Possible involvement of downregulation of the apelin-APJ system in doxorubicin-induced cardiotoxicity

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Doxorubicin (Dox) is an anthracycline antitumor drug that has long been used in cancer chemotherapy. The antitumor action of Dox is based on its intercalation into DNA double helix, which, in turn, leads to inhibition of DNA replication, repair, and transcription. This mechanism inhibits cell growth and exerts a cell-killing effect against diverse malignant tumors. Despite the powerful antitumor effect of Dox, its clinical use is limited by dose-dependent cardiotoxicity, including arrhythmia, acute left ventricular (LV) dysfunction, and degenerative cardiomyopathy (29). Once it occurs, Dox-induced cardiomyopathy is irreversible and often progresses to congestive heart failure. Moreover, this disease has an extremely poor prognosis, as a cohort study (10) has reported that the 3-yr survival rate in patients with heart failure secondary to Dox-induced cardiomyopathy was <50%. Prevention of cardiotoxicity is therefore important in Dox chemotherapy, as is monitoring the onset and progression of cardiomyopathy.

APJ [a putative receptor protein related to ANG II type 1 (AT1) receptor] is a member of a G protein-coupled receptor superfamily with seven transmembrane domains. APJ was first identified as a gene possessing homology with the AT1 receptor but was found not to bind ANG II (23). Thereafter, following a period of orphan designation, APJ was finally deorphanized when it was discovered to be an apelin receptor (31). Apelin peptides exist in multiple short forms of different sizes produced by COOH-terminal cleavage of the 77-amino acid preproapelin. Peptides with amino acid lengths of 12, 13, 17, and 36 have been found in plasma and cardiovascular tissues in rodents and humans and are known to be biologically active (31, 39). In addition, both apelin and APJ mRNAs are highly expressed in the heart, and, therefore, the cardiac apelin-APJ system has been implicated in cardiovascular physiology and pathophysiology (14, 20).

Since the discovery of apelin, APJ has emerged as having a potential role in vasodilation, cardiac contraction, and cardioprotection in rodent models. Apelin stimulates nitric oxide (NO) release from the vascular endothelium through endothelial NO synthase activation, leading to vessel relaxation (12, 32). Apelin exerts a direct positive inotropic action on the heart both ex vivo and in vivo (2, 28). In addition, apelin treatment confers significant cardioprotection in rodent models of cardiac ischemia-reperfusion injury as well as ANG II- or isoproterenol-induced cardiac remodeling (15, 24, 25, 30). More importantly, it has been reported that apelin knockout mice showed impaired cardiac contractility with aging and developed progressive heart failure induced by pressure overload (19). Another study (4) found that both apelin knockout mice and APJ knockout mice showed not only modest declines in cardiac function under basal conditions but also clear decrements in exercise capacity under physiological stress conditions. These studies support the view that the endogenous apelin-APJ system contributes to the maintenance of cardiac function.

Recently, clinical studies have demonstrated that several human cardiovascular diseases are accompanied by changes in the expression of cardiac apelin and APJ. A large cohort study (7) reported that blood apelin levels were decreased in patients with chronic heart failure after LV systolic dysfunction. Implantation of a LV assist device in the patients with severe heart failure resulted in an elevation of APJ mRNA and apelin concentrations in the LV (5). These studies seem to support the notion that the endogenous apelin-APJ system has a cardioprotective role and that APJ is a potential therapeutic target for
cardiovascular diseases. Taken together, we hypothesized that the apelin-APJ system may play a role in the pathogenesis of Dox-induced cardiotoxicity and the development of subsequent heart failure.

To test our hypothesis, we created a mouse model of Dox-induced acute cardiotoxicity and analyzed gene expression. We also assessed protein expressions of apelin and APJ and evaluated the effects of APJ gene deletion on this model. Finally, we examined the effect of apelin on Dox-induced cell toxicity in vitro.

MATERIALS AND METHODS

Experimental animals. Male C57Bl/6J and Balb/cA mice were purchased from CLEA Japan, (Tokyo, Japan). APJ knockout mice were generated as previously described (12). In the present study, we used APJ knockout mice from the F9 generation (backcrossed to C57Bl/6J) to equalize the effect of genetic background. Cross-fertilization between APJ heterozygous mice (APJ+/− + APJ+/− mice) did not produce the expected Mendelian genotype distribution, and few APJ homozygotes were obtained (data not shown). Cross-fertilization between APJ-deficient mice (APJ−/− + APJ−/− mice) produced few offspring due to intrauterine and postnatal deaths. Nurturing offspring by genetic maternal and Balb/cA mice together partially improved the breeding rate. With both types of cross-fertilization, surviving APJ−/− mice grew into adults after weaning and exhibited a normal physical appearance. Although we did not examine the cause of low APJ−/− breeding rates, recent reports (4, 17) have suggested that embryonic deaths of APJ knockout mice are caused by impaired cardiovascular development.

