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p21^{WAF1/Cip1/Sdi1} Knockout Mice Respond to Doxorubicin with Reduced Cardiotoxicity

Jerome Terrand^{1,2}, Beibei Xu¹, Steve Morrissy^{1,3}, Thai Nho Dinh¹, Stuart Williams^{4,5}, and Qin M. Chen^{1,6}

¹Department of Pharmacology, College of Medicine University of Arizona 1501 N. Campbell Ave Tucson, AZ 85724

⁴Biomedical Engineering Program, College of Medicine University of Arizona 1501 N. Campbell Ave Tucson, AZ 85724

Abstract

Doxorubicin (Dox) is an antineoplastic agent that can cause cardiomyopathy in humans and experimental animals. As an inducer of reactive oxygen species and a DNA damaging agent, Dox causes elevated expression of p21^{WAF1/Cip1/Sdi1} (p21) gene. Elevated levels of p21 mRNA and p21 protein have been detected in the myocardium of mice following Dox treatment. With chronic treatment of Dox, wild type (WT) animals develop cardiomyopathy evidenced by elongated nuclei, mitochondrial swelling, myofibrillar disarray, reduced cardiac output, reduced ejection fraction, reduced left ventricular contractility, and elevated expression of ANF gene. In contrast, p21 knockout (p21KO) mice did not show significant changes in the same parameters in response to Dox treatment. In an effort to understand the mechanism of the resistance against Dox induced cardiomyopathy, we measured levels of antioxidant enzymes and found that p21KO mice did not contain elevated basal or inducible levels of glutathione peroxidase and catalase. Measurements of 6 circulating cytokines indicated elevation of IL-6, IL-12, IFN γ and TNF α in Dox treated WT mice but not p21KO mice. Dox induced elevation of IL-6 mRNA was detected in the myocardium of WT mice but not p21KO mice. While the mechanism of the resistance against Dox induced cardiomyopathy remains unclear, lack of inflammatory response may contribute to the observed cardiac protection in p21KO mice.

Keywords

Cell Cycle Inhibitor; antioxidant enzymes; inflammatory response; cardiomyopathy

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⁶To whom correspondence should be addressed. qchen@email.arizona.edu, phone (520)626-9126, fax (520)626-2204..

²Current Address: Université de Strasbourg, Laboratoire de Pharmacologie et Biophotonique, BP24 74, route du Rhin Illkirch, cedex 67401, FRANCE

³Current Address: Cleveland Clinic Foundation R2, Cleveland, OH

⁵Current Address: University of Louisville Department of Surgery, 301 E Muhammad Ali Blvd, Louisville, KY40202

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Introduction

Doxorubicin (Dox) is an anthracycline quinone frequently used during chemotherapy for hematopoietic cancer and a wide range of solid tumors, including breast carcinoma, small-cell lung carcinoma and metastatic thyroid carcinoma. At high doses, Dox causes acute cardiac toxicity manifested by fatal arrhythmia. To reduce cardiac toxicity, formula and administration protocols have been improved and patients are closely monitored in clinics during Dox administration. However, chronic cardiotoxicity develops in certain individuals months or years after initial Dox administration. Such cardiotoxicity is manifested by ventricular dysfunction and dilated cardiomyopathy (Friedman *et al.*, 1978; Lipshultz *et al.*, 1991). Morphological and ultrastructural analyses of Dox induced cardiomyopathy reveal loss of myofibrils, dilation of sarcoplasmic reticulum, cytoplasmic vacuolization, mitochondrial swelling and increased number of lysosomes in cardiomyocytes (Lefrak *et al.*, 1973; Bristow *et al.*, 1978). Dox also induces inflammatory reactions in the heart, leading to thrombosis in the atria and myocarditis (Bristow *et al.*, 1978; Fujihira *et al.*, 1993; Gaudin *et al.*, 1993). Increasing evidence suggests that inflammation is a key element in the progression of heart failure regardless of the type of initial insult (Suleiman *et al.*, 2006; Heymans *et al.*, 2009).

