Cardiac overexpression of A₁-adenosine receptor protects intact mice against myocardial infarction

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Yang, Zequan, Rachael J. Cerniway, Anne M. Byford, Stuart S. Berr, Brent A. French, and G. Paul Matherne. Cardiac overexpression of A₁-adenosine receptor protects intact mice against myocardial infarction. Am J Physiol Heart Circ Physiol 282: H949–H955, 2002.—Previous studies have shown that high-level (300-fold normal) cardiac overexpression of A₁-adenosine receptors (A₁-ARs) in transgenic (TG) mice protects isolated hearts against ischemia-reperfusion injury. However, this high level of overexpression is associated with bradyarrhythmia and increased incidence of arrhythmia during ischemia in intact mice, which interfered with studies to determine whether this line of TG mice might also be protected against myocardial infarction (MI) in vivo. For these studies, we therefore selected a line of TG mice that overexpresses the A₁-AR at more moderate levels (30-fold normal), which affords cardioprotection in the isolated heart while minimizing bradyarrhythmia and arrhythmia during ischemia in intact mice. Wild-type (WT; n = 10) and moderate-level A₁-AR TG (n = 10) mice underwent 45 min of left anterior descending coronary artery occlusion, followed by 24-h reperfusion. Infarct size and region at risk were determined by triphenyltetrazolium chloride and phthalo blue staining, respectively. Infarct size (% region at risk) in WT mice was 52 ± 3%, whereas overexpression of A₁-ARs in the TG mice markedly reduced infarct size to 31 ± 3% (P < 0.05). Furthermore, contractile function (left ventricular ejection fraction) as determined by cardiac magnetic resonance imaging 24 h after MI was better preserved in TG vs. WT mice. Cardiac overexpression of A₁-ARs reduces infarct size by 40% and preserves cardiac function in intact mice after MI.

ischemia-reperfusion; cardioprotection; transgenic mice; magnetic resonance imaging

REPETITIVE A₁-adenosine receptor (A₁-AR) activation can maintain the heart in a protected state against myocardial ischemia-reperfusion injury (6). However, prolonged activation of A₁-ARs with pharmacological agents induces a state of tolerance that blunts this cardioprotective effect (25). One possible explanation for this tolerance is the desensitization of A₁-ARs resulting from continuous stimulation by A₁-AR agonists. Using transgenic techniques, we showed (11–15, 19, 21) that cardiac A₁-AR overexpression activates endogenous protective mechanisms that provide the heart with increased resistance to ischemia-reperfusion injury. Unlike transient ischemic preconditioning that occurs in wild-type (WT) animals, the constitutive preconditioning secondary to transgenic overexpression of the A₁-AR has the potential to provide continuous cardioprotection (21). Although our past work was critical in establishing the long-term cardioprotective potential of A₁-AR overexpression, these studies were conducted in isolated hearts from a line of mice with a 300-fold level of overexpression. In this study, we sought to determine whether A₁-AR overexpression could also protect intact mice against ischemia-reperfusion injury. Although cardiac-specific 300-fold A₁-AR overexpression provided cardioprotection in globally ischemic, isolated heart models of ischemia-reperfusion injury, this was associated with adverse side effects such as significant resting bradycardia [heart rate (HR) of 620 ± 14 beats/min (bpm) vs. 713 ± 8 bpm in WT mice] and a blunted inotropic response to catecholamines (11, 12, 19). These side effects caused high mortality rates in pilot in vivo experiments, making this line of animals unsuitable for whole animal investigations. We therefore selected a line of transgenic (TG) mice with moderate overexpression of the A₁-AR (~30-fold) that exhibited minimal bradycardia (681 ± 11 bpm). If this level of A₁-AR overexpression afforded protection against myocardial infarction (MI) in vivo, then strategies designed to moderately increase the density of A₁-ARs in the myocardium might have clinical potential because this could produce a sustained form of cardioprotection without adverse side effects. In this study, we found that MI size can indeed be substantially reduced by overexpressing A₁-ARs at moderate levels in the heart. Additionally, we used cardiac magnetic resonance imaging (MRI) to show that moderate overexpression of the A₁-AR had no effects on baseline morphology or function yet helped preserve left ventricular (LV) ejection fraction after MI.