Animal experiments were carried out in a humane manner with approval by the Institutional Animal Experiment Committee of the University of Tsukuba. Experiments were performed in accordance with the Regulation of Animal Experiments of the University of Tsukuba and Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Genotyping of APJ knockout mice was performed by PCR using genomic DNA from tail biopsies of 2 mm. The tail genome was extracted with KAPA Express Extract, and multiplex allele-specific PCR was carried out with KAPA2G Robust HotStart ReadyMix with a 5′ specific for the wild-type (WT) allele (APJ coding DNA sequences, CTCAAGGAAGTGTCTTTAGCTGAGC-3′), two reverse primers specific for the wild-type (WT) allele (APJ coding DNA sequences, CTCTAGGATTGCCAGAATCGCACC-3′), and the knockout allele (nuclear localization signal-lacZ cassette, 5′-CGTATAAACGGCTGCTCCGTG-3′). The genotype was determined by the PCR product size as follows: 317 bp for the WT allele and 592 bp for the knockout allele (data not shown).

Intraperitoneal injection of Dox. Experiments were performed using 8- to 12-wk-old male mice. Dox hydrochloride (D1515, Sigma-Aldrich, St. Louis, MO) was resolved in saline (Otsuka Pharmaceutical, Tokushima, Japan) at 3 or 4 mg/ml concentrations and then administered by a single intraperitoneal injection of 5 ml/kg mouse body wt (Dox dose: 15 or 20 mg/kg body wt). In experiments of gene expression analysis and apelin measurements using C57Bl/6J mice, Dox was given to mice at a dose of 20 mg/kg body wt. In experiments using APJ knockout mice, we reduced the dose to 15 mg/kg body wt, because most animals died between days 5 and 7 after Dox injection at the dose of 20 mg/kg body wt. This dose allowed us enough time to analyze survival.

Echocardiographic assessment and survival analysis. On day 5 after the administration of either Dox or saline as vehicle, mice were anesthetized with 1% isoflurane in O2, and chest hair was removed with a depilatory cream. Using a digital ultrasound system Vevo 2100 equipped with a MS550D Microscan transducer with a center frequency of 40 MHz (VisualSonics, Toronto, ON, Canada), M-mode images of the LV short axis at the papillary muscle level were captured with monitoring heart rate. LV fractional shortening (FS) was calculated as follows: FS = [(LV interior diameter at diastole – LV interior diameter at systole)/LV interior diameter at diastole] × 100. For survival analysis of the mice given Dox, we recorded 14-day survival times and performed necropsies on dead mice.

Plasma and tissue preparation. At times of interest (24 h, day 5, and day 14) after Dox injection, blood was collected from the inferior vena cava into 0.5 M EDTA (pH 8.0, final concentration: 7.5–10 mM) or heparin (final concentration: 15–20 U/ml) under inhalational anesthesia with isoflurane. Blood samples were rapidly centrifuged at 800 g for 15 min at 4°C, and the obtained plasma samples were frozen in liquid nitrogen and stored at −80°C. Hearts were then excised, immersed in PBS, and allowed to keep beating for a few seconds to remove remaining blood. After heart weight was measured, the ratio of heart weight to body weight was determined for each animal. For RNA and protein analysis, hearts were immediately transferred into liquid nitrogen and then stored at −80°C. For immunofluorescent analysis, hearts were embedded in OCT compound (Tissue-Tek, Torance, CA) and then frozen in 2-methylbutane precooled with liquid nitrogen. For histomorphological analysis, beating hearts were arrested with 50 mM KCl and PBS, fixed in 4% paraformaldehyde for ~24 h at 4°C, and then processed and embedded in paraffin.

RNA isolation and quantitative real-time RT-PCR. Frozen hearts were ground into a fine powder using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). A subset of frozen tissue powder was homogenized in Isogen (Nippongene, Tokyo, Japan), and total RNA was isolated according to the manufacturer’s protocol. After treatment with DNase I and ethanol precipitation with ephichamine (Nippongene), total RNA was heated at 65°C for 10 min and immediately chilled on ice to denature its secondary structure. Total RNA was then reverse transcribed using ReverTra Ace (RT-101, Toyobo, Osaka, Japan) and random hexamer (Takara Bio, Shiga, Japan). The reaction was carried out as follows: 30°C for 10 min, 42°C for 90 min, and 99°C for 5 min. The obtained sample containing an equivalent of 100 ng of the cDNA was used in a 20-ml volume for RT-PCR containing 10 μl of 2× SYBR Premix Ex Taq (Perfect Real Time, RR081A, Takara Bio) and 0.2 μM forward and reverse primers. The primers used for amplification of target cDNAs are shown in Table 1. Two-step amplification and calculation of the threshold cycle (Ct) value by the second derivative maximum method were performed in duplicate using the Thermal Cycler Dice Real Time System (Takara Bio). Expression levels of target genes were corrected for GAPDH expression levels using the ΔΔCt method. The amplification efficiency of primers for each target gene was confirmed to be equal using serial dilutions of cDNA.

