Early Administration of Carvedilol Protected against Doxorubicin-Induced Cardiomyopathy

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ABSTRACT

This study tested for the benefits of early administration of carvedilol as protection against doxorubicin (DOX)-induced cardiomyopathy. Thirty male, adult B6 mice were categorized into group 1 (untreated control), group 2 [DOX treatment (15 mg/kg/every other day for 2 weeks, i.p.),] and group 3 [carvedilol (15 mg/kg/d, from day 7 after DOX treatment for 28 days)], and euthanized by day 35 after DOX treatment. By day 35, the left ventricular ejection fraction (LVEF) was significantly lower in group 2 than in group 1 and 3, and significantly lower in group 3 than in group 1, whereas the left ventricular (LV) end-diastolic and LV end-systolic dimensions showed an opposite pattern to the LVEF among the three groups. Cardiac microscopic findings of fibrotic and collagen-deposition areas and the numbers of γ-H2AX+ and 53BP1+ cells in the LV myocardium exhibited an opposite pattern, whereas the numbers of endothelial cell (CD31+, vWF+) markers showed an identical pattern to the LVEF among the three groups. Cardiac stem cell markers (KIT+ and Sca-1+ cells) were significantly and progressively increased from group 1 to group 3. Additionally, the in vitro study showed carvedilol treatment significantly inhibited DOX-induced cardiomyoblast DNA (CD90/XRC1+, CD90/53BP1+, and h-rH2AX+ cells) damage. Early carvedilol therapy protected against DOX-induced DNA damage and cardiomyopathy.

Introduction

Hypertrophic cardiomyopathy can be caused by any disease that increases cardiac afterload and volume overload, some myocardiotoxic drugs, and certain primary genetic disorders of the myocardium (Senni et al., 1998; Roura and Bayes-Genis, 2009; Masuda et al., 2012; Maron et al., 2014; Modesto and Sengupta, 2014; Patel et al., 2015). Without appropriate treatment, hypertrophic cardiomyopathy commonly develops into dilated cardiomyopathy, and ultimately decompensated heart failure (Senni et al., 1998; Roura and Bayes-Genis, 2009; Masuda et al., 2012; Patel et al., 2015).

Doxorubicin (DOX) is used to treat a variety of human neoplasms; however, its usage is limited because of its cardiotoxicity (Blum and Carter, 1974; Von Hoff et al., 1979). Long-term treatment with DOX can cause cardiomyopathy and congestive heart failure (CHF) in a process that involves multiple factors, including the generation of free radicals that further damage cellular membranes (Rajagopalan et al., 1988; Keizer et al., 1990); disturbance of adrenergic function; alterations in intracellular Ca2+ homeostasis (Kim et al., 1989); myocardial cell apoptosis/death (Arola et al., 2000; Wu et al., 2000); and selective inhibition of the expression of cardiac muscle-specific proteins (Jeyaseelan et al., 1997). DOX induced cardiac myocyte apoptosis/death through upregulated caspase 3 and downregulated kinase activities of PI 3-kinase and Akt (Negoro et al., 2001).

ABBREVIATIONS: BNP, brain natriuretic peptide; CHF, congestive heart failure; DOX, doxorubicin; ECL, enhanced chemiluminescence; HPF, high-power field; IF, immunofluorescent; LV, left ventricular; LVEF, left ventricular ejection fraction.
Carvedilol, a cardioselective beta blocker/alpha-1 blocker, is widely used to treat hypertension and CHF (Yue et al., 1994; Packer et al., 2002). It reduces morbidity and mortality in CHF (Packer et al., 2002), has antioxidant effects, inhibits lipid peroxidation, and reduces mitochondrial toxicity (Tadolini and Franconi, 1998; Arozal et al., 2010, 2011; Pereira et al., 2011). Therefore, we used an animal model to test the hypothesis that carvedilol would protect against myocardial damage caused by DOX and improve heart function through inhibiting DNA damage, fibrosis, and apoptosis of the left ventricular (LV) myocardium in mice.

Materials and Methods

Echocardiography. Eight-week-old male C57BL/6 mice were purchased from Charles River Technology, BioLASCO Taiwan Co., Ltd., Taiwan, and housed in our hospital in an animal facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care International (Frederick, MD) with controlled temperature (24°C), humidity (50%–70%), and light cycle (12/12) for 2 weeks before administration of DOX. Additionally, animals were given ad libitum access to food (FWUSOW Industry Co., Ltd., Taipei, Taiwan) and autoclaved filtered reverse osmosis water (ELGA MEDICA Pro Water System, West Midlands, United Kingdom). All experimental procedures were approved by the Institute of Animal Care and Use Committee of Kaohsiung Chang Gung Memorial Hospital and performed in accordance with the Guide for the Care and Use of Laboratory Animals. The current investigations were restricted to male mice only in order to avoid the possibility that gender-specific effect differences might increase experimental variation and confound statistical analyses.