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METHODS

Transgenic mice. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996), and the work was approved by the Institutional Animal Care and Use Committee. TG mice overexpressing the rat A1-AR cDNA were produced as described previously (12). Mice were screened for the transgene by PCR amplification of a 550-bp fragment of the transgene with a 350-bp fragment of the endogenous A3-adenosine receptor (A3-AR) gene as a positive control (Fig. 1A). Primers used to detect the rat A1-AR gene were forward: 5'-CTCTGACAGAAGCAGCACTTTACA-TGG-3'; reverse: 5'-CCAGTGACAGGATGAAGCAGAAGGT-GGCAT-3'. Primers used to detect the endogenous mouse A2-AR gene were forward: 5'-CTTTCTGAGAAGAGTCTC-TAAGA-3'; reverse: 5'-GGAGACATAATAGATAGACGTTG-3'. Copy numbers for both high- and moderate-level A1-AR overexpression were determined by Southern analysis (Fig. 1B). The rat A1-AR cDNA probe hybridizes to both the endogenous mouse A1-AR gene (2.6 kb) and the rat A1-AR transgene (1.6 kb).

Experimental mouse model. Surgical procedures were the same as reported previously (26, 27). Briefly, WT and TG mice, comparable in body weight (28.5 ± 1.1 vs. 28.8 ± 1.3 g; P > 0.05), were anesthetized, the upper portion of the trachea was exposed, and an endotracheal tube was inserted orally. Artificial respiration was maintained with a SAR-830/P ventilator (inspired O2 fraction 0.80, rate 100 strokes/min). After intubation, a chest incision was made to open the left pleural cavity. A piece of 7-0 silk suture was passed underneath the left anterior descending (LAD) coronary artery at the level of the lower left atrium, and MI was induced by tying down the suture over PE-10 tubing. Reperfusion was achieved by loosening the suture. Occlusion was confirmed through visualization of pallor of region at risk and by observation of electrocardiogram (ECG) changes such as widening of QRS and ST-T segment elevation (monitored by MacLab). A volume of 1–1.5 ml 5% dextrose was given intraperitoneally to replace fluids. Body temperature was monitored with a rectal probe and maintained between 36.5 and 37.5°C with a heating pad.

Pilot experiments. Experimental MI was initially induced in TG mice (n=5) with high-level A1-AR overexpression (~300-fold). A1-AR overexpression on infarct size. WT (littermate wild-type; n=19) and TG (moderate A1-AR overexpression; n=15) mice underwent 45-min LAD occlusion and 24-h reperfusion. Twenty-four hours after reperfusion, cardiac structure and function (LV ejection fraction, LVEF) in WT (n=5) and TG (n=4) mice were studied by in vivo cardiac MRI. After euthanasia, hearts were excised and stained with triphenyltetrazolium chloride (TTC) and phthalo blue for measurement of MI. Analysis of MI. After excision, hearts were perfused with 0.9% NaCl solution and then 1.0% TTC in phosphate buffer.
Table 1. Study groups

<table>
<thead>
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<th>Groups</th>
<th>Protocol</th>
<th>Measurement</th>
<th>No. of Mice</th>
<th>Mortality and Exclusions</th>
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<td>Infarct size</td>
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<td>All 5 died during ischemia</td>
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<td>MRI and MPO</td>
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<td>2 died and 2 poor staining</td>
</tr>
<tr>
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<td>MRI and MPO</td>
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<td>1 died during followup</td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>MPO</td>
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<td></td>
<td></td>
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<td>Infarct size</td>
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<td></td>
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<td>5</td>
<td>1 died overnight</td>
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<tr>
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<tr>
<td>Total</td>
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High-level A1-AR, 300-fold normal A1-adenosine receptors; WT, wild-type; TG, 30-fold normal A1-adenosine receptors; Ischemia, 45-min left anterior descending (LAD) occlusion, followed by 24 h reperfusion; Sham, 50-min open-chest surgery with no LAD occlusion; MRI, magnetic resonance imaging; MPO, myeloperoxidase activity.

(pH 7.4) to assess tissue viability. The LAD was then reocluded, and the hearts were perfused with 10% phthalo blue to delineate the nonischemic tissue. The hearts were frozen, and the LV was cut into five to seven transverse slices and fixed in 10% buffered formalin. Slices were weighed and photographed from both sides. The images were digitally analyzed for infarct area, ischemic area (risk region, RR), and LV area. The weights of the infarcted area and RR were then calculated as percentages of the total weight of each LV slice.

