Effects of cardiac-restricted overexpression of the A$_{2A}$/adriamycin-induced cardiotoxicity

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Hamad EA, Li X, Song J, Zhang X, Myers V, Funakoshi H, Zhang J, Wang J, Li J, Swope D, Madonick A, Farber J, Radice GL, Cheung JY, Chan TO, Feldman AM. Effects of cardiac-restricted overexpression of the A$_{2A}$/adenosine receptor on adriamycin-induced cardiotoxicity. Am J Physiol Heart Circ Physiol 298: H1738–H1747, 2010. First published April 2, 2010; doi:10.1152/ajpheart.00688.2009.—Activation of the A$_{2A}$/adenosine receptors (A$_{2A}$/R) has been shown to be cardioprotective. We hypothesized that A$_{2A}$/R overexpression could protect the heart from adriamycin-induced cardiomyopathy. Transgenic (TG) mice overexpressing the A$_{2A}$/R and wild-type mice (WT) were injected with adriamycin (5 mg·kg$^{-1}$·wk$^{-1}$ ip, 4 wk). All WT mice survived adriamycin treatment while A$_{2A}$/R TG mice suffered 100% mortality at 4 wk. Telemetry showed progressive prolongation of the QT interval, bradyarrhythmias, heart block, and sudden death in adriamycin-treated A$_{2A}$/R TG but not WT mice. Both WT and A$_{2A}$/R TG demonstrated similar decreases in heart function at 3 wk after treatment. Adriamycin significantly increased end-diastolic intracellular Ca$^{2+}$/concentration in A$_{2A}$/R TG but not in WT myocytes ($P < 0.05$). Compared with WT myocytes, action potential duration increased dramatically in A$_{2A}$/R TG myocytes ($P < 0.05$) after adriamycin treatment. Expression of connexin 43 was decreased in adriamycin treated A$_{2A}$/R TG but not WT mice. In sharp contrast, A$_{2A}$/R overexpression induced after the completion of adriamycin treatment resulted in no deaths and enhanced cardiac performance compared with WT adriamycin-treated mice. Our results indicate that the timing of A$_{2A}$/R activation is critical in terms of exacerbating or protecting adriamycin-induced cardiotoxicity. Our data have direct relevance on the clinical use of adenosine agonists or antagonists in the treatment of patients undergoing adriamycin therapy.

[Ca$^{2+}$/transients; cardiac myocytes; action potential duration; cardiomyopathy]

ANTHRACYCLINE ANTIBIOTICS, such as adriamycin (doxorubicin), are antitumor agents that have been used since the late 1960s for treatment of hematological and solid tumor malignancies (44). Despite their efficacy as anticancer agents, they are associated with significant cardiotoxicity. These deleterious effects are dose dependent and may persist long after the treatment is stopped (36). The effects of adriamycin on the heart have been extensively studied and serve as a model for understanding anthracycline-associated cardiomyopathies (18). Adriamycin causes a decrease in fractional shortening (FS) that is accompanied by myofibril loss and vacuolization (17, 46). Adriamycin-associated arrhythmias have also been documented in case reports and include nonspecific T wave and ST changes and rarely sudden death (8, 20). The mechanisms by which adriamycin induces cardiotoxicity have been proposed to include formation of free radicals (19), inhibition of nucleic acid and protein synthesis (4, 45), lipid peroxidation (29, 35, 39), abnormalities in mitochondria (11), lysosomal changes (39), modifications of sarcolemmal Ca$^{2+}$/transport, diminished activity of adenylate cyclase, Na$^{+}$-K$^{+}$/ATPase, and Ca$^{2+}$/ATPase activities (11, 35, 37, 38), and the release of histamines and catecholamines (3, 22).

