluded due to failure to achieve adequate LVDP (~80 mmHg) at the
end of the equilibration period (immediately before ischemia).

Cardioprotective effects of adenosine. Within age groups, no significant differences were observed before ischemia between vehicle- and adenosine-treated groups with regard to any of the measured parameters. In vehicle-treated adult hearts, recovery of cardiac function (HR, LVDP, dP/dt\(_{\text{max}}\), and CF) ranged from 45 to 55% at the end of 60 min of reperfusion. Administration of 5 μmol/l adenosine to adult hearts at the time of reperfusion significantly reduced HR (P < 0.01 and P < 0.05 vs. vehicle at 10 and 30 min reperfusion, respectively) (Fig. 1, A and B), increased CF, which was significant at all four tested time points during reperfusion (Fig. 1, C and D), and improved LVDP (P < 0.01 at 30 and 60 min reperfusion) (Fig. 2, A and B) and dP/dt\(_{\text{max}}\) (P < 0.01 at 60 min reperfusion) (Fig. 2, C and D). Treatment with adenosine in adult hearts also markedly attenuated myocardial cellular injury, as evidenced by decreased myocardial CK loss (P < 0.01) (Fig. 3). In the aged hearts, HR and LVDP before ischemia were lower than those of adult hearts (P < 0.01 and P < 0.05, respectively). CF and dP/dt\(_{\text{max}}\) were not significantly decreased in the aged hearts (P > 0.05). Perfusion with 5 μmol/l adenosine in the aged hearts attenuated reperfusion injury, as evidenced by significant increases in CF, LVDP, and dP/dt\(_{\text{max}}\), and a decrease in CK loss (Figs. 1–3). However, these protective effects were significantly reduced from those noted in adult hearts. To better compare the degree of protection exerted by adenosine in the two age groups, the percent protection exerted by adenosine treatment was calculated as follows: (individual value in adenosine-treated heart) – (mean value of vehicle-treated hearts)/(mean value of vehicle-treated hearts) × 100. Administration of adenosine in adult hearts caused a 34.9 ± 3.1% (peak change) increase in CF. In contrast, the same concentration of adenosine given to the aged hearts increased CF by only 17 ± 2.1% (peak change, P < 0.01 vs. adult hearts). Adenosine improved LVDP and dP/dt\(_{\text{max}}\) by 39 ± 4.1% and 34 ± 3.9%, respectively, and decreased cardiac CK loss by 40.2 ± 3.4% in the adult hearts. The same concentration of adenosine perfused in aged hearts caused significantly lower protection in postischemic injury compared with protection exerted in adult hearts (LVDP 21.3 ± 3.9%,

![Fig. 1. Effect of adenosine treatment on heart rate and coronary flow rate in adult (A and C) and aged (B and D) hearts subjected to 30 min of global ischemia and 60 min of reperfusion (R). Adenosine was given at the onset of reperfusion.*P < 0.05, **P < 0.01 vs. vehicle.](image-url)
effects of adenosine against myocardial reperfusion injury were markedly attenuated in aged animals.

**Effects of adenosine on NO release after ischemia-reperfusion in adult and aged hearts.** To investigate the mechanism underlying the decreased cardioprotective effects of adenosine in aged hearts and to test the hypothesis that NO may play a significant role in the cardioprotective action of adenosine, NO release (measured as NOx) in isolated perfused hearts and the influence of adenosine were examined. In adult hearts, baseline control NO release in this model ranged from 5.1 to 7.4 nmol·min⁻¹·g⁻¹. The concentration of NO in the coronary effluent increased in the first 5 min of reperfusion. However, the total amount of NO released (nmols per minute per gram) was decreased because of a marked reduction in CF. NO release was partially recovered at the end of 60 min of reperfusion in the vehicle-treated group. Compared with the vehicle-treated group, treatment with adenosine significantly increased NO release at 5 min of reperfusion (Fig. 4), and at the end of 60 min of reperfusion, NO release achieved the control baseline level in the adenosine-treated adult hearts.

In aged hearts, adenosine-stimulated NO release was markedly reduced. During the initial 5 min of reperfusion, NO release in adenosine-treated hearts

![Graph](image-url)
only increased by 0.42 ± 0.12 nmol · min⁻¹ · g⁻¹ compared with an increase of 1.06 ± 0.19 nmol · min⁻¹ · g⁻¹ in the adult hearts. At the end of the reperfusion period, the amount of NO released in response to adenosine treatment was still markedly lower than in adult hearts (P < 0.01), indicating an age-dependant reduction in adenosine-stimulated NO release.