Apelin-12 enzyme immunoassay. A subset of frozen tissue powder was homogenized in 10 eye-measured volumes of 0.1 M acetate on ice. Next, the homogenate was boiled at 100°C for 10 min and rapidly cooled on ice. After centrifugation at 17,800 g for 10 min at 4°C, the clear supernatant was obtained, and its protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s protocol. The supernatant was evaporated to dryness and dissolved with assay buffer from the apelin-12 enzyme immunoassay (EIA) kit (EK-057-23, Phoenix Pharmaceuticals, Burlingame, CA). The obtained crude peptide extract (150 μg equivalent of protein) was applied onto the apelin-12 EIA plate in duplicate. To measure plasma apelin levels, 8- or 16-fold diluted plasma/EDTA samples with assay buffer were directly applied onto the EIA plates in duplicate. The assay was performed according to an EIA protocol recommended by the manufacturer with a slight modification as follows: the first incubation step for the competitive immunoreaction between the sample and biotinylated apelin-12 was

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carried out at 4°C overnight. This process improved the accuracy and reproducibility of the apelin-12 EIA in our laboratory.

**Histology.** Paraffin sections (5 μm thickness) of the short axis of mouse hearts at the papillary muscle level were prepared using a Microtome HM 340 E (Microm, Walldorf, Germany). Deparaffinized sections were stained with hematoxylin and eosin, and images were obtained using a SZ61 microscope, a BX53 microscope, and DP21 digital camera (Olympus, Tokyo, Japan).

**Creatine kinase-MB assay.** Creatine kinase (CK)-MB activities in heparinized plasma samples were measured with a Fuji Dri-Chem 7000Vz (Fujifilm, Tokyo, Japan) according to the manufacturer’s protocol.

**Protein carbonylation assay and Western blot analysis.** A portion of frozen heart powder was homogenized in RIPA buffer containing 20 mM Tris (pH 7.8), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM DTT, 1 mM sodium vanadate, 10 mM sodium fluoride, 20 mM β-glycerophosphate, 1 mM PMSE, 1 μg/ml leupeptin, and 2.1 μg/ml apronin. After centrifugation at 17,800 g for 15 min at 4°C, the supernatant was obtained, and its protein concentration was determined as described above. Protein samples were mixed with Laemmli sample buffer containing 100 mM DTT and treated at 100°C for 3 min. Fifty or one hundred micrograms of the protein sample were subjected to SDS-PAGE and transferred to an Immobilon-P Transfer Membrane (Millipore, Bedford, MA). Protein carbonylation was then detected using the Protein Carbonyls Western Blot Detection Kit (Shima Laboratories, Tokyo, Japan) according to the manufacturer’s protocol. For Western blot analysis, membranes were blocked with skim milk and then blotted with the following primary antibodies: anti-light chain (LC3) antibody (1:1,000, rabbit polyclonal, clone p62 (1:200, rabbit monoclonal, clone Beclin-1 (1:1,000, rabbit polyclonal, clone