Adenosine, an ubiquitous purine nucleotide, is cardioprotective during ischemic pre- and postconditioning (15, 21, 51). The biological actions of adenosine are mediated by a family of G protein-coupled receptors found on the sarcolemmal surface of cardiac myocytes, including the A$_{1}$/, A$_{2A}$/, and A$_{3}$/ adenosine receptors (R) and the A$_{2B}$/R, which is expressed only in the cardiac vasculature. Activation of the A$_{1}$/R or A$_{3}$/R pathways inhibits adenyl cyclase activity through inhibitory guanine nucleotide binding protein αGi, while activation of A$_{2A}$/R enhances cAMP production through αG$\beta$$\gamma$/pathways (6, 15, 42). Activation of the A$_{2A}$/R has been associated with inhibition of the inflammatory response (45, 49). Overexpression of A$_{2A}$/R resulted in enhanced contractility, increased sarcomplastic reticulum (SR) Ca$^{2+}$/uptake, and a higher systolic Ca$^{2+}$/concentration. Therefore, we hypothesize that A$_{2A}$/R overexpression might be protective in adriamycin-induced cardiomyopathy. To test this hypothesis, we induced overexpression of A$_{2A}$/R in transgenic mice (TG), either concomitant with or after adriamycin administration, and compared cardiac performance and survival with adriamycin-treated wild-type (WT) animals.

MATERIALS AND METHODS

TG mouse generation. Mice with inducible, cardiac-specific overexpression of the human A$_{2A}$/R (A$_{2A}$/TG) were engineered on a FVB background as previously described (5, 9). A$_{2A}$/TG mice were crossed with mice that expressed tetracycline transactivator (tTA) in the heart (tTA TG) in the presence of doxycycline (Dox). In this "tet-off"-inducible system, Dox, the stable tetracycline analog, inhibits tTA transactivation and was administered to mice at 300 mg/kg of a mouse diet (Bio-Serv). Dox was removed from mice to induce transgene expression. Mice were housed and fed on a 12:12-h light-dark cycle at the Thomas Jefferson University Animal Facility and were supervised by veterinary staff members. Standard care was provided to all mice used for experiments. All protocols applied to the mice in this study were approved and supervised by the Institutional Animal Care and Use Committee of Thomas Jefferson University.

Chronic mouse model of adriamycin-induced cardiotoxicity. In initial experiments, Dox was removed from the animals’ diets at 3 wk...
of age to assess the concomitant effects of adriamycin administration and activation of the $A_{2A}$R pathways. To this end, 8- to 12-wk-old male $A_{2A}$R TG mice and WT FVB controls (10 mice/group) were injected with adriamycin (5 mg·kg$^{-1}$·wk$^{-1}$ ip for 4 wk; Sigma-Aldrich, St. Louis, MO). This regimen was designed to simulate an adriamycin dose of 1,400 mg/70 kg which is associated with development of cardiomyopathy in humans (36). It should be noted that since adriamycin-treated $A_{2A}$R TG mice had a low survival rate (Fig. 1), measurements of gene and protein expression and cardiac function were made in mice that were killed earlier in the cycle of adriamycin therapy (1 day after the third adriamycin injection when most of treated $A_{2A}$ TG mice were alive). In a second series of experiments, the effects of $A_{2A}$R activation were assessed after adriamycin treatment was complete. Eight-week-old $A_{2A}$R TG mice ($n = 7$) and WT mice ($n = 24$) were maintained on Dox (since birth) and treated with adriamycin (5 mg·kg$^{-1}$·wk$^{-1}$ ip) for 4 wk. Dox was withdrawn after completion of adriamycin treatment, and the animals were monitored for an additional 8 wk.

![Diagram showing the experimental design](image)

**Fig. 1.** A: survival after adriamycin (Ad) treatment. All wild-type (WT) mice survived adriamycin treatment, while adenosine $A_{2A}$ receptor ($A_{2A}$R) transgenic (TG) mice had 100% mortality by the end of 4 wk ($P < 0.05$, log-rank test, $n = 10$). DOX, doxycyclin. B: evaluation of cardiac function after third adriamycin treatment in $A_{2A}$R TG and WT mice. Adriamycin reduced cardiac function in both WT and $A_{2A}$R TG mice. Percent fractional shortening (FS) of indicated mouse groups is shown. *$P < 0.01$ WT baseline vs. WT adriamycin. $+P < 0.001$ $A_{2A}$R TG baseline vs. $A_{2A}$R TG adriamycin. NS, not significant WT adriamycin vs. $A_{2A}$R TG adriamycin (see Table 1 for details). C: electron microscopic images of myocardial sections of WT ($n = 2$) and $A_{2A}$TG ($n = 2$) mouse hearts after third adriamycin injection. m, Mitochondrion. Bar = 500 nM.
In vivo assessment of cardiac function. Left ventricular (LV) function in mice was evaluated with transthoracic two-dimensional echocardiography (TTE). In experiments depicted in Fig. 1 and Table 1, mice were anesthetized with 2% inhaled isoflurane, and LV function at baseline and at 1 day after the third injection of adriamycin was assessed with the VisualSonics VeVo 770 imaging system with a 707 scanhead. This experiment assessed LV function at baseline and at 1 day after the third injection of adriamycin. In experiments depicted in Fig. 5 in which LV function was evaluated 8 wk after cessation of adriamycin treatment, mice were anesthetized with 2.5% Avertin (10 μl/g body wt ip, Aldrich Chemical) and echocardiographic studies were performed using the ACUSON Sequoia C256 system (5). Age-matched, non-TG or tTA mice on a FVB background served as controls. TTE in M-mode was carried out in the parasternal short-axis view at the papillary muscle level to assess LV end-diastolic diameter (LVEDD) and LV end-systolic diameter (LVESD). FS was calculated as

