Regulated Overexpression of the A1-Adenosine Receptor in Mice Results in Adverse but Reversible Changes in Cardiac Morphology and Function

Hajime Funakoshi, MD, PhD; Tung O. Chan, PhD; Julie C. Good, BS; Joseph R. Libonati, PhD; Jarkko Piuholu, MD, PhD; Xiongwen Chen, PhD; Scott M. MacDonnell, PhD; Ling L. Lee, MS; David E. Herrmann, BS; Jin Zhang, PhD; Jeffrey Martini, BS; Timothy M. Palmer, PhD; Atsushi Sanbe, PhD; Jeffrey Robbins, PhD; Steven R. Houser, PhD; Walter J. Koch, PhD; Arthur M. Feldman, MD, PhD

Background—Both the A1- and A3-adenosine receptors (ARs) have been implicated in mediating the cardioprotective effects of adenosine. Paradoxically, overexpression of both A1-AR and A3-AR is associated with changes in the cardiac phenotype. To evaluate the temporal relationship between AR signaling and cardiac remodeling, we studied the effects of controlled overexpression of the A1-AR using a cardiac-specific and tetracycline-transactivating factor–regulated promoter.

Methods and Results—Constitutive A1-AR overexpression caused the development of cardiac dilatation and death within 6 to 12 weeks. These mice developed diminished ventricular function and decreased heart rate. In contrast, when A1-AR expression was delayed until 3 weeks of age, mice remained phenotypically normal at 6 weeks, and >90% of the mice survived at 30 weeks. However, late induction of A1-AR still caused mild cardiomyopathy at older ages (20 weeks) and accelerated cardiac hypertrophy and the development of dilatation after pressure overload. These changes were accompanied by gene expression changes associated with cardiomyopathy and fibrosis and by decreased Akt phosphorylation. Discontinuation of A1-AR induction mitigated cardiac dysfunction and significantly improved survival rate.

Conclusions—These data suggest that robust constitutive myocardial A1-AR overexpression induces a dilated cardiomyopathy, whereas delaying A1-AR expression until adulthood ameliorated but did not eliminate the development of cardiac pathology. Thus, the inducible A1-AR transgenic mouse model provides novel insights into the role of adenosine signaling in heart failure and illustrates the potentially deleterious consequences of selective versus nonselective activation of adenosine-signaling pathways in the heart. (Circulation. 2006;114;&NA;-.)

Key Words: heart failure ■ adenosine ■ cardiomyopathy ■ hypertrophy ■ myocardial contraction ■ remodeling ■ receptors

Adenosine is an endogenous purine nucleoside that plays an important role in protecting the heart during stress. In animal models of ischemia, adenosine reduces infarct size,1,2 affords protection from reperfusion injury after prolonged coronary occlusion,3 and facilitates ischemic preconditioning.4 Furthermore, adenosine infusion has been shown to reduce infarct size in patients with a myocardial infarction.5 Because of its pharmacological effects on neurohormone and cytokine activation,6–10 it was hypothesized that adenosine might also affect ventricular remodeling in models of heart failure. Indeed, adenosine reduced cardiac hypertrophy and improved left ventricular (LV) function in mice with transverse aortic constriction.11 In addition, patients with increased muscle adenosine levels due to mutation in at least 1 allele of the adenosine monophosphate deaminase 1 (AMPD1) gene had a longer survival than patients with the wild-type genotype.12–14 Furthermore, in patients with heart failure, increased levels of adenosine were associated with severity of disease.15 These initial studies led investigators to identify the specific adenosine receptor (AR) subtypes that mediated the salutary benefits of adenosine.

Clinical Perspective

The cardiovascular effects of adenosine are modulated by 4 known G-protein–coupled ARs (A1, A2a, A2b, and A3), all of which are expressed in the heart.16 Activation of the A1- and
A1-ARs inhibits adenylyl cyclase and modulates other signaling pathways regulated by Gβ/γ. By contrast, activation of A2-ARs results in coupling to G1 proteins and activation of adenylyl cyclase.16–18 Pharmacological studies with receptor-subtype–selective agonists suggested that A1- and A2-ARs provide cardioprotection during ischemia and reperfusion,19,20 whereas A2-ARs afford protection after ischemia.21–23 Because pharmacological agonists lack selectivity, and because of significant species differences in the pharmacology of ARs,16 recent studies have assessed the role of AR subtypes using selective gene deletion and cardiac-restricted transgenic overexpression.24–27 Although both A1- and A2-ARs mediate increased myocardial resistance to ischemia, mice overexpressing modest levels of the A1-AR demonstrated a reduction in the response to high doses of catecholamines and an increase in hypertrophy,28 whereas mice with higher levels of A1-AR overexpression did not tolerate myocardial ischemia and had significant atrial arrhythmias.29 Of greater concern was the finding that mice with moderate to high levels of A2-AR overexpression developed a dilated cardiomyopathy.30 These findings raised concerns about the safety of chronic adenosine-AR activation in patients with cardiovascular disease.