**Effect of NO synthesis blockade on the cardioprotective action of adenosine in adult hearts.** To further elucidate the role of NO in adenosine-induced cardioprotective action in postischemia myocardial injury, the effect of NOS inhibition on the cardioprotection exerted by adenosine was studied. N-iminoethyl-l-ornithine (L-NIO), a potent nonselective NOS inhibitor (6, 27, 33), was injected (5 mg/kg ip) into 16 adult rats (8 were administered L-NIO alone, and 8 were given L-NIO + adenosine) 3 h before their hearts were excised. Yang and Mehta (40) previously reported that NOS activity is significantly inhibited in isolated perfused hearts from rats receiving intraperitoneal administration of 10 mg/kg N⁶-nitro-l-arginine methyl ester (L-NAME) 6 h before heart excision. Similarly, we demonstrated in a pilot study that in animals pretreated with 5 mg/kg L-NIO ip 3 h before heart excision, NO release was continuously decreased during the entire 90-min perfusion period (at the end of 90-min sham ischemia-reperfusion: 2.10 ± 0.24 vs. 6.46 ± 0.58 nmol · min⁻¹ · g⁻¹ in control rats without L-NIO pretreatment), and ACh-induced vasorelaxation was still markedly inhibited even at 90 min after in vitro perfusion (maximal vasodilatation to ACh was 19 ± 2.1% vs. 97 ± 1.4% in the control). However, pretreatment with L-NIO 3 h before heart removal only insignificantly decreased the coronary perfusion rate (10.7 ± 0.4 vs. 11.6 ± 0.6 ml/min in hearts not pretreated with L-NIO, P > 0.5). The results of this study are different from those studies where NOS inhibitors were infused directly to the heart in vitro and are consistent with those reported by Yang and Mehta (40). As illustrated in Fig. 5, administration of L-NIO alone slightly decreased LVDP and dP/dt max recovery and significantly increased CK release. Pretreatment with L-NIO blocked adenosine-stimulated NO release to a level comparable to that seen in the aged hearts (0.31 ± 0.07 vs. 0.42 ± 0.1 nmol/l, P > 0.05). Moreover, pretreat-

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**Fig. 4.** Effects of adenosine treatment on nitric oxide (NO) release (measured as NOx) from isolated perfused hearts subjected to 30 min of ischemia and 60 min of reperfusion. Adenosine was administered at the onset of reperfusion at a final concentration of 5 μmol/l. Numbers within the bars represent the number of experiments. **P < 0.01 vs. vehicle.

**Fig. 5.** Effects of NO synthase inhibition on adenosine (Ado) cardioprotection in adult animals. L-NIO, N-iminoethyl-l-ornithine. *P < 0.05, **P < 0.01 vs. vehicle; *P < 0.05, **P < 0.01 vs. Ado alone.
ment with L-NIO also significantly attenuated the cardioprotective effects of adenosine in adult hearts (Fig. 5). The adenosine-mediated improvements in contractility, as indicated by LVDP and dP/dt_max, were reduced from 38.6 ± 4.1% and 34.0 ± 2.9% in control adult hearts to 12.3 ± 2.5% and 20.1 ± 3.1% in L-NIO-treated hearts (P < 0.01). Adenosine-mediated attenuation in myocardial CK loss was reduced from 40.3 ± 3.2% in control adult hearts to 19.5 ± 2.5% in NOS-inhibited hearts (P < 0.01). These results demonstrate that NO plays a significant role in the cardioprotective action exerted by adenosine in the adult heart.

It was noticed that adenosine treatment caused a higher CF in adult rat hearts when compared with aged rat hearts. To determine whether the differences in this CF rate change caused by adenosine are responsible for the varying adenosine cardioprotection in adult versus aged rat hearts, an additional experiment was performed in eight aged rat hearts. At the onset of reperfusion, adenosine was given at the same concentration as described above, and the perfusion pressure was elevated from 60 mmHg to 66 ± 1 mmHg so that the CF rates were increased to a level comparable to those seen in the adult hearts. As summarized in Table 1, raising perfusion pressure and thus increasing CF rate in the aged rat hearts failed to bring the adenosine cardioprotective effects to a level comparable to that observed in the adult rat hearts. This result suggests that the difference in adenosine cardioprotection between adult and aged rats cannot be explained by a different CF response to adenosine.