\[ \%FS = \left( \frac{LVEDD}{LVESD} \right) \times 100 \] (30).

Electron microscopy. WT and A2AR male 8- to 12-wk-old mice (n=2 for each group) were injected with doxorubicin. After the third injection, hearts were perfused with NaCl solution (0.8%) and then with fixative (4% paraformaldehyde, 2% gluteraldehyde in 0.1 M cacodylate buffer) and harvested. Portions of the LV were cut into 1-mm² cubes and washed three times in cacodylate buffer followed by dehydration through graded alcohols and propylene oxide. The samples were embedded in EM bed 812 (Electron Microscopy Sciences, Table 1. Echocardiography of WT and A2AR TG mice before and after adriamycin injection

<table>
<thead>
<tr>
<th></th>
<th>WT (5)</th>
<th>WT Ad (5)</th>
<th>A2AR TG (7)</th>
<th>A2AR TG Ad (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>410 ± 42</td>
<td>476 ± 12</td>
<td>557 ± 11</td>
<td>498 ± 18</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>3.4 ± 0.2</td>
<td>3.5 ± 0.1</td>
<td>3.3 ± 0.1</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>2.0 ± 0.2</td>
<td>2.35 ± 0.2</td>
<td>1.8 ± 0.1</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>%FS</td>
<td>40.1 ± 2.1</td>
<td>33.1 ± 2.6*</td>
<td>45.2 ± 2.7</td>
<td>32.1 ± 2.3*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Numbers in parentheses are number of mice per group (8- to 12-wk male mice). WT, wild-type; WT Ad, WT after 3rd injection of adriamycin (Ad); A2AR TG, adenosine A2A receptor-overexpressing transgenic mice; A2AR TG Ad, A2AR TG mice after 3rd injection of adriamycin; LVEDD and LVESD, left ventricular end-diastolic and -systolic dimensions, respectively; %FS, percentage of fractional shortening. This experiment used mice anesthetized by 2% inhaled isoflurane and the VisualSONICS VeVo 770 imaging system with a 707 scanhead. *P < 0.05 compared with same mouse type without adriamycin.

In vivo assessment of cardiac function. Left ventricular (LV) function in mice was evaluated with transthoracic two-dimensional echocardiography (TTE). In experiments depicted in Fig. 1 and Table 1, mice were anesthetized with 2% inhaled isoflurane, and LV function at baseline and at 1 day after the third injection of adriamycin was assessed with the VisualSonics VeVo 770 imaging system with a 707 scanhead. This experiment assessed LV function at baseline and at 1 day after the third injection of adriamycin. In experiments depicted in Fig. 5 in which LV function was evaluated 8 wk after cessation of adriamycin treatment, mice were anesthetized with 2.5% Avertin (10 μl/g body wt ip, Aldrich Chemical) and echocardiographic studies were performed using the ACUSON Sequoia C256 system (5). Age-matched, non-TG or tTA mice on a FVB background served as controls. TTE in M-mode was carried out in the parasternal short-axis view at the papillary muscle level to assess LV end-diastolic diameter (LVEDD) and LV end-systolic diameter (LVESD). FS was calculated as

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were isolated from WT and A2AR TG mice after the third weekly dose of adriamycin. Indirect immunofluorescence was performed on paraffin-embedded sections of hearts as previously described (7). The sections were incubated with antibodies against connexin 43 and β-catenin overnight at 4°C. The sections were then washed and incubated with either IRDye 700 or 800 secondary antibodies and processed with Odyssey Infrared Imaging System software (Li-Cor, Lincoln, NE).