Pilot Study of DOX-Induced Mouse Cardiomyopathy. The dose dependency of DOX-induced myocardial damage (i.e., cardiomyopathy) was determined in mice. Male, adult C57BL/6 (B6) mice (n = 24), weighing 25–30 mg (Charles River Technology, BioLASCO Taiwan Co., Ltd.), were grouped to receive stepwise increases of DOX doses i.p. [0 mg (group A), 5 mg (group B), 10 mg (group C), and 15 mg (group D) mg] every other day for 2 weeks, for a total of 7 doses. The regimen and total accumulated dosage of DOX used in the present study was based on previous reports (Olson et al., 2003; Miyagawa et al., 2010) with some modification. Additionally, to minimize animal handling and possible discomfort associated with i.p. injections, DOX was administered every other day for 2 weeks.

The mice were anesthetized with an inhaled anesthesia mixture of isoflurane and oxygen 0.8–1 l/min and placed on a temperature-regulated table (25°C) to maintain body temperature. Isoflurane, other day for 2 weeks.

Animal Grouping and Induction of Cardiomyopathy by DOX (15 mg/Every Other Day for 2 Weeks) and Rationale for the Carvedilol Dose in the Current Study. To elucidate the impact of carvedilol therapy on preserving the LVEF, four additional animals were used and categorized into receiving a higher dose (15 mg/kg/day) and a lower dose (5 mg/kg/day) of carvedilol, respectively. The results of the pilot study showed that the LVEF was better preserved in animals (n = 2) that received 15 mg/kg/day of carvedilol than in animals (n = 2) that received 5 mg/kg/day of carvedilol orally, i.e., by lavage (53.0% versus 49.0%; carvedilol was commenced from day 7 after DOX administration and administered for 4 weeks). Additionally, a previous study has shown that the dosage of carvedilol up to 30 mg/kg daily for rodents was still safe without any side effects (Matsu et al., 1999). Thus, 15 mg/kg/day dose of carvedilol was used in the present study.

In the present study, prior to DOX administration, the mice (n = 30) were equally randomized into group 1 (untreated control, n = 10), group 2 (DOX only, i.p.; n = 10), and group 3 (DOX + cardeilol (15 mg/kg/d by lavage), commenced from day 7 after DOX administration for 28 days, n = 10). The present study did not provide a carvedilol-treated control group and was based on the spirit of the Guide for the Care and Use of Laboratory Animals, i.e., replace, reduce, and refine, and the previous study, which had demonstrated that dosage of carvedilol up to 30 mg/kg/day for rodents was safe (Matsu et al., 1999).

In vitro Study for Identifying the Impact of Carvedilol on Protecting the Cardioblasts from DOX-Induced Toxicity. For the in vitro study, H9C2 cells (cardiomyoblast) were purchased from the American Type Culture Collection, Manassas, VA. The cardiomyoblast characteristics of H9C2 cells were verified by immunofluorescent (IF) staining to detect the specific expressions of cardiac troponin T and cardiac sarcomeric α-actinin. Additionally, H9C2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a T-75 culture flask at 37°C with 5% CO₂. For passage, cells at 70% confluence were enzymatically dissociated with 0.25% trypsin/EDTA and subcultured to new flasks with fresh medium.

To determine the impact of carvedilol against the DOX-induced DNA damage, H9C2 cells were first cultured in Dulbecco’s modified Eagle’s medium culture, and then cocultured with 1) stepwise increases of DOX (0, 20, 100, and 500 nM) for 24 hours; 2) stepwise increases of carvedilol (0, 2, 5, and 10 μM) for 24 hours; or 3) DOX (100 nM) and carvedilol (10 μM) for 24 and 48 hours, respectively. The cells were then collected for individual study.

Functional Assessment by Echocardiography. The procedure and protocol for transthoracic echocardiography was based on a previous report (Chua et al., 2014). Transthoracic echocardiography (Vero 2100, Visualsonics Toronto, Ontario, Canada) was performed in each group prior to and on day 35 after DOX treatment by an animal cardiologist blind to the experimental design. M-mode standard two-dimensional left parasternal/long axis echocardiographic examination was conducted. The LV internal dimensions (i.e., LV end-systolic diameter (LVEDd) and LV end-diastolic diameter (LVEDd)) were measured at the mitral valve and papillary levels of the left ventricle, according to the American Society of Echocardiography (Morrisville, NC) leading-edge method using at least three consecutive cardiac cycles. The LVEF was calculated as follows: LVEF (%) = [(LVEDd0 – LVEDd1) / LVEDd0] x 100%

Specimen Collection. Mice in each group were euthanized by day 35 after DOX treatment, and the heart in each mouse was rapidly removed and then immersed in cold saline. The LV tissues were isolated and divided into three parts for 1) cryosections [embedded in compound (Tissue-Tek, Sakura, Netherlands); 2) paraffin sections (fixed with 10% formalin); and 3) protein examination (stored at −80°C refrigeration before using), respectively.