Effect of NO supplement on the cardioprotective action of adenosine in aged hearts. To evaluate the role of decreased adenosine-induced NO production in mediating the loss of cardioprotection in the aged myocardium, we tested whether an NO donor, S-nitroso-N-acetylpenicillamine (SNAP), can restore the protective action of adenosine in hearts of aged rats. In a previous study (23), we administered SNAP at a concentration of 10 μmol/l and found it to exert significant cardioprotective effects in adult hearts subjected to ischemia and reperfusion. In the present study, we infused 1 μmol/l SNAP during reperfusion to hearts of aged rats and found no cardioprotective effect in this severe global ischemia-reperfusion model (Fig. 6). However, when SNAP was administered together with adenosine in aged hearts, a significant synergistic myocardial protective effect of adenosine was observed (Fig. 6). At 60 min of reperfusion, LVDP, an index of cardiac contractile function, was increased to 65 ± 2.1 mmHg in the combined adenosine and SNAP group (P < 0.01 vs. vehicle and SNAP alone group; P < 0.05 vs. adenosine alone group). Moreover, myocardial CK loss was also attenuated in the combined adenosine and SNAP group when compared with the adenosine alone group (Fig. 6). These results therefore support the conclusion that the loss of adenosine-stimulated NO release in aged hearts is at least partially responsible for the decrease in the cardioprotection exerted by adenosine in the aged animal.

Adenosine receptor subtype responsible for adenosine-stimulated NO release in isolated perfused hearts. To define the adenosine receptor subtype that is responsible for NO release in the adult heart, the effects of selective adenosine receptor agonists on NO release were studied in a group of 22 adult hearts. As summarized in Table 2, perfusion with 5 μmol/l adenosine for 5 min resulted in a 1.4-fold increase in NO. Administration of the selective adenosine A1-receptor agonist SPA at 100 nmol/l significantly decreased HR (from 308 ± 15 to 241 ± 18 beats/min, P < 0.01) but only slightly increased NO release (+11%, P > 0.05). However, perfusion with 50 nmol/l of the selective adenosine A2-receptor agonist CPCA, which did not significantly change HR, markedly enhanced NO release (P < 0.05). These results indicate that in the isolated perfused heart preparation, adenosine A2 receptors are primarily responsible for NO release induced by exogenous adenosine.

DISCUSSION

Numerous experimental results have shown that adenosine exerts marked protective effects against ischemic as well as postischemic myocardial injury (10). However, most, if not all, experiments have been performed on adult animals, and the impact of age, a major risk factor for ischemic heart disease, on the

<table>
<thead>
<tr>
<th>Coronary flow, ml/min</th>
<th>Adult Rat Hearts</th>
<th>Aged Rat Hearts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.6 ± 0.6</td>
<td>10.6 ± 0.4</td>
</tr>
<tr>
<td>R5</td>
<td>3.8 ± 0.2</td>
<td>4.1 ± 0.3</td>
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<tr>
<td>R10</td>
<td>6.4 ± 0.3</td>
<td>5.9 ± 0.2</td>
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<tr>
<td>R30</td>
<td>6.5 ± 0.5</td>
<td>6.3 ± 0.3</td>
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<tr>
<td>R60</td>
<td>6.3 ± 0.5</td>
<td>6.2 ± 0.5</td>
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<tr>
<td>Left ventricular developed pressure, mmHg</td>
<td>57 ± 3.1</td>
<td>79 ± 3.8*</td>
</tr>
<tr>
<td>R60</td>
<td>57 ± 3.1</td>
<td>57 ± 2.3</td>
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</table>

Values are means ± SE. L-NIO, N-iminoethyl-L-ornithine; HPP, high perfusion pressure; R, reperfusion time, in min. *P < 0.01 vs. vehicle.
cardioprotective effects of adenosine in myocardial ischemia-reperfusion injury has not been determined. Our results provide clear evidence demonstrating that the protective effects of adenosine in ischemia-reperfusion myocardial injury are markedly reduced in aging hearts, and that the loss in the ability of adenosine to stimulate NO release in these hearts is partially responsible for this age-related vulnerability of the heart to injury. Thus these results suggest that decreased cardiovascular responses to cardioprotectants such as adenosine in elderly patients may contribute to the increased mortality rate in elderly subjects suffering from ischemic heart disease.