<p>| Table 1. Primers used for quantitative real-time relative PCR analysis |
|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th><strong>Gene Name</strong></th>
<th><strong>Accession</strong></th>
<th><strong>Forward Primer</strong></th>
<th><strong>Reverse Primer</strong></th>
<th><strong>Product Size, bp</strong></th>
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<tr>
<td>GAPDH</td>
<td>NM_008084.2</td>
<td>5'-TCAGCTGGGATGGCCCTGCG-3'</td>
<td>5'-CAAGGCGGGATGGCCCTGCG-3'</td>
<td>65</td>
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<tr>
<td>APJ</td>
<td>NM_031784.3</td>
<td>5'-CGGCAGGATGGCCCTGCG-3'</td>
<td>5'-CAAGGCGGGATGGCCCTGCG-3'</td>
<td>99</td>
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<td>Apelin</td>
<td>NM_010332.2</td>
<td>5'-GCTGTTGGGTGCTGCTGCTG-3'</td>
<td>5'-CAAGGCGGGATGGCCCTGCG-3'</td>
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<tr>
<td>Angiotensinogen</td>
<td>NM_002478.3</td>
<td>5'-AAGTGGCCCTGCG-3'</td>
<td>5'-CAAGGCGGGATGGCCCTGCG-3'</td>
<td>119</td>
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<td>Ang II type 1a receptor</td>
<td>NM_177322</td>
<td>5'-ACTGCAGAAGACCTGATGATG-3'</td>
<td>5'-TCAAGGACGGAGAGAGGAC-3'</td>
<td>61</td>
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<td>Ang II type 1b</td>
<td>NM_175086</td>
<td>5'-GGGTGTGCTGCTGCTGCTG-3'</td>
<td>5'-CGAGGCGGGATGGCCCTGCG-3'</td>
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<td>Endothelin type A receptor</td>
<td>NM_010332.2</td>
<td>5'-GCTGTTGGGTGCTGCTGCTG-3'</td>
<td>5'-CAAGGCGGGATGGCCCTGCG-3'</td>
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<td>Endothelin type B receptor</td>
<td>NM_007904</td>
<td>5'-TCAAGAAGACCTGATGATG-3'</td>
<td>5'-TCAAGGACGGAGAGAGGAC-3'</td>
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<td>Proendothelin-1</td>
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<td>5'-TCAAGGACGGAGAGAGGAC-3'</td>
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<td>β1-Adrenergic receptor</td>
<td>NM_004719.2</td>
<td>5'-GCTGTTGGGTGCTGCTGCTG-3'</td>
<td>5'-TCAAGGACGGAGAGAGGAC-3'</td>
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<td>β2-Adrenergic receptor</td>
<td>NM_004720.3</td>
<td>5'-GCTGTTGGGTGCTGCTGCTG-3'</td>
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<td>β-Myosin heavy chain</td>
<td>NM_008728.5</td>
<td>5'-AACAGACGACCTGAGAGGAC-3'</td>
<td>5'-CGAGGCGGGATGGCCCTGCG-3'</td>
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<td>Brain natriuretic peptide</td>
<td>NM_008726.5</td>
<td>5'-GGGTGTGCTGCTGCTGCTG-3'</td>
<td>5'-CGAGGCGGGATGGCCCTGCG-3'</td>
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<td>Atrial natriuretic peptide</td>
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<td>Light chain 3B</td>
<td>NM_026160.4</td>
<td>5'-AAGTGGATGGTACGAGCTGAG-3'</td>
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<td>p62</td>
<td>NM_010104.2</td>
<td>5'-AAGCAGATCCAGACCGAGAGGAC-3'</td>
<td>5'-AAGTGGATGGTACGAGCTGAG-3'</td>
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<tr>
<td>Beclin-1</td>
<td>NM_019584.3</td>
<td>5'-CGGGGAGGATGGCCCTGCG-3'</td>
<td>5'-AAGTGGATGGTACGAGCTGAG-3'</td>
<td>141</td>
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Cell viability assay. Cells were plated at 1,000 cells/well into poly-D-lysine-coated 96-multiwell plates and allowed to adhere over-night in growth medium at 37°C (5% CO₂ and 95% humidity). Cells were starved for 12 h in DMEM supplemented with 0.5% FBS followed by drug treatment. Cells were incubated with pE-apelin-13 for 1 h before exposure to Dox. After Dox treatment at 1 μM for 24 h, cell viability was determined by measurement of ATP levels using a CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). Sample luminescence was measured on a Wallac ARVOSX Multilabel Counter (Perkin-Elmer, Tokyo, Japan).

Statistics. All data are presented as means ± SE. Statistical analysis was performed using Prism 5 (version 5.0a, GraphPAD Software, San Diego, CA). Unpaired data were compared by Student's t-test, Welch's t-test, nonparametric Mann-Whitney's U-test, or one-way ANOVA with a Bonferroni test. Survival rates were compared by a log rank (Mantel-Cox) test.

RESULTS

Dox-induced downregulation of cardiac apelin and APJ mRNA expression in mice. Because Dox has been known to acutely alter the expression of multiple genes, we first performed a quantitative real-time relative PCR analysis of hormones, receptors, and cardiac stress markers in mouse hearts on days 1 and 5 after the administration of Dox (20 mg/kg body wt). Figure 1, A and B, shows that both apelin and APJ mRNA drastically declined on days 1 and 5 after Dox injection. In contrast, mRNAs of angiotensinogen and β-myosin heavy chain (known as a cardiac stress marker) were increased on day 1 and further strengthened on day 5 (Fig. 1, C and D). mRNA of brain natriuretic peptide (also
known as a cardiac stress marker) was significantly reduced on day 1 but was increased on day 5 (Fig. 1E). mRNA of the endothelin (ET) type A receptor showed small increases on days 1 and 5 after Dox injection (Fig. 1F). mRNA of the AT1a receptor was slightly decreased on day 1 but was increased to the same level as the control group on day 5 (Fig. 1G). mRNA of the ET type B receptor was not altered on day 1 but fractionally increased on day 5 after Dox injection (Fig. 1H). Besides the above, mRNA levels of the AT1b receptor, preproET-1, β1-adrenergic receptor, and β2-adrenergic receptor were unaffected at the observed time points (Fig. 1, I–L). Taken together, these results show that Dox administration causes changes in the expression of cardiac genes and, in particular, downregulates mRNA expression of apelin and APJ.