Western Blot Study. The procedure and protocol for western blot analysis were based on previous reports (Chen et al., 2014a,b). In brief, equal amounts (50 μg) of protein extracts were loaded and separated by SDS-PAGE using acrylamide gradients. After electrophoresis, the separated proteins were transferred electrophoretically to a
polyvinylidene difluoride membrane (Amersham Biosciences, Freiburg, Germany). Non-specific sites were blocked by incubation of the membrane overnight in blocking buffer (5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20). The membranes were incubated for 1 hour at room temperature with the indicated primary antibodies (Bax (1:1000, Abcam Cambridge, MA); cleaved poly (ADP-ribose) polymerase (PARP) (1:1000, Cell Signaling Danvers, MA); caspase 3 (1:1000, Cell Signaling Danvers, MA); Smad3 (1:1000, Cell Signaling); transforming growth factor (TGF)-β (1:500, Abcam Cambridge, MA); Smad1/5 (1:1000, Cell Signaling); bone morphogenetic protein (BMP)-2 (1:1000, Abcam); phosphorylation of histone H2AX (γ-H2AX) (1:1000, Cell Signaling); α-MHC (1:300, Santa Cruz); β-MHC (1:1000, Santa Cruz Santa Cruz, CA); brain natriuretic peptide (BNP) (1:800, Abcam); Akt (1:1000, Cell Signaling); Ku-70 (1:1000, Cell Signaling); and cytosolic (1:2000, BD San Jose, CA) and mitochondrial (1:2000, BD San Jose, CA) cytochrome C. Horseradish peroxidase-conjugated anti-rabbit IgG (1:2000, Cell Signaling) was used as a secondary antibody for 1-hour incubation at room temperature. The washing procedure was repeated eight times within 1 hour. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) (Amersham Biosciences) and exposed to Biomax L film (Kodak). For quantification, ECL signals were digitized using the Labwork software (UVP Upland, CA).

In our western blot study for the assessment of specific protein expressions, we loaded the lysate in the same SDS/polyacrylamide gel and then transferred it to the polyvinylidene difluoride membrane, followed by hybridization with different antibodies and with the use of β-tubulin as the control for each lane in order to assess the expressions of the proteins, which means the β-tubulin expression of each lane was used to compare the expressions of different proteins on the same lane.

Oxidative Stress Measurement of LV Myocardium. The procedure and protocol for assessing the protein expression of oxidative stress have been detailed in previous reports (Chen et al., 2014a,b). The Oxyblot Oxidized Protein Detection Kit was purchased from Chemicon (Billerica, MA). 2,4-dinitrophenylhydrazine (DNPH) derivatization was carried out in 6 μg protein for 15 minutes according to the manufacturer’s instructions. One-dimensional electrophoresis was carried out in 10% SDS/polyacrylamide gel after DNPH derivatization. Proteins were transferred to nitrocellulose membranes, which were then incubated in the primary antibody solution (anti-dinitrophenyl [DNP] 1:150) for 2 hours, followed by incubation in secondary antibody solution (1:300) for 1 hour at room temperature. The washing procedure was repeated eight times within 40 minutes. Immunoreactive bands were visualized by ECL (Amersham Biosciences) and exposed to Biomax L film (Kodak Rochester, NY). For quantification, ECL signals were digitized using the Labwork software (UVP, Upland, CA).

IF and Immunohistochemical Staining. IF staining was performed using the respective primary antibodies for examination of CD31 (1:100, Abcam), γ-H2AX (1:500, Abcam), Ku-70 (1:100, Abcam), CD90(1:100, abcam), XRCC1 (1:200, Abcam), and 53BP1 (1:100, Abcam; 1:300, Novus Littleton, CO) cells and actinin-phalloidin (1:500, Life Technologies Carlsbad, CA); β-MHC (1:500, Abcam Cambridge, MA); phosphorylation of histone H2AX (γ-H2AX) (1:1000, Cell Signaling); α-MHC (1:300, Santa Cruz, CA); brain natriuretic peptide (BNP) (1:800, Abcam); Akt (1:1000, Cell Signaling); Ku-70 (1:1000, Cell Signaling); and cytosolic (1:2000, BD San Jose, CA) and mitochondrial (1:2000, BD San Jose, CA) cytochrome C. Horseradish peroxidase-conjugated anti-rabbit IgG (1:2000, Cell Signaling) was used as a secondary antibody for 1-hour incubation at room temperature. The washing procedure was repeated eight times within 1 hour. Immunoreactive bands were visualized by ECL (Amersham Biosciences) and exposed to Biomax L film (Kodak). For quantification, ECL signals were digitized using the Labwork software (UVP, Upland, CA).

Histologic Quantification of Myocardial Fibrosis and Collagen Deposition. The procedure and protocol have been described in previous reports (Leu et al., 2011; Chua et al., 2014). Masson’s trichrome staining was used to identify fibrosis of the LV myocardium. Three serial sections of LV myocardium in each animal at the same levels were prepared at 4 μm thickness by cryostat (Leica, Singapore). The integrated areas (μm²) of fibrosis in each section were calculated using the ImageTool 3 software, version 3.0 (University of Texas, Health Science Center, San Antonio, TX). Three randomly selected high-power fields (HPFs) (100 × ) were analyzed in each section. After determining the number of pixels in each infarct and fibrotic area per HPF, the numbers of pixels obtained from three HPFs were summed. The procedure was repeated in two other sections for each animal. The mean pixel number per HPF for each animal was then determined by summing all pixel numbers and dividing by 9. The mean integrated area (μm²) of fibrosis in LV myocardium per HPF was obtained using a conversion factor of 19.24 (where 1 μm² represented 19.24 pixels).