Because activation of the A1-AR in pregnant mice inhibits cardiac cell proliferation and leads to cardiac hypoplasia,31 we hypothesized that the changes in the cardiac phenotype that result from moderate A1-AR overexpression might be due, at least in part, to activation of the A1-AR transgene in the early heart tube.32 To test this hypothesis, we created mice with inducible overexpression of the A1-AR using a tetracycline-based system in which expression could be regulated throughout cardiac development.33 Surprisingly, both constitutive and controlled overexpression of the A1-AR resulted in the development of a reversible dilated cardiomyopathy.

**Methods**

**Transgenic Mouse Generation**

The human A1-AR cDNA was cloned into a cardiac-specific and inducible controlled vector (TREMHC) composed of a modified mouse α-myosin heavy chain (α-MHC) minimal promoter fused with nucleotide binding sites for tetracycline transactivating factor (tTA).33 A1-AR transgenic (TG) mice, engineered on an FVB background (PolyGene, Zurich, Switzerland), were crossed with mice that expressed tTA in the heart (MHC-tTA; Figure 1A). In this “tetracycline-off” inducible system, the stable tetracycline analog doxycycline (DOX) inhibits tTA transactivation, and it was administered to mice at 300 mg/kg of mouse diet (Bio-Serv, Frenchtown, NJ). Survival studies were performed with both male and female mice and found no statistical significant differences between sexes; therefore, only male mice were used for subsequent studies. All protocols were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University.

**Echocardiography and ECG**

Echocardiographic studies on A1-AR TG mice were performed with an ultrasonographic system (Acuson Sequoia C256, Acuson, Siemens, Mountain View, Calif) as described previously34,35 and detailed in the online Data Supplement. To measure conscious heart rate, ECG recordings were obtained with PowerLab (ML866) and Bio Amp (ML136; ADInstruments, Colorado Springs, Colo). Briefly, animals were anesthetized with inhalation of isoflurane, placed in a supine position, and restrained. Mice returned to consciousness within 2 minutes. An ECG was recorded for 2 minutes and analyzed with Clar package software (ADInstruments). To normalize the recordings, mice were trained 3 times a day for 7 days before measurement.

**Mouse Langendorff Heart Perfusion**

As we described previously,36–38 anesthetized mice were euthanized by cervical dislocation. The abdominal cavity was opened immediately and the heart cooled with ice-cold perfusion fluid. The aorta was cannulated above the aortic valve, and retrograde perfusion was begun with a modified Krebs-Henseleit bicarbonate buffer, pH 7.4, equilibrated with 95% O2/5% CO2 at 37°C. Buffer composition (in mmol/L) was NaCl 113.8, NaHCO3 22, KCl 4.7, KH2PO4 1.2, MgSO4 1.1, CaCl2 2.0, and glucose 11.0. The hearts were perfused with a Langendorff apparatus and paced at 400 bpm with a Grass stimulator (9 V, 0.5 ms, Grass Instruments, Quincy, Mass). All hearts were immersed in a water-jacketed organ chamber to maintain a temperature of 37°C. A constant-pressure protocol was used to compare the acute response between wild-type and A1-TG mouse hearts. For hemodynamic measurements, a balloon was inserted into the LV, and the balloon volume was adjusted to 8 to 11 mm Hg of LV end-diastolic pressure (LVEDP) for stabilization. Initially, each isolated heart was perfused with a constant pressure of 55 mm Hg for 15 minutes. Then, the perfusion pressure was elevated to 120 cm H2O for 15 minutes. After stabilization, no further alterations in balloon volume were made. LV pressure (LVp), the maximum rate of positive and negative change in LVP (±dP/dt), and coronary perfusion pressures were recorded continuously (Powerlab/8SP, ADInstruments, Colorado Springs, Colo).
ADInstruments). Coronary perfusion pressure was measured at heart level via a fluid-filled pressure transducer. At the end of the protocol, left and right ventricles were flash-frozen with liquid nitrogen.