Table 2. Effects of adriamycin on [Ca$^{2+}$]o, transients and SR Ca$^{2+}$ uptake

<table>
<thead>
<tr>
<th>[Ca$^{2+}$]o, mM</th>
<th>WT (18)</th>
<th>WT Ad (13)</th>
<th>A2AR TG (20)</th>
<th>A2AR TG Ad (18)</th>
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</thead>
<tbody>
<tr>
<td>Systolic [Ca$^{2+}$]o, nM</td>
<td>202.3 ± 8.6</td>
<td>210.1 ± 20.6†</td>
<td>308.5 ± 20.7*</td>
<td>318.5 ± 19.7*</td>
</tr>
<tr>
<td>Diastolic [Ca$^{2+}$]o, nM</td>
<td>88.3 ± 3.9</td>
<td>105.8 ± 9.7*</td>
<td>88.3 ± 2.7</td>
<td>132.54 ± 9.9††</td>
</tr>
<tr>
<td>[Ca$^{2+}$]o transient amplitude, % increase in fura 2 signal</td>
<td>19.7 ± 1.0</td>
<td>16.03 ± 1.7††</td>
<td>33.7 ± 2.3*</td>
<td>23.8 ± 1.7††</td>
</tr>
<tr>
<td>t1/2 of [Ca$^{2+}$]o transient decline, ms</td>
<td>142.8 ± 8 (16)</td>
<td>205 ± 20† (7)</td>
<td>134.4 ± 13 (8)</td>
<td>176.8 ± 21 (5)</td>
</tr>
</tbody>
</table>

Values are means ± SE. For calcium transient measurements, numbers in parentheses are myocytes pooled from 3 mice/treatment group. [Ca$^{2+}$]o and [Ca$^{2+}$]i, intracellular and extracellular Ca$^{2+}$ concentration, respectfully. *P < 0.05 compared with WT. †P < 0.05 compared to A2AR TG. ‡P < 0.05 WT-Ad vs. A2AR TG Ad.
shown). Electron microscopy showed a striking noninflammatory myopathy (sarcotomer loss, disarray of myofibrils, and increase in the number of mitochondria) consistent with Adriamycin cardiotoxicity in both WT and A2AR TG mice exposed to Adriamycin (Fig. 1C). Telemetry demonstrated progressive prolongation of the QT interval, runs of ventricular tachycardia, bradyarrhythmias, and first- and second-degree heart block in A2AR TG mice treated with Adriamycin (Fig. 2). Asystole and sudden death were also recorded in A2AR TG mice and was preceded by runs of nonsustained ventricular tachycardia (Fig. 2B). By contrast, WT mice treated with Adriamycin did not demonstrate any arrhythmias.

**Effects of Adriamycin on [Ca^{2+}]_{i} transients in A2AR TG and WT mice.** To explore cellular mechanisms responsible for sudden death in A2AR-overexpressed mice treated with Adriamycin, we measured [Ca^{2+}]_{i} transients in isolated WT and A2AR TG myocytes after exposure to vehicle or 5 μM Adriamycin for 18 h. Preliminary studies showed that at this dose, Adriamycin-induced lethality was similar between WT and A2AR TG myocytes (data not shown). In the absence of Adriamycin, myocytes from A2AR TG mice had significantly higher (P < 0.001) systolic [Ca^{2+}]_{i} compared with WT myocytes (Table 2, Fig. 3A). Adriamycin caused a significant prolongation in t_{1/2} of [Ca^{2+}]_{i} transient decline in both WT and A2AR TG myocytes. However, Adriamycin induced higher end-diastolic [Ca^{2+}]_{i} in the A2AR TG group compared with the WT group (Table 2). Adriamycin depolarized membrane potential and prolonged action potential in A2AR TG but not in WT mice. Another cellular mechanism that may explain sudden death in A2AR-overexpressed mice exposed to Adriamycin is prolongation of the action potential. Treatment with Adriamycin depolarized membrane potential in A2AR TG but not WT myocytes (Table 3). At
baseline, A2AR TG myocytes exhibited slightly prolonged action potential duration at both 50 (APD₅₀) and 90% repolarization (APD₉₀) compared with WT myocytes (P < 0.05) (Table 3, Fig. 3B). In addition, exposure to adriamycin resulted in dramatic prolongation in APD₉₀ in A2AR TG but not in WT myocytes (P < 0.005; Table 3).