Changes in plasma and cardiac tissue levels of apelin and suppression of APJ expression in Dox-treated mice. We examined plasma and cardiac tissue apelin contents and found that plasma apelin levels were clearly diminished on day 5 after Dox injection (Fig. 2A). Cardiac apelin peptide levels were significantly reduced on day 1 but were increased to the same levels as the control group on day 5 after Dox injection (Fig. 2B). Next, we performed an immunofluorescent staining of heart tissues on day 5 after Dox injection (Fig. 2C). In the saline-treated group, immunoreactivity against anti-APJ antibody was observed in association with cardiomyocytes (dystrophin positive) in a patchy fashion. In contrast, in Dox-treated and knockout groups, most of immunoreactivity against anti-APJ antibody disappeared. These data suggest that Dox administration impacts on contents of systemic and cardiac apelin and suppresses APJ expression in mouse hearts.

Effects of APJ deletion on Dox-induced cardiac contractile dysfunction in mice. To evaluate the potential involvement of the apelin-APJ system in Dox-induced cardiotoxicity, we examined the effects of APJ gene deletion on Dox-induced cardiac dysfunction in mice. It has been well established that Dox administration impairs cardiac function in rodent models, and we therefore measured fractional shortening by echocardiography on day 5 after Dox injection (15 mg/kg body wt). First, we confirmed that lowering of the Dox dose to 15 mg/kg body wt had a similar effect as high-dose Dox (20 mg/kg body wt) on mRNA levels of apelin and APJ in hearts. The administration of Dox (15 mg/kg body wt) resulted in persistent downregulation of mRNA expression of apelin and APJ in APJ+/+ mouse hearts at all observed time points (Fig. 3, A and B). In contrast, in APJ−/− mouse hearts, the apelin mRNA level was significantly reduced on day 1 but showed an increasing tendency to the same level as the saline-treated group through day 14 from day 5 after Dox administration (Fig. 3A). In addition, the APJ mRNA level in APJ+/− mouse hearts was significantly reduced on days 1 and 5 but showed an increasing tendency to the same level as the saline-treated group on day 14 after Dox administration (Fig. 3B). We also observed a significant decrease in the apelin mRNA level in APJ−/− mouse hearts on day 5 after Dox injection (Fig. 3A). The Dox-treated group of APJ+/+ mice showed a significant decrease in FS compared with the saline-treated group of APJ+/+ mice (average percent decrease: 36.38 ± 4.45%, mean ± SE; Fig. 3, C and D). On the other hand, FS in the saline-treated group of APJ−/− mice was substantially lower than that in the saline-treated group of APJ+/+ mice. Furthermore, FS in the Dox-treated group of APJ−/− mice was even lower than that in the Dox-treated group of APJ+/+ mice, but these groups showed a similar decreasing ratio in FS (average percent decrease: 33.74 ± 3.50%, mean ± SE).

To determine whether the Dox-induced FS reduction in APJ−/− mice was caused at least in part by lack of the APJ gene or not, we also measured FS in APJ+/− mice. The saline-treated group of APJ−/− mice showed normal FS, similar to the saline-treated group of APJ+/+ mice, but the Dox-
of fractional shortening (FS; echocardiography on APJ susceptibility of APJ and APJ mRNA, echocardiographic assessment of left ventricular (LV) function, and survival rate in APJ KO mice. APJ KO mice were treated with saline or Dox (15 mg/kg, single intraperitoneal injection), and hearts were then subjected to quantitative real-time relative PCR analysis. The graphs show relative expression levels of apelin (A) and APJ (B) mRNAs in saline- and Dox-treated groups. Each bar represents means ± SE for 5–6 mice/group in all cases. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the corresponding saline-treated group. LV function was assessed by echocardiography on day 5, and 14-day survival rates after Dox administration were analyzed. Representative echocardiographic images (C) and quantified data are shown. The saline-treated group of APJ+/− mice exhibited a substantial decline in FS compared with saline-treated groups of APJ+/+ and APJ−/− mice. Dox-treated groups of APJ+/+ and APJ+/− mice showed a severe deterioration in FS compared with APJ+/− mice. Values are expressed as means ± SE for 8–12 mice/group in each genotype. **P < 0.001 vs. the corresponding saline-treated group; ###P < 0.001 vs. the saline-treated group of APJ+/− mice; †P < 0.05 and ‡P < 0.01 vs. the Dox-treated group of APJ+/− mice. Effects of APJ deletion on survival of Dox-treated mice. To investigate the effect of APJ gene deletion on the prognosis after Dox treatment, we analyzed survival rates of APJ+/+, APJ+/−, and APJ−/− mice. Fourteen-day survival rates did not differ between APJ+/+ and APJ+/− mice but were markedly decreased in APJ−/− mice (Fig. 3E and Table 3). In most dead mice, there were visible signs related to heart failure at necropsy, such as pleural effusion and ascite retention. These findings led us to consider that Dox-administered mice died mainly from acute heart failure and that APJ−/− mice were susceptible to Dox-induced heart failure. Therefore, it is suggested that a defect of the apelin-APJ system can facilitate the development of Dox-induced heart failure in mice associated with its cardiotoxicity. Hence, we used APJ−/− mice to inves-