**Real-Time Quantitative Polymerase Chain Reaction**

Real-time quantitative polymerase chain reaction analysis determined both genomic copies of inserted transgene and transgene expression with the human and mouse conserved \( \text{A1-AR} \) primer set (see Data Supplement). Briefly, 40 ng of genomic DNA from mouse tail was used to quantify the number of transgenes inserted into the genome. Reverse-transcribed cDNA from myocardium RNA was used to determine the expression of \( \text{A1-AR} \), atrial natriuretic peptide (ANP), sarcoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA), phospholamban, cFos, early growth response factor-1 (EGR-1), and collagen 1a, 3a and 6a genes with specific primer sets (see Data Supplement).

**Membrane Preparation and \( \text{A1-AR} \) Receptor Binding Assay**

Radioligand binding of \( \text{A1-AR} \) in crude cardiac membranes was performed as described previously\(^{50,40}\) and detailed in the Data Supplement.

**Immunoblotting and Histopathology of Myocardium**

Immunoblotting of ventricular protein extracts was digitally detected with an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, Neb) as described previously\(^{41}\) and detailed in the Data Supplement. Picrosirius red staining for assessment of fibrosis was performed by the Research Animal Diagnostic Laboratory, University of Missouri. To determine fibrosis, 5 independent high-power fields of stained images from each animal were analyzed with Image-Pro Plus software (MediaCybernetics, Silver Spring, Md).

**Surgical Procedure for Aortic Banding**

Six-week-old male wild-type and TG FVB mice were used for aortic banding and are described in the online Data Supplement. Aortic banding was created by placing a ligature (7-0 nylon suture) securely between the origin of the right innominate and left common carotid arteries with a 27-gauge needle as a guide. The sham procedure was identical except that the aorta was not ligated. The authors had full access to and take full responsibility for the integrity. All authors have read and agree to the manuscript as written.

**Results**

**Inducible and Cardiac Specific Expression of \( \text{A1-AR} \)**

We generated human \( \text{A1-TG} \) mice controlled by an inducible cardiac-specific promoter with binding sites for tTA. Gene expression was initiated by crossing 6 founder \( \text{A1-TG} \) lines with mice that expressed tTA in the heart (MHC-tTA; Figure 1A). By immunoblotting, 4 of the 5 founder lines showed robust \( \text{A1-AR} \) protein expression (Figure 1B). Expression of \( \text{A1-AR} \) was confirmed by radioligand binding in cardiac membranes with the \( \text{A1-AR} \) ligand DPCPX (Figure 1C). Quantification of immunoblots and radioligand binding indicated that these \( \text{A1-AR TG} \) lines expressed the \( \text{A1-AR} \) at 500- to 1000-fold above the endogenous \( \text{A1-AR} \) level. Specificity was confirmed by competition with \( \text{A1-AR} \) antigenic peptide and with excess nonradioactive \( \text{A1-AR} \) binding ligand (Figure 1C and data not shown).

\( \text{A1-TG} \) lines B and C were chosen for further characterization. Real-time polymerase chain reaction assays showed that both lines had similar genomic copy numbers and expressed transgene messages at similar levels (Data Supplement Figures I and II). Human \( \text{A1-AR} \) mRNA was not detected in other organs, such as brain, lung, kidney, and liver, and gene expression of other AR subtypes (\( \text{A2a}, \text{A2b}, \text{and A3} \)) was expressed at similar levels (Data Supplement Figure 1C and data not shown).

**DOX-Regulated \( \text{A1-AR} \) Expression**

The stable tetracycline analog DOX inhibits tTA transactivation. We determined the minimum dose of DOX (300 mg/kg of mouse diet) that attenuated \( \text{A1-AR} \) expression, which in turn prevented cardiomyopathy. As shown in Figure 2A, when DOX was continuously administered to pregnant mothers and subsequently to the offspring, cardiac \( \text{A1-AR} \) expression was significantly attenuated at 6 weeks of age compared with constitutively expressed \( \text{A1-AR TG} \) mice (\( \text{A1-TGCon} \)). To generate inducible \( \text{A1-AR} \) transgenic mice (\( \text{A1-TGInd} \)), DOX was removed when mice reached 3 weeks of age. Time-course studies showed that \( \text{A1-AR} \) was fully reexpressed at 6 weeks of age. At 6 weeks of age, \( \text{A1-AR} \) protein expression in \( \text{A1-TGCon} \) and \( \text{A1-TGInd} \) mice was identical (Figures 2B and 2C). DOX inhibition and \( \text{A1-AR} \) induction were confirmed by \( \text{A1-AR} \) binding assays (Figure 2D).