To analyze the extent of collagen synthesis and deposition, cardiac paraffin sections (6 μm) were stained with Picrosirius Red (1% Sirius Red in saturated picric acid solution) for 1 hour at room temperature using standard methods. The sections were then washed twice with 0.5% acetic acid. The water was physically removed from the slides by vigorous shaking. After dehydration three times in 100% ethanol, the sections were cleaned with xylene and mounted in a resins medium. The HPFs (×100) of each section were used to identify the Sirius Red–positive area in each section. Analyses of collagen deposition area in LV myocardium were identical to the description of the calculations of the infarct and fibrotic areas.

Statistical Analysis. Quantitative data are expressed as means ± S.D. Statistical analysis was performed by analysis of variance followed by the Bonferroni multiple-comparison post hoc test. The SAS statistical software for Windows, version 8.2 (SAS institute, Cary, NC) was used. A probability value <0.05 was considered statistically significant.

Results

In Vitro Studies Identifying Protective Effect of Carvedilol on DNA Damage Induced by DOX (Figs. 1–4). To elucidate the impact of DOX therapy on DNA damage, H9C2 cells (cardioblast cell line) were cocultured with stepwise increases of DOX (0, 20, 100, and 500 nM) for 24 hours, followed by collection of cells for western blot. The results showed that the protein expression of γ-H2AX, a marker of DNA damage, progressively increased, whereas that of Ku-70 and phosphorylated Akt, two indicators of DNA repair, progressively decreased (Fig. 1).

To assess whether carvedilol treatment would activate DNA repair in H9C2 cells, the cells were cocultured with stepwise increases of carvedilol concentration (0, 2, 5, and 10 μM) for 24 hours. The protein expressions of Ku-70 and phosphorylated Akt were progressively enhanced as the dose of carvedilol was increased (Fig. 1).

To assess whether carvedilol could prevent DNA damage induced by DOX, H9C2 cells were cocultured with DOX (100 nM) and carvedilol (10 μM) for 24 and 48 hours, respectively. The protein expressions of phosphorylated Akt, Ku-70, and TNNI3K, three indicators of DNA repair, were preserved, whereas protein expression of γ-H2AX was suppressed by carvedilol (Fig. 2). Additionally, the IF microscopic findings demonstrated that the numbers of CD90/XRCC1⁺, CD90/53BP1⁺ (Fig. 3), and γ-H2AX⁺ cells (Fig. 4), three markers of DNA damage, were significantly higher, whereas the number of Ku-70⁺ cells (Fig. 4), an indicator of DNA repair, was significantly lower in the DOX-treated group compared with the control group and the group treated with DOX + carvedilol.

DOX Treatment of Dose-Dependent Myocardial Damage and Alternation of Heart Weight and LV Function in Living Animals (Fig. 5). By day 35 after DOX treatment, the protein expressions of Bax, cleaved caspase 3, and cleaved PARP, three indicators of apoptosis in LV myocardium,
progressively increased as the DOX dose was increased (Fig. 5, A–C). Additionally, the lower dose of DOX (i.e., 10 mg) notably induced cardiac hypertrophy, whereas the higher dose of DOX caused a significantly lower ratio of the heart weight to tibial length (Fig. 5D) and total heart weight (Fig. 5E), an indirect indicator of loss of myocardium and cell death. Furthermore, the LVEF notably and progressively decreased as the DOX dose was increased stepwise (Fig. 5F).

The results of the immunohistochemical staining showed that the fibrotic area (Fig. 5G) and collagen-deposition area in LV myocardium (Fig. 5H) progressively increased as the DOX dose was increased (please see the illustrations of the microscopic findings in the Supplemental Material). The results of the IF staining displayed that 53BP1 cells (Fig. 5I) and γ-H2AX cells (Fig. 5J), two markers of DNA damage, were identical to the expression of fibrosis in LV myocardium (please see the illustrations of microscopic findings in Supplemental Material).

Carvedilol Therapy Preserved LV Function and Inhibited LV Remodeling by Day 35 after DOX Treatment (15 mg/Every Other Day for 2 Weeks) (Fig. 6). By day 35 after DOX treatment, the LVEF was significantly lower in group 2 (DOX) than in group 1 (untreated control) and group 3 (DOX + carvedilol), and significantly lower in group 3 than in group 1. Conversely, the LV end-diastolic and end-systolic diameters showed a reversed pattern of LVEF among the three groups. These findings suggest that carvedilol treatment protected against DOX-induced myocardial damage.

Carvedilol Prevented Apoptosis, DNA, and Mitochondrial Damage Caused by DOX Treatment (15 mg/Every Other Day for 2 Weeks) in LV Myocardium (Fig. 7). By day 35 after 15 mg of DOX therapy, the protein expressions of Bax, cleaved caspase 3 and PARP, γ-H2AX, and cytosolic cytochrome C (i.e., an indicator of mitochondrial damage) were significantly higher in group 2 than in groups 1 and 3, and significantly higher in group 3 than in group 1. On the other hand, mitochondrial cytochrome C, an indicator of mitochondrial integrity, revealed an opposite pattern to apoptosis among the three groups.