Adriamycin decreased Cx43 and N-cadherin in A2AR TG but not in WT mice. Cx43 is the predominant gap junction protein expressed in the myocardium. Loss of the gap junction protein Cx43 has been shown to slow myocardial conduction velocity and induce unidirectional block, resulting in an arrhythmogenic substrate and sudden cardiac death in mice (12–14). After three doses of adriamycin treatment, immunofluorescence imaging showed sparse Cx43 signals at A2AR TG myocyte junctions compared with WT controls (Fig. 4A). Immunoblotting Cx43 from ventricular extracts not exposed to adriamycin and from posttreatment extracts showed that Cx43 protein expression was significantly less (P < 0.05) in hearts overexpressing A2AR than in those from WT controls (Fig. 4B). This decrease was associated with a decrease in Cx43 phosphorylation.

A2AR overexpression after adriamycin treatment improved cardiac performance and survival. Our data thus far indicated that A2AR overexpression concomitant with adriamycin administration not only did not afford protection but was actually detrimental. Another clinically relevant protective strategy is to activate A2AR signaling after adriamycin treatment is complete. When Dox was removed after completion of adriamycin treatment, A2AR expression was induced in a time-dependent manner (Fig. 5A). In contrast to increased mortality associated with simultaneous activation of A2AR and adriamycin treatment (Fig. 1A), induction of A2AR overexpression after cessation of adriamycin administration enhanced survival (Fig. 5B) and improved cardiac function compared with the WT adriamycin-treated controls (Fig. 5C).

**DISCUSSION**

Adriamycin is known to cause a dose-dependent cardiomyopathy (36) with a decrease in FS associated with myofibril loss and vacuolization in the myocardium (17, 46). In agreement, our current results demonstrated noninflammatory myopathy with decreased FS in both WT and A2AR TG mice treated with adriamycin. Oxidative damage has been shown to contribute to the toxic effects of adriamycin in mitochondria, the SR, and myofibrils. Damage to cardiomyocytes contributes to remodeling of the ventricle with a globular geometry that can increase wall stress. In addition, adriamycin has been shown to decrease SR Ca²⁺ uptake activity and enhance Ca²⁺ release by the ryanodine receptors in rabbits (33), which can lead to abnormalities in <span class="red">Ca²⁺</span> homeostasis and excitation contraction coupling (38). Our current results are in agreement with these earlier studies in that SR Ca²⁺ uptake (as estimated by 1/2 of [Ca²⁺] transient decline) was decreased in both WT and A2AR TG myocytes exposed to adriamycin. In addition, adriamycin resulted in significantly elevated end-diastolic <span class="red">Ca²⁺</span> [Ca²⁺], likely explained by decreased SR Ca²⁺ uptake and enhanced SR Ca²⁺ release by ryanodine receptors. Adriamycin has been associated with a variety of arrhythmias in both animal models and humans (8, 20). However, the mechanisms responsible for these arrhythmias remain undetermined. Many transient nonspecific EKG abnormalities have been described including nonspecific ST T-wave changes, a decrease in QRS voltage, right-axis deviation, T-axis abnormalities, and prolongation of the QT interval. There are a few case reports of heart block, ventricular ectopy, and rare cases of sudden death after adriamycin treatment (8, 20).

Given the known cardioprotective properties of the A2AR in ischemia, we hypothesized that cardiac-restricted A2AR overexpression would be beneficial in adriamycin cardiotoxicity. We initially induced A2AR overexpression concomitant with adriamycin administration. Surprisingly, this maneuver proved to be deleterious rather than cardioprotective, as exposure to adriamycin resulted in sudden death at 4 wk. Thus one of the major findings of our present study is that concomitant activation of A2AR signaling and adriamycin resulted in progressive prolongation in QT interval, heart block, development of ventricular arrhythmias, and sudden death. Since sudden death is rare after adriamycin treatment in WT animals (5, 16), the deleterious effects of adriamycin observed in the present study were most likely mediated by A2AR signaling. A second major finding is that when A2AR overexpression was induced after completion of adriamycin treatment, both survival and cardiac function were better than that seen in the WT controls.