As reported previously,\(^{33}\) the MHC-tTA mouse line expressed tTA at very low levels. We found tTA expression in wild-type mice did not affect mouse heart weight and function up to 12 weeks. Similarly, the amount of DOX used in the study did not affect wild-type mouse heart size or function (Data Supplement Figures III and IV).

**Constitutive and Induced Overexpression of \( \text{A1-AR} \)**

As shown by the induction scheme (Figure 3A), \( \text{A1-AR} \) was either expressed constitutively in the absence of DOX (\( \text{A1-TGCon} \)) or expression was induced (\( \text{A1-TGInd} \)) by removing DOX at 3 weeks of age. Constitutive \( \text{A1-AR} \) overexpression in 2 \( \text{A1-TG} \) lines led to development of a dilated cardiomyopathy and high mortality rate in both male and female mice. Almost all \( \text{A1-TGCon} \) mice died within 6 to 12 weeks, depending on the founder line (Figure 3B). The higher mortality rate of line B was associated with a higher \( \text{A1-AR} \) protein expression (Figure 1B). Mice died of apparent congestive heart failure (postmortem cardiac hypertrophy and dilatation; pleural effusion). Both male and female mice showed similar mortality rates and phenotypes (data not shown). In contrast, when \( \text{A1-AR} \) expression was delayed until mice reached 3 weeks of age, >90% of \( \text{A1-TGInd} \) mice survived 30 weeks or longer despite comparable levels of \( \text{A1-AR} \) overexpression. We chose to use line C for the remaining physiological and biochemical studies because this
line had the lowest transgene expression level and afforded longer survival.

We assessed cardiac functions and physical cardiac parameters when A1-TGCon or A1-TGInd mice reached 6 weeks of age. A1-TGCon mice developed early and profound cardiac dilatation, diminished ventricular function, and marked bradycardia (Figure 4A; Table 1). However, we did not detect significant arrhythmia using surface ECG measurements (data not shown). In addition, we determined the expression of genes frequently associated with cardiomyopathy. Data showed that expression of ANP and collagen genes was enhanced in A1-TG myocardium (Figure 4A; Table 1). On the other hand, expression of the calcium-handling genes, SERCA and phospholamban, was decreased (Table 1). These mice also demonstrated extensive fibrosis by picrosirius red staining and enhanced expression of collagen genes (Figure 4A). In contrast, when A1-AR induction was delayed until 3 weeks of age, A1-TGInd mice had a normal phenotype at 6 weeks of age, as demonstrated by ventricular weight and cardiac function. There was, however, a small but statistically significant decrease in heart rate (Table 1). The marked reduction in heart rate was detected in both anesthetized resting mice and in conscious restrained mice. When A1-AR expression was delayed until mice reached 3 weeks of age, A1-TGInd mice showed a significantly improved heart rate and cardiovascular parameters compared with A1-TGCon mice. To assess the effects of A1-AR overexpression on Akt phosphorylation, we probed heart extracts from 6-week-old male mice using antibodies that recognize phosphorylated Akt at Thr308. A1-AR overexpression decreased significantly in basal Akt phosphorylation in both A1-TGCon and A1-TGInd mice (Figure 4C). Consistently, phosphorylation of Akt Ser473 was also decreased (Data Supplement Figure V). In contrast, phosphorylation of map kinases (Erk1/2, JNK1/2, and p38) was not altered (Data Supplement Figure V).

Figure 3. A, A1-AR induction scheme. B, Kaplan-Meier survival curve for TG line B and line C constitutively expressing A1-AR (A1-TGCon) or with A1-AR induced at 3 weeks of age by removal of DOX (A1-TGInd). For both line B and line C, P<0.01 for A1-TGInd vs A1-TGCon. Also, P<0.01 for line B A1-TGCon vs line C A1-TGCon. WT indicates wild type.