Receptor-mediated cardioprotection and the impact of aging. Recent experiments demonstrate that most of the cardiovascular effects of adenosine are mediated through membrane-bound receptors, which are classified as $A_1$, $A_2$, and $A_3$. Considerable evidence supports the notion that $A_1$-receptor-mediated myocardial protection (via decreasing heart work and improving cellular energy metabolism) is predominantly exerted during ischemia, whereas adenosine $A_2$-receptor-mediated cardioprotection (dilating coronary resistance vessels, which increases oxygen and nutrition supply; decreasing neutrophil and platelet adhesion, which reduces capillary plugging and embolism; and decreasing oxygen-derived free radical-induced damage) is primarily exerted during reperfusion (41). However, most of the evidence suggesting $A_2$-receptor-mediated anti-reperfusion effects is obtained from in vivo studies. It is generally believed that $A_2$-receptor activation exerts its cardiac protection against reperfusion injury by reducing inflammation and leukocyte-mediated damage. To date, it has not been conclusively demonstrated that adenosine-activated $A_2$ receptors on endothelial cells and cardiomyocytes directly protect coronary endothelial and myocardial injury associated with reperfusion. To address this question, we observed the cardioprotective effects of adenosine when administered only during reperfusion in a cell-free, crystalloid-perfused preparation. Our results demonstrated that administration of adenosine to isolated perfused hearts significantly improved cardiac function recovery and reduced myocardial cellular injury after 30 min of ischemia and 60 min of reperfusion, suggesting that adenosine may protect

Table 2. Effects of adenosine or selective $A_1$ or $A_2$ adenosine receptor agonists on nitric oxide release in adult hearts

|                     | Adenosine (5 mmol/l) $n = 8$ | SPA (100 mmol/l) $n = 7$ | CPCA (50 mmol/l) $n = 7$
|---------------------|-----------------------------|--------------------------|---------------------------
| Pretreatment, mmol·min$^{-1}$·g$^{-1}$ | 6.31 ± 0.56 | 6.44 ± 0.59 | 6.38 ± 0.63 |
| Posttreatment, mmol·min$^{-1}$·g$^{-1}$ | 8.85 ± 0.63† | 7.15 ± 0.61 | 8.36 ± 0.59* |

Values are means ± SE of $n$ independent experiments. Adenosine, $N^\omega$-p-sulfophenyladenosine (SPA, a selective $A_1$-receptor agonist), or 5’-$\text{N}$-cyclopropyl)-carboxamidoadenosine (CPCA, a selective $A_2$-receptor agonist) was infused at the final concentrations specified. Coronary effluent was collected for 5 min before and after drug treatment. Nitric oxide was measured by gas phase chemiluminescence after reaction with ozone; see MATERIALS AND METHODS for description. *$P < 0.05$, †$P < 0.01$ vs. pretreatment.
against myocardial reperfusion injury via mechanisms that are independent of blood components, such as neutrophils. While this paper was in preparation, Cargnini et al. (5) published a paper that also demonstrated that administering adenosine only during reperfusion is cardioprotective in a constant-flow crystalloid-perfused rabbit heart model. They proposed that this protection may be achieved by $A_2$-receptor-mediated activation of K$^+$ channel conductance (thus reducing superoxide production by endothelial cells and myocytes), activation of ATP-sensitive K$^+$ channels (thus decreasing intracellular calcium), and inhibition of tumor necrosis factor-$\alpha$ release.

The impact of age on adenosine receptor function is complicated, and the existing experimental results are contradictory. Earlier studies by Di Gennaro et al. (7) demonstrated that adenosine receptor sensitivity is significantly decreased with aging. Recently, we demonstrated a substantial decline in adenosine receptor function in the heart during aging (4, 12, 13). In contrast, Dobson and Fenton (8) reported that cardiac responses to adenosine are markedly increased in the aging heart. Moreover, Headrick (15) recently reported that aging significantly increases adenosine levels and produces opposing changes in adenosine $A_1$ and $A_2$ responses (i.e., increasing $A_1$ and decreasing $A_2$ receptor sensitivity) in the rat cardiovascular system.

In the present study, we attempted to determine the effects of age on adenosine responses by comparing the cardioprotective effects of adenosine on postischemic myocardial injury. Because adenosine possesses significant anti-ischemic effects in adult hearts, administration of the same concentration of adenosine would exert more cardioprotection in aged hearts if adenosine receptor function were increased with aging. We have clearly demonstrated in the present study that the cardioprotective effects of adenosine in ischemia-reperfusion-elicited damage are markedly decreased in the aged heart. These results provide additional key evidence that age significantly decreases adenosine receptor function and thus attenuates the cardioprotective actions of adenosine.