Improper calcium regulation is likely responsible for ventricular arrhythmias seen in mice in which A2AR overexpression was induced concurrent with adriamycin administration. In the canonical Gs-coupled signaling, the A2AR activates adenyl cyclase, generates intracellular cAMP, and activates PKA. Then, PKA activates SERCA2 and elevates systolic <span class="red">Ca²⁺</span> [Ca²⁺], by phosphorylating both the SERCA2a inhibitor phospholamban (PLN) and the PLN inhibitor complex (PP1/inhibitor 1) (10, 53). At the cellular level, myocytes isolated from A2AR TG hearts and treated with adriamycin demonstrated significantly higher end-diastolic [Ca²⁺], decreased SR Ca²⁺ uptake as indicated by longer 1/2 of [Ca²⁺] transient decline, depolarized membrane potential, and prolongation of APD₉₀. Elevated diastolic [Ca²⁺], promotes forward Na⁺/Ca²⁺ exchange (3 Na⁺ in: 1 Ca²⁺ out), which generates an inward current during diastole, leading to membrane depolarization and afterdepolarizations. Action potential prolongation and afterdepolarizations are commonly incriminated as cellular

**Table 3. Effects of adriamycin on action potential in A2AR TG and WT mice**

<table>
<thead>
<tr>
<th></th>
<th>WT (7)</th>
<th>WT Ad (4)</th>
<th>A2AR TG (12)</th>
<th>A2AR TG Ad (13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting Emv, mV</td>
<td>−65.2 ± 2.7</td>
<td>−68.7 ± 2.2</td>
<td>−66.7 ± 2.2</td>
<td>−57.89 ± 1.81 ‡</td>
</tr>
<tr>
<td>AP amplitude, mV</td>
<td>110.3 ± 6.4</td>
<td>106.6 ± 5.7</td>
<td>106.3 ± 3.9</td>
<td>103.12 ± 4.98</td>
</tr>
<tr>
<td>APD₉₀, ms</td>
<td>4.9 ± 0.71</td>
<td>5.6 ± 0.45</td>
<td>9.9 ± 1.8*</td>
<td>10.22 ± 1.45*</td>
</tr>
<tr>
<td>APD₉₀, ms</td>
<td>25 ± 3.6</td>
<td>25.2 ± 4.5</td>
<td>43.8 ± 3.8*</td>
<td>64.89 ± 5.57 ‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. For action potential measurements, numbers in parentheses are myocytes pooled from 3 mice/treatment group. Emv, membrane potential; AP, action potential; APD₉₀ and APD₉₀, action potential duration at 50 and 90% repolarization, respectively. Cells were paced at 1 Hz. *P < 0.05 compared with WT. †P < 0.05 compared to A2AR TG. ‡P < 0.05 WT-Ad vs. A2AR TG Ad.

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substrates for arrhythmogenesis. Thus the sudden death seen in mice overexpressing the A2A R and treated with Adriamycin may be explained by development of cardiac arrhythmias caused by altered [Ca^{2+}]	extsubscript{i} homeostasis.

Another cellular mechanism that may account for increased arrhythmogenesis resulting in sudden death is structural changes in gap junctions (16). In the present study, the finding that Adriamycin significantly altered the levels of the junctional protein Cx43 provided an alternative explanation for the marked increase in sudden death. Cx43 mechanically and electrically couples cardiomyocytes to ensure rhythmic contraction. Cardiac-specific loss of Cx43 slows myocardial conduc-