Determination of cardiac structure and function by cardiac MRI. Cardiac MRI was used to assess cardiac function before and after MI. Imaging was performed with a Helmholtz RF coil on a Varian Inova 4.7-T MRI equipped with Magnex gradients (80 G/cm maximum strength). An ECG-triggered two-dimensional cine FLASH sequence was used with a slice thickness of 1 mm and an in-plane resolution of 100 × 100 μm². The flip angle was 20° with a repetition time of 12–20 ms. During each session, the LV was scanned using six to nine contiguous short-axis slices. Raw data from the imaging were scaled and converted for image analysis with ARGUS (Siemens Medical Systems, Iselin, NJ). End-systolic and end-diastolic phases from each set of contiguous short-axis slices were used to determine LVEF and LVEDV so that LVEF could be calculated.

Measurement of tissue MPO activity. The heart was flushed with cold phosphate-buffered saline to remove blood from the vasculature. The LV was homogenized in a solution containing 0.5% hexadecyltrimethylammonium bromide and centrifuged for 30 min at 20,000 g and 4°C. An aliquot of the supernatant was reacted with a solution of tetramethylbenzidine (1.6 mmol/l) and 0.1 mmol/l H₂O₂. The rate of the change in absorbance was measured with a spectrophotometer at 650 nm. MPO activity was calculated as described previously (27).

Statistical analysis. Data are reported as means ± SE. Infarct size, RR, and cardiac function were analyzed with ANOVA, followed by Student’s t-tests for unpaired data with Bonferroni correction. MPO activity was analyzed by ANOVA, followed by Tukey’s post hoc test. P < 0.05 was considered significant.

RESULTS

Hemodynamics. In our pilot studies with TG mice overexpressing the A1-AR at high levels (300-fold), conscious resting HR recorded by a tail cuff BP 2000 Analysis System (Visitech, Apex, NC) was found to be significantly lower than in WT controls (620 ± 14 vs. 713 ± 8 bpm; P < 0.05). During coronary occlusion, bradycardia was aggravated in the high-level A1-AR overexpressing mice, resulting in 100% mortality within 10 min after the LAD was occluded (Fig. 2). To overcome this problem, we used a different line of TG mice that overexpresses the A1-AR at more moderate levels (30-fold). Resting bradycardia (681 ± 11 bpm) in this line was much less severe than in the line with 300-fold overexpression of the A1-AR (620 ± 14 bpm; P < 0.05).

There was no significant difference in mean arterial blood pressure between moderate-level A1-AR-overexpressing TG and WT mice (69 ± 5 vs. 67 ± 10 mmHg) under anesthesia. Furthermore, there was no significant difference between TG and WT mice in anesthetized HR monitored by ECG before the LAD occlusion (baseline) (368 ± 17 vs. 398 ± 21 bpm; Fig. 2). HR increased in response to LAD occlusion and reperfusion to similar degrees in both TG and WT mice (Fig. 2). There was no difference in perioperative mortality between the TG and WT mice when the moderate-level A1-AR-overexpressing mice were used (2 of 24 in WT vs. 1 of 20 in TG).

Cardiac overexpression of A1-AR protects intact heart against MI. Moderate (30-fold) overexpression of the A1-AR significantly reduced infarct size in intact mice after 45-min LAD occlusion and 24 h of reperfusion. Histological examination of the infarcted hearts after phthalo blue staining revealed that there was no difference in the region at risk (39 ± 2% vs. 42 ± 2%) between WT and TG mice. However, as illustrated in Fig. 3, infarct sizes (pale areas) in TG mice were significantly smaller than in WT mice, even though the ischemic regions (sum of pale and red areas) were comparable. Infarct size was 52 ± 3% in WT mice compared with 31 ± 3% in TG mice (P < 0.05; Fig. 4). No differences were found between WT and TG mice in heart weight after MI (148 ± 7 vs. 145 ± 8 mg).

Tissue MPO activity. To assess the role of inflammation in this model, MPO activity was measured 24 h after MI as an indicator of neutrophil infiltration (Fig. 5). To control for any potential effect of the operative procedure on myocardial MPO activity, TG (n = 5) and WT (n = 5) mice were subjected to a sham operation in which the chest was opened for 50 min and a suture was passed around the LAD but not tightened. As expected, MPO activity was low in both the WT and TG sham-operated groups, with no difference observed. Twenty-four hours after MI, MPO activity was mark-
edly increased in both groups, but to a significantly lower level in TG compared with WT mice (Fig. 5).