Carvedilol Prevented Fibrosis and Myocardial Hypertrophy Caused by DOX Treatment (15 mg/Every Other Day for 2 Weeks) in LV Myocardium (Fig. 8). By day 35 after DOX treatment, the protein expressions of Smad3 and TGF-β were significantly higher in group 2 than in groups 1 and 3, and significantly higher in group 3 than in group 1. Conversely, the protein expressions of Smad1/5 and BMP-2, two antifibrotic biomarkers, exhibited an opposite pattern to fibrosis among the three groups. Additionally, the protein expressions of BNP and β-MHC, two indicators of pressure overload/heart failure, showed an identical pattern of fibrosis among the three groups. Conversely, protein expression of α-MHC, a reverse myocardial hypertrophic biomarker, showed an opposite pattern to β-MHC among the three groups.

**Fig. 1.** DOX-induced DNA damage and carvedilol therapy upregulated the capacity of DNA repair. (A) The protein expression of γ-H2AX was progressively enhanced, whereas the protein expressions of Ku-70 and phosphorylated (p)-Akt were progressively attenuated, after stepwise increased DOX dosage (i.e., 0, 20, 100, and 500 nM). (B) The protein expressions of Ku-70 and phosphorylated Akt (p-Akt) (i.e., two indicators of DNA repair) were progressively enhanced as the dose of carvedilol increased (i.e., 0, 2, 5, and 10 μM).

**Fig. 2.** In vitro study of the protective effect of carvedilol on DNA damage induced by DOX. (A) Demonstrates the dosage and timing of DOX and carvedilol to be used in coculture with H9C2 cells. (B) The protein expressions of Ku-70, phosphorylated Akt (p-Akt), and cardiac troponin I-interacting kinase (TNNI3K). Three indicators of DNA repair were notably and progressively downregulated at two time interval points (i.e., at 24 hours or extended to 48 hours) in DOX (100 nM) treatment, whereas these three parameters were stepwise upregulated in carvedilol (10 μM) treatment at two time interval points.
Carvedilol Prevented Microscopic Findings of Fibrosis and Collagen Deposition caused by DOX Treatment (15 mg/Every Other Day for 2 Weeks) in LV Myocardium (Fig. 9). By day 35 after DOX treatment, Mason’s trichrome staining showed that the fibrotic area was significantly higher in group 2 than in groups 1 and 3, and significantly higher in group 3 than in group 1. Additionally, the results of the Sirius Red staining exhibited that the collagen deposition area displayed an identical pattern of fibrosis among the three groups.

Fig. 3. IF stain for identifying DOX-induced DNA damage that was reversed by carvedilol therapy. (A–D) The IF microscopic finding (400×) illustrates the CD90/XRCC1+ cells. The red color indicates positively stained XRCC1, the green color indicates positively stained CD90, and the positively double stain (i.e., red and green colors) indicates CD90/XRCC1+ cells. (E) Analytical result of CD90/XRCC1+ cells (*) versus other groups with different symbols (*, †, ‡), P < 0.0001. (F–I) The IF microscopic finding (400×) illustrates the CD90/53BP1+ cells. The red color indicates positively stained 53BP1, the green color indicates positively stained CD90, and the positively double stain (i.e., red and green colors) indicates CD90/53BP1+ cells. (J) Analytical result of CD90/53BP1+ cells (*) versus other groups with different symbols (*, †, ‡), P < 0.0001. Scale bars in right, lower corner represent 20 μm. All statistical analyses were performed by one-way analysis of variance, followed by the Bonferroni multiple comparison post hoc test (n = 6 for each group). Symbols (*, †, ‡) indicate significance (at the 0.05 level). Dox = doxorubicin; CAR = carvedilol.
Cardvedilol Prevented the Expressions of \( \gamma \)-H2AX\(^+\) and 53BP1\(^+\) Cells Caused by DOX (15 mg/Every Other Day for 2 Weeks) Expressions and Upregulation of Ku-70\(^+\) Cell Expression in LV Myocardium (Fig. 10). By day 35 after DOX treatment, the numbers of \( \gamma \)-H2AX\(^+\) and 53BP1\(^+\) cells, two indices of DNA damage markers, were significantly increased in group 2 compared with groups 1 and 3, and significantly higher in group 3 than in group 1. On the other hand, the cellular expression of Ku-70, a DNA repair biomarker, exhibited an opposite pattern of DNA damage markers among the three groups.