Effect of Induced A1-AR Expression Responding to Pressure Overload

Because A1-TGInd mice had normal cardiac morphology and function at 6 weeks, we assessed whether A1-AR overexpression was cardioprotective in the presence of pressure overload induced by aortic banding. We measured LV systolic pressure immediately before and after banding, and pressure
gradients between wild-type and A1-TGInd mice were similar (data not shown). As shown in Figures 5A, 5B, and 5C, 4 weeks after surgery, aortic banding accelerated the hypertrophic response to pressure overload and further decreased fractional shortening as compared with nontransgenic controls. In addition, aortic banding markedly reduced the level of expression of the calcium-handling genes SERCA and phospholamban (Figure 5D), markedly enhanced fibrosis, and effected a significant decrease in heart rate in mice overexpressing the A1-AR transgene (Figure 5E).

On the basis of these results, we evaluated cardiac morphology and function in older A1-TGInd mice. Consistent with the maladaptive effects of A1-AR overexpression in young mice with aortic banding, at 20 weeks of age, A1-TGInd mice developed ventricular enlargement, fibrosis, decreased fractional shortening, and changes in the expression of calcium-handling genes (Figure 4B; Table 2). Importantly, at this age, a marked reduction in heart rate was detected in anesthetized resting mice compared with heart rates of 6-week-old mice (Table 2). Interestingly, heart rates in conscious restrained mice did not differ between the 2 age groups.

Finally, to obviate the effects of heart rate on the changes in cardiac biology in the transgenic mice, we assessed the expression of early-response genes, cFos and EGR-1, in Langendorff-perfused A1-TGInd hearts. Under identical pacing conditions, hemodynamic parameters (LV developed pressure, diastolic pressure, +dP/dt, and −dP/dt) were similar between wild-type control hearts and A1-TGInd hearts (Data Supplement Table I). In preliminary studies, real-time quantitative polymerase chain reaction showed that serum rapidly induced cFos and EGR-1 expression in a neonatal myocyte-derived cell line, H9C2, within 30 minutes (data not shown). As seen in Figures 5F and 5G, at steady state, levels of cFos and EGR-1 in mouse hearts were low, and no differences were observed between wild-type and A1-TG mice. However, the administration of a high perfusion pressure and therefore of increased cardiac load resulted in the rapid induction of cFos and EGR-1 expression. Importantly, the induction of cFos and EGR-1 expression was twice as high in A1-TG mice as in wild-type controls.

Effects of DOX Treatment
By 3 weeks after birth, A1-TGCon mice demonstrated enlargement of the LV cavity and fibrosis (Figure 6A). To determine whether the cardiomyopathy induced by A1-AR overexpression was reversible, A1-TGCon mice were fed DOX beginning at 3 weeks to inhibit A1-AR transgene expression (Figure 6B). Assessment of cardiac function at 12 weeks demonstrated
that attenuation of A1-AR expression in the A1-TGCon mice normalized ventricular weight, increased fractional shortening, and modulated LV dimension (Figures 6C and 6D). In addition, DOX reversed collagen staining, corrected the gene expression profile toward normal levels, and enhanced the survival of A1-TGCon mice (Figures 6E and 6F and Data Supplement Figure VII), which indicates that the functional pathology in this model was largely reversible.

**Discussion**

To understand the effects of temporal changes in A1-AR expression on cardiac morphology and function, we created TG mice in which A1-AR expression could be temporally regulated, by crossing mice that harbor the α-MHC promoter, which drives very low levels of tTA, with mice harboring a TG construct that consists of the human A1-AR gene linked to a banded wild-type nontransgenic controls. These physiological changes were accompanied by diminished expression of calcium-handling genes and enhanced expression of ANP and collagen genes. Importantly, the profound adverse consequences of constitutive A1-AR overexpression could be partially reversed by “turning off” the A1-AR transgene with the administration of DOX. To address concerns that the level of A1-AR overexpression in the present experiments might be “superphysiological,” we evaluated A1-TG mice in which expression was activated at 3 weeks, were not able to tolerate pressure overload because banding resulted in markedly enhanced hypertrophy and diminished cardiac function, changes that were not observed in banded wild-type nontransgenic controls. These physiological changes were accompanied by diminished expression of calcium-handling genes and enhanced expression of ANP and collagen genes.
The present studies differ from earlier reports in which higher or comparable levels of constitutive overexpression of the A1-AR had less deleterious effects on cardiac morphology or function. For example, 1000-fold constitutive overexpression of rat A1-AR resulted in a decrease in the intrinsic heart rate of Langendorff-perfused hearts of 7- to 9-week-old TG mice (248 versus 318 bpm) and a lower developed tension.26 A1-AR overexpression in these mice also dampened the contractile response to high doses of catecholamines and significantly increased the heart weight/body weight ratio; however, no morphological changes were noted.28 Lower levels of constitutive A1-AR overexpression (300-fold) did not appear to be associated with changes in cardiac morphology or function and provided cardioprotection in globally ischemic, isolated heart models of ischemia-reperfusion injury. However, when studied in vivo, these mice had mildly decreased conscious resting heart rates compared with wild-type controls and died during even brief coronary occlusion.25