**Cardiac overexpression of A1-AR preserves cardiac function 24 h after MI.** Cardiac function was evaluated by cardiac MRI before MI and 24 h after reperfusion. There was no significant difference in heart rate during MRI. At baseline (i.e., under anesthetic but with no experimental intervention), there were no significant differences in LVEDV, LVESV, or LVEF between WT and TG mice (Fig. 6). MI increased LVESV to a similar extent in the two groups and showed a trend toward elevated LVEDV in TG mice (Fig. 6, A and B). As shown in Fig. 6C, LVEF was similar between the two groups at baseline (69 ± 4% in WT and 63 ± 4% in TG). Both groups suffered a significant loss of LVEF as a result of MI; however, LVEF 24 h after MI was significantly better in TG mice (47 ± 2%) than it was in WT mice (34 ± 2%; P < 0.05).

**DISCUSSION**

It is now well established that the autacoid adenosine functions as a potent intrinsic cardioprotectant (8). The binding of adenosine to the A1-AR is the initial step in triggering this protective mechanism against myocardial stunning and infarction (2, 3, 6, 9, 17, 22, 23). Recently we demonstrated (19) that the effects of adenosine are limited by receptor number rather than by agonist concentration in ischemic murine myocardium. Thus transgenic overexpression of A1-ARs improves tolerance to ischemia-reperfusion injury, whereas continuous exogenous activation of A1-AR is less effective. In our previous studies with isolated heart models, we found that TG hearts with >300-fold A1-AR overexpression demonstrated significantly increased resistance to myocardial ischemia-reperfusion injury. This cardioprotection was due in part to an improved metabolic and/or bioenergetic state in response to ischemia-reperfusion injury (13). The increased myocardial A1-AR density in these mice constitutively activates endogenous signaling pathways, which includes activation of mitochondrial ATP-sensitive potassium (KATP) channels, and continuously protects the heart against ischemia-reperfusion injury (14, 15). This cardioprotective effect of A1-AR overexpression was blocked by selective A1-AR antagonist (19, 21). Although our previous studies conducted in isolated heart models were critical in exploring the mechanisms underlying the cardioprotective potential of A1-AR manipulation, it nevertheless remained to be demonstrated that tissue-specific overexpression of A1-ARs in the heart could produce clinically relevant cardioprotection in vivo.

However, adverse side effects associated with high-level overexpression of the A1-AR made it difficult to
use this line of mice for studying regional MI in vivo. In particular, hearts overexpressing A1-ARs at levels 300-fold above normal exhibited resting bradycardia and a blunted inotropic response to catecholamines (13–15). In a pilot study conducted with this line of TG mice, bradycardia was worsened on anesthesia and a 100% mortality rate was incurred during the LAD occlusion (Table 1; Fig. 2). The purpose of the present study, therefore, was to examine a different line of TG mice with a more moderate level of A1-AR overexpression (Fig. 1) to determine the effect of A1-AR overexpression on MI in an in vivo model.

**Moderate myocardial A1-AR overexpression protects heart against MI.** Moderate overexpression of myocardial A1-AR caused mild resting bradycardia that disappeared under anesthesia compared with mice with high-level (300-fold) overexpression (Fig. 2). There was also no significant difference in blood pressure or cardiac structure and function measured at baseline by cardiac MRI. Thus the moderate-level A1-AR-overexpressing TG mice were comparable to the littermate WT mice before the surgery. In contrast, moderate overexpression of myocardial A1-ARs in TG mice markedly reduced infarct size and preserved cardiac function at 24 h after MI (Figs. 3, 4, and 6). This protective effect on myocardial function may be attributed not only to a smaller infarction but also to a better preserved noninfarct myocardial compliance, as indicated by the trend of higher LVEDV in TG mice (Fig. 6A).

To examine the role of inflammation in this model, we measured tissue MPO activity in TG and WT mice 24 h after infarction or a sham operation. MPO is generated by neutrophils as they accumulate in the infarct and peri-infarct regions early after ischemia-reperfusion injury in response to the degranulation of mast cells and other inflammatory stimuli (10). We found that TG mice with moderate overexpression of A1-ARs had lower levels of tissue MPO activity compared with WT mice (Fig. 5). It is likely that the observed reduction in the inflammatory mediator MPO is secondary to the decrease in cell death, because dying and necrotic cells provide potent stimuli for neutrophil recruitment after MI. Additional studies, however, will be needed to determine whether the reduction in neutrophil infiltration is a direct result of smaller infarct size or whether other mechanisms related to A1-AR overexpression are involved.