**Cardvedilol Therapy Enhanced the Expressions of Cardiac Stem Cells and Endothelial Cells in LV Myocardium (Fig. 11).** By day 35 after DOX treatment
(15 mg/every other day for 2 weeks), the numbers of Sca-1⁺ and C-kit⁺ cells, two indicators of cardiac stem cells, were significantly higher in group 2 and significantly increased in group 3 compared with group 1. We suggest that the increased numbers of cardiac stem cells after DOX treatment could be an intrinsic response to cardiotoxicity and DNA damage. Additionally, carvedilol therapy had an extrinsic capacity to enhance the increase of cardiac stem cells for myocardial repair. Conversely, the number of CD31⁺ cells, an indicator of endothelial cells, was significantly lower in group 2 than in

Fig. 5. DOX treatment of dose-dependent augmentation of myocardial damage and alternation of heart weight and LV function in living animals. (A) Protein expression of Bax; * versus other groups with different symbols (*, †, ‡), P < 0.0001. (B) Protein expression of cleaved caspase 3 (c-Casp 3); * versus other groups with different symbols (*, †, ‡), P < 0.0001. (C) Protein expression of cleaved Poly (ADP-ribose) polymerase (c-PARP); * versus other groups with different symbols (*, †, ‡), P < 0.0001. (D) The ratio of heart weight to tibial length; * versus other groups with different symbols (*, †, ‡), P < 0.0001. (E) The analytical results of total heart weight; * versus other groups with different symbols (*, †, ‡), P < 0.0001. (F) The analytical results of the LVEF; * versus other groups with different symbols (*, †, ‡), P < 0.0001. (G) Analytic result of the fibrosis area; * versus other groups with different symbols (*, †, ‡), P < 0.0001. (H) Analytic result of the collagen deposition; * versus other groups with different symbols (*, †, ‡), P < 0.0001. (I) Analytic result of 53BP1⁺ cells (*) versus other groups with different symbols (*, †, ‡), P < 0.0001. (J) Analytic result of γ-H2AX⁺ cells (*) versus other groups with different symbols (*, †, ‡), P < 0.0001. The lysate was loaded in the same SDS/polyacrylamide gel and then transferred to the polyvinylidene difluoride membrane, followed by hybridization with different antibodies and with the use of β-tubulin as the control for each lane to assess the protein expressions of Bax, c-caspase 3, and c-PARP. All statistical analyses were performed by one-way analysis of variance, followed by the Bonferroni multiple comparison post hoc test (n = 6 for each group). Symbols (*, †, ‡) indicate significance (at the 0.05 level). Dox = doxorubicin.

Fig. 6. Carvedilol therapy against DOX-induced deterioration of heart function and enhancement of LV remodeling by day 35 after DOX treatment (15 mg/every other day for 2 weeks). (A) Analytical results of the LVEF; * versus other groups with different symbols (*, †, ‡), P < 0.0001. (B) Analytical results of the LV end-diastolic dimension (LVEDd); * versus other groups with different symbols (*, †, ‡), P < 0.0001. (C) Analytical results of the LV end-systolic dimension (LVESd); * versus other groups with different symbols (*, †, ‡), P < 0.0001. All statistical analyses were performed by one-way analysis of variance, followed by the Bonferroni multiple comparison post hoc test (n = 10 for each group). Symbols (*, †, ‡) indicate significance (at the 0.05 level). Dox = doxorubicin; CAR = carvedilol.
groups 1 and 3, and significantly lower in group 3 than in group 1.

Discussion

This study, which investigated the protective effect of carvedilol therapy on myocardium against DOX damage, yielded several striking implications. First, DOX caused significant LV dysfunction and increased LV remodeling. Second, DOX-induced LV dysfunction and remodeling were shown to be mainly through fibrosis, apoptosis, DNA damage, mitochondrial dysfunction, and oxidative stress. Third, DOX therapy was associated with cumulative and dose-dependent cardiomyopathy. Fourth, these molecular-cellular and functional perturbations caused by DOX were significantly reversed by carvedilol, suggesting that such therapy could be effective for patients with neoplasms and DOX-induced cardiotoxicity/caridiomyopathy.

Undoubtedly, accumulating doses of DOX therapy are associated with irreversible dilated cardiomyopathy. Once

Fig. 7. Protein expressions of apoptotic, NDA-damaged, and mitochondria-damaged markers in LV myocardium by day 35 after DOX treatment (15 mg/every other day for 2 weeks). (A) Protein expression of Bax; * versus other groups with different symbols (*, †, ‡), P < 0.0001. (B) Protein expression of cleaved caspase 3 (c-Casp 3); * versus other groups with different symbols (*, †, ‡), P < 0.0001. (C) Protein expression of cleaved polyADP-ribose polymerase (c-PARP); * versus other groups with different symbols (*, †, ‡), P < 0.0001. (D) Protein expression of γ-H2AX; * versus other groups with different symbols (*, †, ‡), P < 0.0001. (E) Protein expression of cytosolic cytochrome C (cyt-Cyt C); * versus other groups with different symbols (*, †, ‡), P < 0.0001. (F) Protein expression of mitochondrial cytochrome C (mit-Cyt C); * versus other groups with different symbols (*, †, ‡), P < 0.0001. The lysate was loaded in the same SDS/polyacrylamide gel and then transferred to the polyvinylidene difluoride membrane, followed by hybridization with different antibodies and with the use of β-tubulin as the control for each lane to assess the protein expressions of Bax, c-caspase 3, and c-PARP, and for each lane to assess the protein expressions of γ-H2AX and cyt-Cyt C, respectively. All statistical analyses were performed by one-way analysis of variance, followed by the Bonferroni multiple comparison post hoc test (n = 10 for each group). Symbols (*, †, ‡) indicate significance (at the 0.05 level). Dox = doxorubicin; CAR = carvedilol.