The disparity between the marked adverse effects of even moderate constitutive overexpression of A1-AR in the present experiments and the limited toxicity associated with moderate overexpression in earlier studies may be due to 2 fundamental differences in the studies: (1) Earlier studies created TG mice in the C57/B16 background (G.P. Matherne, personal communication), whereas the present studies were performed in the FVB strain; and (2) earlier studies utilized rat A1-AR cDNA, whereas the present studies used human A1-AR cDNA. It is unlikely that differences between the present results and those reported previously are due to the use of human rather than rat (or mouse) cDNA, because the A1-AR cDNA from mice and humans shared 95% amino acid identity (Data Supplement Figure VI). That the disparities could be explained by the difference in strain is supported by the fact that the genetic background of TG mice can significantly alter morphology, function, and survival. For example, survival was markedly better in mice overexpressing tumor necrosis factor-α in an FVB background than on a mixed FVB/C57/B16 background.42,43 In addition, strain can markedly influence cardiac responsiveness to adrenergic and cholinergic agents. For example, baseline heart rates are significantly higher in C57/Bl6 mice than in FVB mice.43 Importantly, whereas isoproterenol and atropine increase heart rate in C57/Bl6 mice, isoproterenol and atropine have
TABLE 2. Organ Weights and Echocardiographic and Real-Time PCR Data of 20-Week-Old Mice

<table>
<thead>
<tr>
<th>Organ weights</th>
<th>WT</th>
<th>A1-TGInd</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>30.0 ± 1.4</td>
<td>28.6 ± 0.7</td>
</tr>
<tr>
<td>Ventricular/body weight ratio</td>
<td>3.94 ± 0.17</td>
<td>5.12 ± 0.19*</td>
</tr>
<tr>
<td>Lung/body weight ratio</td>
<td>6.06 ± 0.17</td>
<td>6.45 ± 0.12</td>
</tr>
</tbody>
</table>

Echocardiographic data

<table>
<thead>
<tr>
<th>n</th>
<th>5</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>467 ± 31</td>
<td>144 ± 3*</td>
</tr>
<tr>
<td>Heart rate, bpm (conscious)</td>
<td>591 ± 4</td>
<td>333 ± 8*</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>3.64 ± 0.10</td>
<td>5.03 ± 0.13*</td>
</tr>
<tr>
<td>LVEDS, mm</td>
<td>1.81 ± 0.15</td>
<td>3.43 ± 0.10*</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>50.4 ± 3.2</td>
<td>31.8 ± 1.1*</td>
</tr>
</tbody>
</table>

Real-time PCR (relative expression)

<table>
<thead>
<tr>
<th>n</th>
<th>5</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERCA</td>
<td>1.00 ± 0.05</td>
<td>0.53 ± 0.02*</td>
</tr>
<tr>
<td>PLB</td>
<td>1.00 ± 0.03</td>
<td>0.68 ± 0.05*</td>
</tr>
<tr>
<td>ANP</td>
<td>1.00 ± 0.1</td>
<td>138.5 ± 31.9*</td>
</tr>
</tbody>
</table>

Abbreviations as in Table 1.

Organ weights and echocardiographic and gene expression data in 20-week-old mice after DOX was removed from TG mice at 3 weeks of age to induce A1-AR expression (A1-TGInd).

