Effects of cardiac-restricted overexpression of the A2A adenosine receptor on adriamycin-induced cardiotoxicity

Eman A. Hamad, Xue Li, Jianliang Song, Xue-Qian Zhang, Valerie Myers, Hajime Funakoshi, Jin Zhang, Ju-Fang Wang, Jifen Li, David Swope, Ashley Madonick, John Farber, Glenn L. Radice, Joseph Y. Cheung, Tung O. Chan, and Arthur M. Feldman

Center for Translational Medicine, Department of Medicine, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania

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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 sarcoplasmatic Ca2+ transport, diminished activity of adenylate cyclase, Na+-K+-ATPase, and Ca2+-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 A1, A2A, and A3 adenosine receptors (R) and the A2BR, which is expressed only in the cardiac vasculature. Activation of the A1R or A2R pathways inhibits adenylyl cyclase activity through inhibitory guanine nucleotide binding protein αGi, while activation of A2AR enhances cAMP production through αGs (6, 15, 42). Activation of the A2AR has been associated with inhibition of the inflammatory response (45, 49). Overexpression of A2AR resulted in enhanced contractility, increased sarcoplasmic reticulum (SR) Ca2+ uptake, and a higher systolic Ca2+ concentration. Therefore, we hypothesized that A2AR overexpression might be protective in adriamycin-induced cardiomyopathy. To test this hypothesis, we induced overexpression of A2AR 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 A2AR (A2A TG) were engineered on a FVB background as previously described (5, 9). A2A 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

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 sarcoplasmatic Ca2+ transport, diminished activity of adenylate cyclase, Na+-K+-ATPase, and Ca2+-ATPase activities (11, 35, 37, 38), and the release of histamines and catecholamines (3, 22).

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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 A2AR pathways. To this end, 8- to 12-wk-old male A2AR 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 A2AR 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 A2A TG mice were alive). In a second series of experiments, the effects of A2AR activation were assessed after adriamycin treatment was complete. Eight-week-old A2AR 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.

Fig. 1. A: survival after adriamycin (Ad) treatment. All wild-type (WT) mice survived adriamycin treatment, while adenosine A2A receptor (A2AR) 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 A2AR TG and WT mice. Adriamycin reduced cardiac function in both WT and A2AR TG mice. Percent fractional shortening (FS) of indicated mouse groups is shown. *P < 0.01 WT baseline vs. WT adriamycin. +P < 0.001 A2AR TG baseline vs. A2AR TG adriamycin. NS, not significant WT adriamycin vs. A2AR TG adriamycin (see Table 1 for details). C: electron microscopic images of myocardial sections of WT (n = 2) and A2AR 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). Fractional shortening (FS) was calculated as %FS = [(LVEDD - LVESD)/LVEDD] × 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>
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<tr>
<td>Heart rate, bpm</td>
<td>410 ± 2</td>
<td>476 ± 12</td>
<td>557 ± 11</td>
<td>498 ± 18</td>
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<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>
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<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).Fractional shortening (FS) was calculated as %FS = [(LVEDD - LVESD)/LVEDD] × 100 (30).

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 Hatfield, PA). Longitudinal and transverse sections were cut on an UltraCut E ultramicrotome and stained with uranyl acetate and lead citrate (Electron Microscopy Sciences). Images were collected with an AMT XR41-B 4 megapixel camera on a Hitachi H-7000 electron microscope.

Isolation of adult murine cardiac myocytes. Cardiac myocytes were isolated from the septum and LV free wall of WT and A2AR TG mice (male, 8–12 wk old) as previously described (32). Briefly, mice were heparinized (1,500 U/kg ip) and anesthetized (pentobarbital sodium, 50 mg/kg ip). Excised hearts were mounted on a steel cannula and retrograde perfused (100 cmH2O, 37°C) with Ca2+-free bicarbonate buffer followed by enzymatic digestion (collagenases B and D, protease XIV). Isolated myocytes were cultured on laminin-coated glass coverslips, and the Ca2+ concentration of the buffer was incrementally increased from 0.05 to 0.5 mM (0.05, 0.125, 0.25, 0.5 mM) with 10 min of exposure at each Ca2+ concentration. The 0.5 mM Ca2+ buffer was then aspirated and replaced with MEM (Sigma-Aldrich) containing 1.2 mM Ca2+, 2.5% FBS, and antibiotics (1% penicillin/streptomycin). After 1 h (4°C, CO2, 37°C), media were replaced with FBS-free MEM. Preliminary studies were performed to establish the appropriate adriamycin dose to use in single-cell studies. Adriamycin at 5 μM induced lethality in ~50% of myocytes at 24 h (data not shown). Based on these studies, myocytes from A2AR TG and WT mice were treated with 5 μM adriamycin for 18 h (n = 3 mice for each group).

Intracellular Ca2+ concentration transient measurements. Myocytes from A2AR TG and WT mice pretreated with adriamycin or vehicle were exposed to 0.67 mM fura 2-AM for 15 min at 37°C. Fura 2-loaded myocytes were field-stimulated to contract (1 Hz, 37°C) in medium 199 containing 1.8 mM extracellular Ca2+. Myocytes were then incubated with either IRDye 700 or 800 secondary antibodies and IGEPAL CA-630, and 10 NaF and freshly supplemented with (in mM) 4 KEGTA, 3 Na2ATP, and 0.6 NaH2PO4, 7.5 HEPES, 7.5 Na+–HEPES, and 5 glucose, pH 7.4.

Immunofluorescence analysis of connexin 43 and β-catenin. Hearts 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 β-catenin and connexin 43 (Cx43; Zymed Labs, South San Francisco, CA) overnight at 4°C. Secondary antibodies were Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes, Carlsbad, CA) for 1 h. The double-stained sections were imaged with a Zeiss Meta confocal microscope (Thornwood, NY).