Fig. 8. Protein expressions of fibrotic, antifibrotic, and hypertrophic biomarkers in LV myocardium by day 35 after DOX treatment (15 mg/every other day for 2 weeks). (A) Protein expression of Smad3; * versus other groups with different symbols (*, †, ‡), P < 0.0001. (B) Protein expression of transforming growth factor (TGF)-β; * versus other groups with different symbols (*, †, ‡), P < 0.0001. (C) Protein expression of Smad1/5; * versus other groups with different symbols (*, †, ‡), P < 0.0001. (D) Protein expression of bone morphogenetic protein (BMP)-2; * versus other groups with different symbols (*, †, ‡), P < 0.0001. (E) Protein expression of BNP; * versus other groups with different symbols (*, †, ‡), P < 0.0001. (F) Protein expression of β-MHC; * versus other groups with different symbols (*, †, ‡), P < 0.0001. (G) Protein expression of α-MHC; * versus other groups with different symbols (*, †, ‡), P < 0.0001. The lysate was loaded in the same SDS/polyacrylamide gel and then transferred to the polyvinylidene difluoride membrane, followed by hybridization with different antibodies and with the use of β-tubulin as the control for each lane to assess the protein expressions of p-Smad3, Smad1/5, and BMP-2. All statistical analyses were performed by one-way analysis of variance, followed by the Bonferroni multiple comparison post hoc test (n = 10 for each group). Symbols (*, †, ‡) indicate significance (at the 0.05 level). Dox = doxorubicin; CAR = carvedilol.
Fig. 9. Fibrosis and collagen deposition in LV myocardium by day 35 after DOX treatment (15 mg/every other day for 2 weeks). (A–C) The microscopic findings (100×) of Masson’s Trichrome staining illustrate the fibrosis in LV myocardium among the three groups. (D) Analytic result of the fibrosis area; * versus other groups with different symbols (*, †, ‡), $P < 0.0001$. (E–G) The microscopic findings (100×) of Sirius Red staining illustrate the collagen deposition in LV myocardium among the three groups. (H) Analytic result of the collagen deposition; * versus other groups with different symbols (*, †, ‡, •), $P < 0.0001$. Scale bars in right, lower corner represent 100 μm. All statistical analyses were performed by one-way analysis of variance, followed by the Bonferroni multiple comparison post hoc test ($n = 10$ for each group). Symbols (*, †, ‡) indicate significance (at the 0.05 level). Dox = doxorubicin; CAR = carvedilol.
established, medical therapy is mostly ineffective. Therefore, prevention of cardiotoxicity has great clinical importance.

One important finding in the present study was that cardiotoxicity was strongly associated with stepwise increases in DOX dosage. Additionally, cardiotoxicity/cardiomyopathy occurred at an early stage of DOX therapy (by day 28). Intriguingly, one clinical observational study previously revealed that subclinical systolic dysfunction occurs in almost 50% of patients early after anthracycline (DOX is one kind of anthracycline) therapy (Lotrionte et al., 2007). Similarly, another observational study showed that changes in LV longitudinal peak systolic strain and LV remodeling were observed early in patients receiving anthracycline chemotherapy (Poterucha et al., 2012). Accordingly, the results of our experimental study (i.e., preclinical study) support the findings of those previous studies (Lotrionte et al., 2007; Poterucha et al., 2012) (i.e., clinical observational study) that cardiotoxicity quite often developed at an early stage of DOX therapy.

The benefit of carvedilol on preserving heart function has been extensively discussed by numerous clinical observational studies (Kalay et al., 2006; Elitok et al., 2014). The most important finding in the present study was that early administration of carvedilol significantly preserved LV function and inhibited LV remodeling. Our findings strengthen previous work (Kalay et al., 2006; Elitok et al., 2014) and highlight that early administration is essential for protecting against DOX-induced cardiomyopathy. This finding has important preclinical relevance that can be extrapolated to the clinical setting: early provision of carvedilol for chemotherapy patients may be crucial to prevent cardiotoxicity, which in turn preserves heart function and inhibits LV remodeling.

The cardiotoxicity/cardiomyopathy caused by DOX is due to multiple mechanisms, including generation of free oxygen radicals (Olson and Mushlin, 1990), apoptosis/cell death (Kalyanaraman et al., 2002), mitochondrial dysfunction (Olson and Mushlin, 1990; Wallace et al., 1997), activation of matrix metalloproteinase (Bai et al., 2004), DNA damage/abnormal protein processing (Shi et al., 2011), and decreased vasculogenesis (Shi et al., 2011). Another important finding in the present study was that the fibrotic and collagen deposition areas were remarkably higher in the DOX-treated group than in the untreated control group. Additionally, both in vitro and in vivo studies exhibited that the DNA damage markers, apoptotic and fibrotic biomarkers, as well as the oxidative stress and mitochondrial dysfunction markers (cytosolic cytochrome C increased and mitochondrial cytochrome C decreased) were substantially increased in DOX-treated animals compared with those of untreated control animals. Accordingly, our findings corroborate the findings of those previous studies (Olson and Mushlin, 1990; Kalyanaraman et al., 2002; Shi et al., 2011). However, all of these perturbation parameters were markedly reversed and those of the antifibrotic biomarkers were notably increased after carvedilol therapy. Therefore, our findings, in addition to supporting the findings of previous studies (Olson and Mushlin, 1990; analysis of variance, followed by the Bonferroni multiple comparison post hoc test (n = 10 for each group). Symbols (*, †, ‡) indicate significance (at the 0.05 level). Dox = doxorubicin; CAR = carvedilol.