Mechanism of decreased adenosine protection in the aged heart: role of NO. It is well documented that adenosine and NO are two potent cardioprotective molecules produced by endothelial cells and myocytes, and that these two autacoids share remarkably similar cardioprotective mechanisms against ischemia-reperfusion injury (39). However, the relative importance and interrelationship of these two molecules in protecting the myocardium from reperfusion injury is presently unclear. Accumulating evidence indicates that the cardiovascular effects of adenosine are in part mediated by NO. Adenosine stimulates NO production by vascular endothelial cells (22), smooth muscle cells (9, 16), and cardiac myocytes (17). Blocking NO production with l-arginine analogs markedly decreases the vasodilatory effect of adenosine both in vivo (19, 28, 35) and in vitro (38). In addition, NO has also been found to mediate adenosine-induced airway smooth muscle relaxation (1) and contribute to adenosine regulation of serotonin transport in cultured cells (29).

Our present study investigated several aspects of the relationship between NO and adenosine. By using a highly sensitive and selective chemiluminescence method, we directly measured NO release from coronary circulation and clearly demonstrated that adenosine increases NO production in isolated perfused rat hearts. Moreover, we demonstrated that the cardioprotective effects of adenosine are partially mediated by NO. In adult animals, pretreatment with L-NIO, under conditions that were shown to reduce NO production, significantly blunted the cardioprotective effects of adenosine, whereas in aged hearts, supplementation of a low NO concentration markedly enhanced the cardioprotective effects of adenosine. It should be noticed that L-NIO pretreatment alone insignificantly decreased cardiac function recovery and significantly increased CK release (Fig. 5). However, its partial adverse effect on adenosine cardioprotection cannot be explained entirely as a simple negative additive effect.

For instance, L-NIO pretreatment alone increased cardiac CK loss from $159 \pm 4.6$ IU/100 mg protein in the vehicle-treated group to $174 \pm 5.2$ IU/100 mg protein (an increase of 15 IU or 9.4%). However, L-NIO pretreatment increased cardiac CK loss from $95 \pm 3.9$ IU/100 mg protein in the adenosine-treated group to $128 \pm 3.8$ IU/100 mg protein in the L-NIO + adenosine group (an increase of 33 IU or 34.7%). These results suggest that the cardioprotective actions of adenosine may operate via a direct NO-independent pathway and an indirect NO-dependent pathway. In this regard, Peralta et al. (32) reported recently that in hepatic ischemia-reperfusion, inhibition of NO production with $N^G$-monomethyl-l-arginine markedly decreased the hepatic protective effects of adenosine, suggesting that NO plays a significant role in the protective action of adenosine in ischemia-reperfusion injury. Therefore, in adult animals, adenosine itself and NO released by adenosine stimulation may protect tissue from reperfusion injury either additively or synergistically. In contrast, in aged animals, adenosine-stimulated NO release is markedly reduced. Thus the NO-dependent cardioprotective action of adenosine is compromised, resulting in a myocardium that is increasingly vulnerable to ischemia-reperfusion injury in the aged animal.

Considerable evidence suggests that adenosine-elicited NO release into the coronary circulation is primarily mediated via adenosine $A_2$ receptors (9, 16, 35, 38). The present results support these observations. Recent studies by Headrick (15) and Jiang et al. (18) demonstrated that $A_2$-receptor function is markedly decreased in the senescent rat. Therefore, it is conceivable that in the aged heart, $A_2$-receptor dysfunction is likely responsible for the loss of adenosine-stimulated NO release and the consequent loss of NO-mediated cardioprotection. Numerous previous studies demonstrated that adenosine acting via the adenosine $A_1$ receptor exerts important cardioprotective actions in ischemia-reperfusion injury (20, 26, 30), and that cardiac adenosine $A_1$-receptor function is impaired in the
aging rat (4, 12, 13). Thus the age-related reduction in the cardioprotective actions of adenosine that we demonstrated in the present study may be mediated via adenosine A₁ as well as A₂ receptors. It remains to be demonstrated whether NO plays a role in A₂-receptor-mediated protective effects of adenosine.

In summary, we demonstrate that the cardioprotective responses to adenosine are markedly blunted in aged hearts, and NO, a highly recognized cardioprotective second messenger molecule, appears to mediate, at least in part, this age-related loss of adenosine function. Our results may be of significant practical value in suggesting novel therapeutic modalities for achieving the best cardioprotection in elderly patients with ischemic heart disease.

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