treated group of APJ+/− mice suffered from a marked decline in FS to almost the same level as the Dox-treated group of APJ−/− mice (average percent decrease: 51.95 ± 2.65%, mean ± SE; Fig. 3, C and D). It was therefore hypothesized that FS in the Dox-treated groups of APJ+/− and APJ+/− mice had bottomed out. All these changes in FS were caused by increases in LV internal diameter at systole, not diastole (Table 2), indicating systolic dysfunction. Heart rate, body weight, and heart weight were reduced to similar levels among all Dox-treated groups, but heart weight-to-body weight ratios were not affected in either saline- or Dox-treated groups (Table 2). These findings indicate that a deficiency in the apelin-APJ system can underlie Dox-induced cardiac contractile dysfunction in mice.

Table 2. Echocardiographic assessment of LV function and other parameters

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<tr>
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<th>Saline</th>
<th>Doxorubicin</th>
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<tr>
<td></td>
<td>APJ+/+</td>
<td>APJ−/−</td>
</tr>
<tr>
<td>LV internal diameter at diastole, mm</td>
<td>3.558 ± 0.2699</td>
<td>3.629 ± 0.1951</td>
</tr>
<tr>
<td>LV internal diameter at systole, mm</td>
<td>1.908 ± 0.1888</td>
<td>1.917 ± 0.2151</td>
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<tr>
<td>Fractional shortening, %</td>
<td>46.7 ± 3.5</td>
<td>47.3 ± 3.9</td>
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<tr>
<td>Heart rate, beats/min</td>
<td>452.1 ± 63.55</td>
<td>436.6 ± 61.14</td>
</tr>
<tr>
<td>Heart weight, mg</td>
<td>115.0 ± 6.481</td>
<td>115.5 ± 6.704</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>24.48 ± 1.092</td>
<td>24.71 ± 1.066</td>
</tr>
<tr>
<td>Heart weight/body weight, mg/g</td>
<td>4.698 ± 0.2343</td>
<td>4.683 ± 0.3502</td>
</tr>
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</table>

Values are expressed as means ± SE for 8–12 mice/group in each genotype. LV, left ventricular. *P < 0.05 and **P < 0.001 vs. the corresponding saline-treated group; †P < 0.05 and ‡P < 0.01 vs. the saline-treated group of APJ+/− mice; ††P < 0.05 and ‡‡P < 0.01 vs. the doxorubicin-treated group of APJ+/− mice.
tigate the effects of loss of the apelin-APJ system on Dox-induced cardiotoxicity.

Effects of APJ deletion on Dox-induced cardiac damage in mice. We observed histological sections stained with hematoxylin and eosin from hearts of APJ knockout mice on day 5 after Dox administration. Saline-treated groups of APJ+/+ and APJ−/− mice showed normal cardiac morphology and myofibril architecture, but Dox-treated groups of APJ+/+ and APJ−/− mice underwent atrophy with local deterioration of myofibrils (Fig. 4, A and B). To assess Dox-induced cardiac damage quantitatively, we measured plasma CK-MB activity, the most specific biomarker for myocardial necrosis. CK-MB activity in plasma was significantly increased in the Dox-treated group of APJ+/+ mice but were significantly elevated in the Dox-treated group of APJ−/− mice (Fig. 4, D and E). These results suggest that a compromised apelin-APJ system can aggravate Dox-induced cardiac damage in mice.

Effects of APJ gene deletion on autophagy in mouse hearts. Recent reports (18, 21, 26) have suggested that Dox triggers cardiotoxicity is well characterized by oxidative damage, including lipid peroxidation and protein carbonylation (6). We therefore examined carbonyl formation in heart proteins of APJ+/+ and APJ−/− mice on day 5 after Dox administration. Protein carbonylation levels were similar among saline- and Dox-treated groups of APJ+/+ mice but were significantly elevated in the Dox-treated group of APJ−/− mice (Fig. 4, D and E). These results suggest that a compromised apelin-APJ system can aggravate Dox-induced cardiac damage in mice.