Fig. 10. The expressions of DNA-damaged and DNA-repaired cells in LV myocardium by day 35 after DOX treatment (15 mg/every other day for 2 weeks). (A–C) The IF microscopic findings (400×) illustrate the expression of γ-H2AX⁺ cells in LV myocardium (pink color). (D) Analytical result of γ-H2AX⁺ cells (*) versus other groups with different symbols (*, †, ‡), P < 0.0001. Scale bars in right, lower corner represent 20 μm. (E–G) The IF microscopic findings (200×) illustrate the expression of 53BP1⁺ cells in LV myocardium (red color). (H) Analytical result of 53BP1⁺ cells * versus other groups with different symbols (*, †, ‡), P < 0.0001. Scale bars in right, lower corner represent 50 μm. (I–K) The IF microscopic findings (400×) illustrate the expression of Ku-70⁺ cells in LV myocardium (green color). (L) Analytical result of Ku-70⁺ cells (*) versus other groups with different symbols (*, †, ‡), P < 0.0001. Scale bars in right, lower corner represent 20 μm. All statistical analyses were performed by one-way
Kalyanaraman et al., 2002; Shi et al., 2011), could lend partial explanation to why LV function was preserved and LV remodeling was inhibited in DOX-treated animals after re-receiving carvedilol therapy. In this way, the results of our studies encourage the early use of carvedilol for patients with the requirement of DOX treatment.

Interestingly, in the present study we found that DOX-induced cardiotoxicity enhanced the generation of cardiac stem cells in myocardium. We suggest that such progenitor cell renewal could be an intrinsic response to myocardial damage for regeneration of myocardium. Of importance was that carvedilol therapy further enhanced this phenomenon of progenitor cell renewal. This finding could, at least in part, explain why carvedilol therapy preserved heart function and abrogated LV remodeling in DOX-treated animals.

An essential finding in the present study was that the number of CD31 cells was significantly reduced in DOX-treated animals than in those of the untreated control group, indicating that DOX therapy not only induced cardiotoxicity but also destroyed endothelial cells/endothelial function and angiogenesis. Importantly, this endothelial cell was significantly reversed (i.e., from 0.5% to 2.5% with an increment of 5.0 times) after carvedilol treatment. This increment in CD31+ cells may indicate that the microcirculation and blood supply in myocardium was preserved after carvedilol treatment; therefore, protecting cardiomyocytes against ischemia and death. In this way, our findings could also once again explain why carvedilol treatment preserved LV function and ameliorated LV remodeling.

A principal finding in the present study was that the protein expressions of BNP and β-MHC were significantly enhanced, whereas the protein expression of α-MHC was notably reduced in the DOX group compared with the untreated controls. In fact, numerous studies have shown a correlation between an increase in circulating levels of BNP and CHF/pressure overload and prognostic outcome in patients after ischemic myocardial infarction (Wu et al., 2006). Additionally, cardiac hypertrophy is characterized by a switch from α- to

Fig. 11. Expressions of cardiac stem cells and endothelial cells in LV myocardium by day 35 after DOX treatment (15 mg/every other day for 2 weeks). (A–C) The microscopic findings (400×) of immunohistochemical (IHC) staining identify the expression of Sca-1+ cells in LV myocardium (red arrows). (D) Analytical result of Sca-1+ cells (*) versus other groups with different symbols (*, †, ‡), P < 0.001. Scale bars in right, lower corner represent 20 μm. (E–G) The microscopic findings (400×) of IHC staining identify the expression of Sca-1+ cells in LV myocardium (red arrows). (H) Analytical result of Sca-1+ cells (*) versus other groups with different symbols (*, †, ‡), P < 0.001. Scale bars in right, lower corner represent 20 μm. (I–K) The IF microscopic findings (200×) illustrate the expression of CD31+ cells in LV myocardium (yellow arrows). Scale bars in right, lower corner represent 50 μm. All statistical analyses were performed by one-way analysis of variance, followed by the Bonferroni multiple comparison post hoc test (n = 10 for each group). Symbols (*, †, ‡) indicate significance (at the 0.05 level). Dox = doxorubicin; CAR = carvedilol.
β-MHC mRNA expression (i.e., reactivation of fetal gene program) (Sun et al., 2014). Intriguingly, these biomarkers were remarkably reversed after carvedilol therapy. In this way, our findings, in addition to being consistent with that of previous studies (Wu et al., 2006; Sun et al., 2014), once more explain why carvedilol therapy preserves LV function and ameliorates LV remodeling.

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