**Models of cardioprotection utilizing genetically manipulated mice.** The study presented here is the first step toward using genetic manipulation to harness an intrinsic cardioprotective mechanism against ischemia-reperfusion injury. Other recently developed murine models using TG and knockout technology have also demonstrated promise by improving myocardial function and reducing MI as a result of ischemia-reperfusion injury. These include TG mice overexpressing the inducible heat shock protein 70 (24) and the antioxidant enzyme manganese superoxide dismutase (5). Furthermore, overexpression of glutathione peroxidase is protective (28), and knockout of the glutathione peroxidase gene has been shown to increase susceptibility to myocardial ischemia-reperfusion injury (29).

**Signaling mechanisms involved in cardioprotection by A1-AR overexpression.** Genetically manipulated mouse models contribute substantially to our understanding of the mechanisms of pathophysiological processes.
The signaling mechanisms involved in A1-adenosine receptor activation have been extensively studied, but there is still no universal agreement regarding the specific pathway(s) or end-effectors responsible for the observed cardioprotection. Our previous studies with isolated heart models have shown that A1-AR overexpression increases myocardial resistance to ischemia by constitutively activating endogenous signaling pathways, with mitochondrial KATP channels being critical effectors (14, 21). Because A1-AR-mediated cardiac protection shares at least some common mechanisms with ischemic preconditioning (17, 21), it is logical that other signaling intermediates [such as protein kinase C (16, 18) and tyrosine kinase] are also held in common. Recently, p38 mitogen-activated protein kinase was found to be an important signaling component in the cardioprotection induced by preconditioning and adenosine (4, 7, 20). Furthermore, it is likely that other end-effectors implicated in cardioprotection from preconditioning, such as the activation of heat shock proteins or inhibition of apoptosis (1, 18), will be involved in A1-AR-mediated cardiac protection.

By examining a line of TG mice with moderate overexpression of myocardial A1-ARs (30-fold above normal), we could extend the conclusions drawn from our previous isolated heart studies into intact animals. This line of A1-AR TG mice exhibits minimal bradycardia and normal hemodynamic responses to MI. The moderate overexpression of A1-ARs makes the heart more resistant to ischemia-reperfusion injury, as indicated by decreased infarct size and reduced contractile dysfunction after MI. The results of these in vivo studies establish a foundation for future research aimed at evaluating the potential of A1-AR manipulation in altering the myocardial response to ischemia-reperfusion injury. Ultimately, these studies may have clinical relevance because the direct transfer of genes encoding A1-ARs to the heart may provide for an effective and durable form of cardioprotective gene therapy.
23. Thornton JD, Liu GS, Olsson RA, and Downey JM. Intra-
venous pretreatment with A₁-selective adenosine analogues pro-
Covell JW, and Dillmann WH. Protection against myocardial 
dysfunction after a brief ischemic period in transgenic mice 
expressing inducible heat shock protein 70. *J Clin Invest* 101: 
25. Tsuchida A, Thompson R, Olsson RA, and Downey JM. The 
anti-infarct effect of an adenosine A₁ selective agonist is dimin-
ished after prolonged infusion as is the cardioprotective effect of 
ischemic preconditioning in rabbit heart. *J Mol Cell Cardiol* 26: 
26. Yang Z, Zingarelli B, and Szabo C. Effect of genetic disrup-
tion of poly (ADP-ribose) synthetase on delayed production of 
inflammatory mediators and delayed necrosis during myocardial 
27. Yang Z, Zingarelli B, and Szabo C. The crucial role of endog-
28. Yoshida T, Maulik N, Engelman RM, Ho YS, Magnenat JL, 
Rousou JA, Flack JE 3rd, Deaton D, and Das DK. Glutathi-
one peroxidase knockout mice are susceptible to myocardial 
ischemia reperfusion injury. *Circulation* 96, Suppl: II-216–II-
29. Yoshida T, Watanabe M, Engelman DT, Engelman RM, 
Schley JA, Maulik N, Ho YS, Oberley TD, and Das DK. Transgenic mice overexpressing glutathione peroxidase are re-
sistant to myocardial ischemia reperfusion injury. *J Mol Cell 