Immunoblotting. Frozen ventricular tissues were homogenized on ice using a nonionic detergent-based lysis buffer containing (in mM) 25 Tris-HCl (pH 7.6), 137 NaCl, 10% glycerol, 1% NP-40 or IGEPA LT-630, and 10 NaF and freshly supplemented with (in mM) 1 sodium pyrophosphate, 1 EDTA, 10 PMSF, and 1 NaVO4 and leupeptin, and aprotinin (5 mM) 1 sodium pyrophosphate, 1 EDTA, 10 PMSF, and 1 NaF and freshly supplemented with (in mM) 4 KEGTA, 3 Na2ATP, and 0.6 NaH2PO4, 7.5 HEPES, 7.5 Na+–HEPES, and 5 glucose, pH 7.4.

Statistics. All results are expressed as means ± SE. Kaplan-Meier survival curves were compared between groups using log-rank tests. One-way analysis of variance was used to analyze [Ca2+]i, transients and action potential parameters. Mann-Whitney nonparametric methods were used to compare between two groups. A commercial software package was used for all statistical analysis (Graph Pad Software, La Jolla, CA). A P value of <0.05 was considered statistically significant.

RESULTS

Increased mortality in A2AR TG mice after treatment with adriamycin. When A2AR overexpression was induced 5 wk before adriamycin treatment, A2AR-R-overexpressed mice suffered 100% mortality 1 wk after the fourth adriamycin injection while all WT mice survived adriamycin treatment (P < 0.05, Fig. 1A). In a separate cohort, cardiac function was evaluated at baseline and 1 day after the third injection of adriamycin (when >50% of A2A TG mice were still alive). Baseline %FS was significantly higher in A2A TG compared with WT mice (Fig. 1B, Table 1). After three injections of adriamycin, both WT and A2AR TG mice demonstrated significantly reduced FS (Fig. 1B, Table 1). The heart weight/body weight ratio was not different in the two groups (data not shown).

Table 2. Effects of adriamycin on [Ca2+]i, transients and SR Ca2+ uptake

<table>
<thead>
<tr>
<th>[Ca2+]i, nM</th>
<th>WT (18)</th>
<th>WT Ad (13)</th>
<th>A2AR TG (20)</th>
<th>A2AR TG Ad (18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic</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</td>
<td>88.3 ± 3.9</td>
<td>105.8 ± 9.7*</td>
<td>88.3 ± 2.7</td>
<td>132.54 ± 9.1‡ ‡</td>
</tr>
<tr>
<td>[Ca2+]i 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 [Ca2+]i transient decline, ms</td>
<td>142.8 ± 8 (16)</td>
<td>203 ± 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. [Ca2+]i and [Ca2+]o, intracellular and extracellular Ca2+ 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 (sarcomere 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 [Ca2+]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 [Ca2+]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 [Ca2+]i compared with WT myocytes (Table 2, Fig. 3A). Adriamycin caused a significant prolongation in t1/2 of [Ca2+]i transient decline in both WT and A2AR TG myocytes. However, adriamycin induced higher end-diastolic [Ca2+]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 TG 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).

Fig. 3. Intracellular Ca2+ concentration ([Ca2+]i) transients and action potential durations. After adult myocytes from WT and A2AR were isolated, 5 μM adriamycin was added to the culture medium and measurements were made 18 h after exposure. A: representative tracing of myocyte calcium transients in A2AR TG and WT mice with and without adriamycin. B: representative tracing of action potential duration measurements in A2AR TG and WT mice with and without adriamycin.
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 myocardial injury 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 Ca2+ homoeostasis and excitation contraction coupling (38). Our current results are in agreement with these earlier studies in that SR Ca2+ uptake (as estimated by t1/2 of [Ca2+]i, transient decline) was decreased in both WT and A2AR TG myocytes exposed to adriamycin. In addition, adriamycin resulted in significantly elevated end-diastolic [Ca2+]i, 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-axi 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 [Ca2+]i 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+]i, decreased SR Ca2+ uptake as indicated by longer t1/2 of [Ca2+]i, transient decline, depolarized membrane potential, and prolongation of APD90. Elevated diastolic [Ca2+]i 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 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>
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<tr>
<td>Resting Em, 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>
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<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. Em, 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.
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\(^{2+}\)]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

Fig. 5. Effect of A2AR expression after cessation of adriamycin treatment. A: schematic diagram showing DOX inhibition and induction of A2AR in 8-wk-old TG mice. Mice were harvested at indicated times after DOX removal. Total ventricular protein extracts were immunoblotted with anti-A2AR antibody. B: schematic diagram showing induction of A2AR expression after cessation of adriamycin administration. Survival curve showed that control mice treated with adriamycin (n = 24) had over 30% mortality, while all A2AR TG mice (n = 7) survived. C: relative cardiac function in A2AR TG and control mice 8 wk after adriamycin administration. As detailed in MATERIALS AND METHODS, this echocardiographic experiment was performed using the ACUSON Sequoia C256 system and Avertin anesthesia. Exact %FS: WT no adriamycin (53.0 ± 1.4, n = 8), WT post-adriamycin (45.2 ± 1.9, n = 12); A2AR TG post-adriamycin (52.7 ± 1.3, n = 6). *P < 0.05 vs. WT post-adriamycin.

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absence of adriamycin. Therefore, while changes in Cx43 may increase susceptibility to arrhythmia in the presence of adria-
mycin 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 con-
current A2AR activation and adriamycin treatment, we found
that induced overexpression of A2αR after adriamycin therapy
was completed was beneficial, both in terms of cardiac func-
tion 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 adminis-
tration of herceptin after completion of adriamycin therapy
with diminished LV function secondary to anthracyline ther-
apy.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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