Table 3. Survival rate analysis and necropsy findings of doxorubicin-treated mice

<table>
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<th>Survival (Alive/Total)</th>
<th>Pleural Effusions</th>
<th>Ascites</th>
</tr>
</thead>
<tbody>
<tr>
<td>APJ+/+</td>
<td>26/41</td>
<td>12/15</td>
</tr>
<tr>
<td>APJ−/−</td>
<td>29/41</td>
<td>11/12</td>
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<td>APJ−</td>
<td>3/12</td>
<td>8/9</td>
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Fig. 4. Aggravation of Dox-induced cardiac damages in APJ−/− mouse hearts. Tissue damage of hearts from APJ KO mice on day 5 after Dox injection (15 mg/kg) was analyzed. A and B: representative photomicrographs of hematoxylin and eosin-stained transverse heart sections. A: saline-treated groups of APJ+/+ and APJ−/− mice showed normal cardiac morphology, but Dox-treated groups of APJ+/+ and APJ−/− mice appeared have cardiac atrophy. Scale bar = 2 mm. B: at the LV myocardial area, Dox-treated groups of APJ+/+ and APJ−/− mice showed myofibrillar loss (arrowheads). Scale bar = 50 μm. Sections were analyzed for 3–4 mice/group. C: creatine kinase (CK)-MB activity in plasma of APJ KO mice was measured. APJ gene deletion promoted the Dox-induced elevation of plasma CK-MB activity. Values are expressed as means ± SE for 3 mice/group in all cases. \(* * * P < 0.001\) vs. saline-treated groups of APJ+/+ mice. \(* P < 0.05\) vs. Dox-treated group of APJ+/+ mice.

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tophy substrate, was also significantly increased in the Dox-treated group of APJ−/− mice. Protein expression levels of beclin-1, required for autophagosome formation, were not changed in any group. To determine whether p62 protein and ubiquitinated proteins had accumulated in cardiomyocytes, we carried out immunofluorescent staining. We found that p62-positive and ubiquitin-positive aggresome-like structures appeared in cardiomyocytes, in much higher numbers in the Dox-treated group of APJ−/− mice compared with the Dox-treated group of APJ+/+ mice, whereas saline-treated groups showed no such structures (Fig. 5B). In Dox-treated groups, mRNA levels of LC3B and p62 were significantly increased to a similar extent compared with the saline-treated group of APJ−/− mice, suggesting that Dox administration showed the same upregulatory effect on expression levels of LC3B and p62 in these mice (Fig. 5C). Levels of beclin-1 mRNA did not change significantly in any group. As reported in a previous study (18), Dox-induced myocardial accumulation of LC3 and p62 proteins indicates a possible impairment of autophagic degradation. Thus, our results indicate that Dox-induced cardiotoxicity can be accompanied by the activation of myocardial autophagy in the presence or absence of the APJ gene, but that...
APJ gene deletion can accelerate dysfunctional changes in the autophagic degradation pathway after Dox treatment. Furthermore, we performed a TUNEL assay on heart sections. On day 5 after Dox administration, TUNEL-positive cells were increased in mouse hearts of each genotype, but no significant difference was found among Dox-treated groups (Fig. 5D), indicating that APJ gene deletion did not affect Dox-induced cardiac apoptotic cell death.

Effects of APJ overexpression and apelin treatment on Dox-induced cell death in vitro. We next evaluated a potential role of the apelin-APJ system in cardioprotection from Dox-induced cardiotoxicity in vitro. We verified the abundant APJ mRNA expression in neonatal mouse hearts, but it was suppressed in vitro primary cultured neonatal mouse cardiac myocytes when prepared (data not shown). Therefore, we used immortalized cardiac myoblasts from embryonic rat H9c2 cells as a commonly used model for the study on Dox toxicity. We also detected little expression of APJ in H9c2 cells and thus established the transformants stably expressing human APJ. The cell viability of control H9c2/mock cells with 1 μM Dox for 24 h was remarkably reduced in the presence or absence of pE-apelin-13. In contrast, the Dox-induced decrease in cell viability of H9c2/hAPJ cells was attenuated with pE-apelin-13 in a dose-dependent manner (Fig. 6). Interestingly, overexpression of APJ showed a tolerance to Dox-induced cardiotoxicity in the absence of pE-apelin-13. Taken together, these results indicate that the apelin-APJ system has preventive effect against Dox-induced cell death.