*P<0.01 vs WT.

no effect on heart rate in FVB mice. Thus, mice with A1-AR overexpression on a FVB background may be more susceptible to AR-mediated activation of the Gs-adenylyl cyclase owing to a limited ability to increase heart rate through autonomic nervous system activity. Indeed, adenosine attenuated cardiac hypertrophy and prevented heart failure in mice with LV pressure overload in C57/Bl6 mice and had no effect on heart rate.11

By contrast with constitutive overexpression of A1-AR in C57/Bl6 mice, constitutive overexpression of the A1-AR in FVB mice effected changes in the cardiac phenotype that were consistent with the results in the present experiment. Founders with high levels of A1-AR died of cardiac failure within 4 weeks of birth, whereas heterozygote mice with moderate overexpression of the A1-AR demonstrated cardiac hypertrophy, dilation, decreased function, recapitulation of the fetal gene program, and a significant decrease in heart rate at 14 to 17 weeks of age.30,44 By contrast, low levels of A1-AR expression were not associated with significant changes in morphology or function at young ages; however, they did demonstrate a significant reduction in adenylyl cyclase activity,30 first-degree AV block,44 and altered sinus nodal and AV nodal function.29 A1-AR and A2-AR are both G-protein receptors coupled to Gi. Interestingly, cardiac-specific and adult-induced expression of a synthetic recombinant, Gi-coupled receptor (Ro1), induced a high rate of mouse mortality that was associated with arrhythmia and cardiomyopathy.45,46 However, increased Gi-dependent signaling may not account for all the phenotypes seen in A1-AR TG mice, because A1-AR can also associate directly with other cell signaling molecules.47

Consistent with other rodent models of dilated cardiomyopathy, cardiac failure in mice overexpressing A1-AR was associated with the development of hypertrophy, fibrosis, ANP induction, and decreased SERCA and phospholamban expression. However, a marked decrease in the expression of SERCA was observed in the A1-TGInd mice even before the onset of any changes in LV size or function. Thus, A1-AR-mediated alterations in calcium handling might be a key step in the development of cardiac dilation and dysfunction in these animals. In contrast to other heart failure models, the development of LV hypertrophy and dysfunction was not associated with a change in mitogen-activated protein kinase activity, a key signaling protein in the development of cardiac hypertrophy (Data Supplement Figure V). In addition, the activity of Akt decreased (Figure 4). Enhanced phosphatidylinositol-3 (PI3) kinase and Akt kinase signaling plays an important role in both the “physiological” hypertrophy process and in the regulation of cell survival. The latter finding differs from an earlier report in which adenosine activated PI3 kinase in rabbit hearts through transactivation of receptor tyrosine kinases, whereas stimulation of the A1-AR effected enhanced phosphorylation of Akt.48 However, Akt activation does not appear to be necessary for A1-AR-mediated cardioprotection.49,50

The development of cardiac hypertrophy and dilatation in FVB mice overexpressing A1-AR is closely related to the A1-AR-induced decrease in heart rate. Indeed, the profound bradycardia seen in mice with both constitutive and inducible overexpression of A1-AR may be the most likely explanation for the development of cardiomyopathy in these animals. This finding is consistent with other reports in which selective overexpression of A1-AR or A2-AR appears to have salutary benefits on cardioprotection without adversely affecting cardiac morphology or function when heart rate does not change; however, cardiac dysfunction is present when heart rate is either moderately or markedly decreased.50 Assessment of the role of heart rate in effecting changes in cardiac function and morphology in the presence of A1-AR overexpression is challenging, because pacemakers small enough for a mouse are not commercially available. However, we took advantage of the earlier finding that natriuretic peptides (ANP and brain natriuretic peptide) were induced in the presence of increased wall stress and cardiac hypertrophy and that their expression is regulated by the early-response genes such as cFos and EGR-1.51-57 We hypothesized that in the presence of increased load, A1-AR TG hearts would demonstrate a rapid and robust increase in early-response gene expression that was independent of heart rate. Consistent with this hypothesis, in the presence of ventricular pacing, Langendorff-perfused hearts from both A1-AR TG and wild-type mice demonstrated an increase in cFos and EGR-1 expression; however, the level of expression induced in the A1-AR TG mice was twice that seen in the wild-type controls (Figures 5F and 5G). Thus, intrinsic molecular changes occur in response to an increase in pressure load in the presence of A1-AR overexpression that are independent of heart rate, and these changes are associated with alterations in the fetal gene program that are a characteristic feature of the heart failure phenotype. However, heart-rate related changes in cardiac...
function and morphology may also contribute to the cardiomyopathy observed in these animals, particularly the profound cardiac dysfunction seen in older animals.