Fig. 4. Connexin 43 (Cx43) staining patterns and expression in A2A R TG and WT mice treated with Adriamycin. A: after the third dose of Adriamycin, ventricular myocardium from A2A R TG and WT mice was immunostained with Cx43 and β-catenin (n = 3 for each group). B: ventricular extracts were prepared from hearts not exposed to Adriamycin (left) and from hearts after 3 injections of Adriamycin (right). Ten- to 12-wk-old male WT hearts (n = 4) and A2A R TG hearts (n = 4–6) were probed for Cx43 and dephosphorylated (De-P Cx43, 13-8300). Cx43 signals were normalized to GAPDH. Values are means ± SE. *P < 0.05 vs. WT.
tion velocity, which leads to arrhythmias and sudden cardiac death (12–14). Recent studies have also demonstrated that cardiac-specific loss of Cx43 or change in Cx43 localization can induce unidirectional block, resulting in an arrhythmogenic substrate and sudden cardiac death in mice (12–14). Arrhythmic susceptibility in N-cadherin/Cx43 compound heterozygous mice was associated with a reduced Cx43 protein level and an increase in the dephosphorylated Cx43 level (23, 24). Changes in Cx43 phosphorylation regulate Cx43 trafficking, assembly, gating, and turnover in the cell (41), and progressive dephosphorylation of Cx43 is associated with electrical uncoupling (1, 2). Thus the significant decrease in Cx43 and accompanying changes in intercellular coupling may lead to the high incidence of bradyarrhythmias and sudden death observed in A2AR TG mice exposed to adriamycin. However, levels of Cx43 were significantly reduced in A2AR mice even in the absence of A2AR expression, suggesting that the decrease in Cx43 level is not solely due to the presence of A2AR.
absence of adriamycin. Therefore, while changes in Cx43 may increase susceptibility to arrhythmia in the presence of adriamycin and A2AR activation, our present study did not go into sufficient depths to accept or refute this hypothesis.

We cannot exclude the possibility that alterations in K+ channel activity or levels might also have contributed to the sudden death seen in A2AR TG animals exposed to adriamycin; however, this explanation is less likely. Abnormalities in K+ channels have been implicated in long QT syndrome, short QT syndrome, and familial atrial fibrillation, all of which lead to tachyarrhythmias rather than bradyarrhythmias or heart block as seen in our A2AR TG mice treated with adriamycin.

In contrast to the catastrophic survival associated with concurrent A2AR activation and adriamycin treatment, we found that induced overexpression of A2AR after adriamycin therapy was completed was beneficial, both in terms of cardiac function and survival. There are clinical precedents to this finding. For example, the coadministration of adriamycin and herceptin results in as much as a 27% incidence of heart failure in women with metastatic breast cancer (40). By contrast, the administration of herceptin after completion of adriamycin therapy substantially lowers the risk (31). The reason for this differential response remains undefined but may be related to the acute ability of adriamycin to induce free radicals, alter oxidation-related genes (48, 52), and elevate diastolic calcium (this paper and Ref. 43). After cessation of adriamycin exposure, oxidation-related gene alterations rapidly return to normal (within 1–3 days).

Although we did not elucidate the exact cellular mechanisms by which induced A2AR overexpression after cessation of adriamycin therapy afforded cardioprotection, some insights can be gleaned from the known effects of A2AR activation. A2AR signaling may ameliorate adriamycin cardiotoxicity by enhancing SERCA2 activity. Analogous to the proposed mechanisms for SERCA2 overexpression in heart failure (10), A2AR activation may enhance systolic function, reduce hypertrophy, and improve cardiac energy metabolism. Alternatively, A2ARs could also mediate cardiac protection independently of the canonical Gs/cAMP/PKA/SERCA2 pathway, as A2AR binds other proteins and forms heteromeric complex with other G protein-coupled receptors (53). Other signaling pathways activated by A2AR include MAPKs, PKC, and Akt and nitric oxide (27, 50, 53). The Gi-independent mechanism is likely mediated through the unique A2AR C terminus that binds accessory proteins such as α-actinin, ARNO, USP4, and translin-associated protein-X. It is through ARNO (the guanine nucleotide exchange factor for ARF6 small G proteins) that A2AR regulates Gi-independent functions such as endocytosis, arrestin interaction, and MAPK activation (53).

In summary, our study demonstrated that the concurrent activation of A2AR potentiated the cardiotoxic effects of adriamycin. Increased cardiotoxicity was not due to additional reduction in cardiac contractility but was associated with elevated end-diastolic [Ca2+]i, altered [Ca2+]i homeostasis, decreased Cx43 in gap junctions, and prolonged APD with the subsequent development of cardiac arrhythmias and sudden death. By contrast, activation of A2AR signaling after completion of anthracycline therapy had salutary benefits. Our findings have significant clinical relevance regarding the use of A2A adenosine agonists or A1 adenosine antagonists in patients with diminished LV function secondary to anthracycline therapy.

GRANTS
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DISCLOSURES
No conflicts of interest are declared by the authors.

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