**DISCUSSION**

In the present study, we demonstrated that Dox administration decreased the expression of apelin and APJ mRNA in mouse hearts followed by a significant reduction of APJ at the protein level. Genetic deletion of APJ in mice exacerbated Dox-induced cardiotoxicity, including LV contractile dysfunction, myocardial necrosis, oxidative damage, and impaired autophagy. Furthermore, genetic deletion of APJ in mice increased the mortality from heart failure after Dox administration.
mice led to marked enhancement of Dox-induced carbonyl formation in cardiac proteins, cardiac necrosis, cardiac dysfunction, and mortality. Taken together, these observations suggest a possible protective role of the endogenous apelin-APJ system in cardiac oxidative stress and the onset or progression of heart failure secondary to Dox-induced cardiotoxicity in mice.

Cardiac autophagy serves an essential role in not only maintenance of cardiac homeostasis but also in the clearance of damaged organelles and aggregated proteins under pathological conditions such as cardiac ischemia-reperfusion injury and heart hypertrophy (22, 35). Recently, several animal studies have reported the possible involvement of autophagy in Dox-induced cardiotoxicity. It has been suggested that Dox can promote cardiac autophagy to protect the heart from Dox-related toxicity (26). However, another study (21) has suggested that Dox-induced excessive autophagy causes cardiomyocyte cell death. In addition, it has also been reported that Dox impairs cardiac autophagosome formation and its degradation, resulting in cardiac dysfunction (18). Although it has not yet been completely elucidated whether autophagy has beneficial or causal effects on Dox-induced cardiotoxicity, our study suggests that downregulation of the apelin-APJ system by Dox treatment may progress Dox-induced cardiotoxicity through a functional disturbance of the autophagic degradation pathway. More recently, it has been reported that apelin treatment can attenuate glucose deprivation-induced excessive autophagy in rat cardiomyocytes (16). Excess ROS activate autophagy by producing damages to mitochondria and can cause necrosis due to ATP depletion (22). Therefore, the endogenous apelin-APJ system may play a suppressive role in cardiac oxidative stress, and Dox-induced downregulation of the apelin-APJ system may consequently accelerate cardiac autophagic cell death and necrosis.

Since there is no effective means of preventing or treating Dox-induced cardiotoxicity, we figured out a part of the mechanisms underlying this toxicity, which involves the breakdown of the apelin-APJ system. In the present study, despite the reduced apelin mRNA levels, apelin contents in the heart were increased to basal levels on day 5 after Dox injection. The discrepancy between apelin expression and its peptide contents may be attributed to the suppression of the number of APJ molecules available for apelin binding, resulting in an apparent recovery in apelin contents. At this moment, neither clear preventive effects nor improvement have been observed in our experimental model involving systemic coadministration of apelin-13 (data not shown), which can be explained by concomitant downregulation of APJ expression. Correspondingly, we observed notable adverse effects in APJ knockout mice, suggesting that the degree of APJ loss can impact the pathophysiological changes in mouse hearts after Dox administration.

An important issue raised by our findings is why APJ<sup>−/−</sup> mice showed higher Dox-induced mortality rates regardless of similar levels of cardiac dysfunction compared with APJ<sup>+/+</sup> mice. In this regard, we found that mRNA levels of apelin and APJ in APJ<sup>−/−</sup> mouse hearts were restored to basal levels on day 14 after Dox injection (Fig. 3, A and B), suggesting a functional recovery of the apelin-APJ system. Accordingly, we consider that the apelin-APJ system has the potential to delay the progression of heart failure arising from Dox treatment when cardiac APJ is expressed above certain thresholds. Furthermore, our data using cultured cell lines indicated that apelin did mitigate Dox-induced cytotoxicity.

Because of the broad tissue distribution of Dox, it can affect a variety of tissues, and its toxicity is not confined to the heart (1, 34), which may be responsible in part for the increased mortality of Dox-treated APJ<sup>−/−</sup> mice. In addition to susceptibility to Dox-induced cardiotoxicity, the apelin-APJ system may have some further aspects of sensitivity against Dox-induced systemic toxicity, including nephrotoxicity and hepatotoxicity. At this time, it still remains to be unsettled whether the basal FS reduction in APJ<sup>−/−</sup> mice can affect on Dox-induced cardiotoxicity. On this point, the use of APJ<sup>+/−</sup> and/or APJ-overexpressing mice treated with apelin-13 will have a great advantage for further study, which is required to elucidate the pathophysiological relevance of the apelin-APJ system to Dox-induced cardiotoxicity.

Based on our observations in vivo and in vitro, we concluded that Dox-induced cardiotoxicity is associated with a risk of impairment of the apelin-APJ system in the heart, which is responsible, at least in part, for the aggravation of Dox-induced cardiotoxicity. Although the acute model used in this study differed from the actual clinical case in Dox chemotherapy, our work generates important findings in the field of Dox-induced cardiotoxicity. Modulating of apelin-APJ signaling and the expression of apelin and APJ without a simultaneous reduction in Dox’s antitumor efficacy may improve the outcomes of cancer patients receiving Dox chemotherapy.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