Although the A1-AR TG mice displayed profound heart rate reduction, several cardiomyopathy mouse models, such as Gq overexpression, calcineurin overexpression, and tumor necrosis factor-α overexpression, were also associated with significant but less profound heart rate reduction.43,58,59 In contrast, heart rate was not altered in cardiomyopathies caused by calsequestrin, a mutant MHC, and muscle LIM protein knockout.60–63 Interestingly, these cardiomyopathy models with unaffected heart rate all responded favorably to adrenergic pathway inhibition with a β-adrenergic receptor kinase inhibitor.60–63 In contrast, β-adrenergic receptor kinase inhibition did not improve cardiomyopathy caused by Gq overexpression.64 Thus, it remains to be determined whether the coupling of heart rate and cardiomyopathy alters the response to β-adrenergic blockade therapeutics. These findings have important implications on the potential therapeutic use of selective AR agonists in patients with cardiovascular disease. For example, when used chronically, doses of selective adenosine agonists that do not decrease heart rate should be chosen for clinical investigation, and individual patients should be observed carefully to ensure that variations in genetic background do not result in unique effects on heart rate in selected patient populations. In addition, the present results suggest that therapeutic agents that have a more balanced effect, ie, those that activate both A1- and A2-ARs, may be safer in patients with normal or compromised cardiac function. This hypothesis will require further evaluation in experimental animals.

Sources of Funding

This study was supported by National Institutes of Health grants HL61690 and HL075443 (Dr Koch), the Pennsylvania Research Formulary Fund (Dr Feldman), the Pennsylvania Research Formulary Fund and the American Heart Association, SDG F64702 (Dr Chan), and the British Heart Foundation (Dr Palmer).

Disclosures

None.

References


Adenosine is an endogenous nucleoside that plays a critical role in the process of ischemic preconditioning through activation of a group of G-protein-coupled receptors that includes the A$_1$, A$_2$, and A$_3$-adenosine receptors. Acute administration of either adenosine or of an A$_3$-selective adenosine receptor agonist decreases infarct size after coronary ligation in experimental animals, the same salutary benefit that is seen in transgenic mice in which the A$_1$-adenosine receptor after maturity markedly impairs the heart’s ability to withstand non-ischemic stress. In the present study, we demonstrate that cardiac-selective overexpression of the A$_1$-adenosine receptor after maturity markedly impairs the heart’s ability to withstand the hemodynamic stress of aortic banding. Furthermore, prolonged overexpression results in a marked decrease in heart rate and in cardiac function. However, the decrease in function appears to be independent of the change in heart rate. These results raise important cautions about the use of selective adenosine receptor agonists (or potentially selective antagonists) in patients with heart muscle dysfunction. Furthermore, the finding that the development of cardiac dysfunction in these transgenic mice was associated with a decrease in heart rate suggests that heart rate should be carefully monitored in patients undergoing investigational studies of agents that alter the balance between activation of the various adenosine receptor subtypes.

**CLINICAL PERSPECTIVE**

Adenosine is an endogenous nucleoside that plays a critical role in the process of ischemic preconditioning through activation of a group of G-protein-coupled receptors that includes the A$_1$, A$_2$, and A$_3$-adenosine receptors. Acute administration of either adenosine or of an A$_3$-selective adenosine receptor agonist decreases infarct size after coronary ligation in experimental animals, the same salutary benefit that is seen in transgenic mice in which the A$_1$-adenosine receptor after maturity markedly impairs the heart’s ability to withstand non-ischemic stress. In the present study, we demonstrate that cardiac-selective overexpression of the A$_1$-adenosine receptor after maturity markedly impairs the heart’s ability to withstand the hemodynamic stress of aortic banding. Furthermore, prolonged overexpression results in a marked decrease in heart rate and in cardiac function. However, the decrease in function appears to be independent of the change in heart rate. These results raise important cautions about the use of selective adenosine receptor agonists (or potentially selective antagonists) in patients with heart muscle dysfunction. Furthermore, the finding that the development of cardiac dysfunction in these transgenic mice was associated with a decrease in heart rate suggests that heart rate should be carefully monitored in patients undergoing investigational studies of agents that alter the balance between activation of the various adenosine receptor subtypes.
Regulated Overexpression of the A₁-Adenosine Receptor in Mice Results in Adverse but Reversible Changes in Cardiac Morphology and Function

Circulation. published online November 6, 2006;
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/early/2006/11/06/CIRCULATIONAHA.106.620211.citation

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2006/11/06/CIRCULATIONAHA.106.620211.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/