Cardiac specific toxicity of Dox is associated with the fact that cardiomyocytes have the highest content of mitochondria among all cell types. About 40% cell volume is occupied by mitochondria in cardiomyocytes (Barth *et al.*, 1992). When accepting electrons from oxoreductive enzymes in the mitochondria, Dox forms semiquinone free radicals, which can initiate a chain of redox reactions (Myers *et al.*, 1977). With mitochondria isolated from cardiac tissue, incubation with Dox *in vitro* leads to generation of superoxide anion and H₂O₂ (Doroshov and Davies, 1986). Administration of pharmacological agents with antioxidant properties or overexpressing antioxidant proteins or enzymes have been shown to reduce cardiac toxicity of Dox (Kotamraju *et al.*, 2000; Ludke *et al.*, 2009; Kang *et al.*, 1996; Sun *et al.*, 2001). In addition to producing reactive oxygen species (ROS), Dox can intercalate DNA, inhibit topoisomerase II and block cell proliferation.

Like most DNA damaging agents or ROS inducers, Dox elicits stress response at the cellular level, inducing the expression of p21^{WAF1/Cip1/Sdi1} (p21) gene. Although cardiomyocytes are mostly non-proliferative cells, elevated levels of p21 have been observed under several disease conditions, including myocardial infarction (Kuhn *et al.*, 2007). Whereas p21 suppresses cell proliferation due to its function as a cyclin-dependent kinase inhibitor, little is known about the role of p21 in nonproliferative cells such as cardiomyocytes. Expecting Dox to cause an elevation of p21 in the myocardium, we examined whether p21 is involved in Dox induced cardiomyopathy.

Materials and Methods

Animals

All animal studies were reviewed and approved by the University of Arizona Institutional Animal Care and Use Committee. The breeding pairs of wild type (B6; 129 SF1) or p21KO mice (B6; 129 S2 Cdkn1a^{tm1Tyj}) were purchased from the Jackson laboratory. The p21KO

mice were back crossed with wild type animals to generate offspring of wild type or homozygous, which were bred in parallel so the same generation was used for experimental comparisons. The mice used for experiments were 2 to 5 generations from the heterozygous parents.

Dox Treatment

Wild type (WT) or p21KO animals (male, 5-7 weeks old, 18-22 g) were treated with Dox, (Sigma, St Louis, MO) according to Sun *et al.* (Sun *et al.*, 2001). Animals were dosed with 4 mg/kg Dox (i.p. 10 ml/kg in saline, controls received the same volume of saline without Dox) twice a week for a total of 10 injections. p21KO mice were slightly smaller than WT and were selected for bigger sizes within the same litter for treatment of an equal concentration of Dox, i.e. 85 µg per injection, as WT animals. The animals were not treated for 2 weeks between the first 4 injections and last 6 injections to allow recovery from bone marrow depression. Various measurements were taken 2 weeks after the final injection. After collecting the blood from the abdominal vena cava, serum was prepared by centrifugation at 2000×g for 5 min at 4°C. Upon excision, the hearts were immediately frozen in liquid nitrogen and were ground in liquid nitrogen bath using a mortar and pestle. The resulting tissue powders were divided into 3 parts for dissolving in 1) TRIzol for RNA isolation; 2) Phosphate buffered saline (PBS, pH 7.4) for enzymatic activity assays; and 3) Lysis buffer (Tris 0.05M, pH 6.8, Sodium Dodecyl Sulfate 2%, Glycerol 40%) for Western blots.

Hemodynamic Measurements

Two weeks after final Dox injection, mice were anesthetized with urethane (1 g/kg, i.p.) and α-chloralose (50 mg/kg, i.p, Calbiochem, CA.) and placed in a supine position on a temperature-controlled surgical mat for a closed chest protocol (Lips et al, 2004). Animals were ventilated via a cannula connected to a pressure-control respirator (Harvard Apparatus, Holliston, MA) after tracheotomy. Upon exposure and isolation from the internal jugular vein and the vagus nerve, the right common carotid artery was clamped with a microvessel clip (FST, Foster city, California) to the caudal end and sutured to the cranial end. The artery was incised with a 27 Gauge needle and the incision was enlarged with microscissors for insertion of a Millar 1.4 Fr catheter (SPR-719, ADInstruments, Colorado Springs, CO). The catheter containing four conductance electrodes was introduced into the left ventricle via the carotid artery and aortic valve. The external jugular vein was cannulated for volume administration with a maximum 300 µl of 50% albumin in saline. In order to acquire the pressure-volume loops, the respirator was paused for 5-6 sec (Nemoto *et al.*, 2003). The conductance of catheter was expressed in Volts⁻ by the Millar system (Millar MCS-100, ADInstruments, Colorado Springs, CO), which quantifies the voltage difference between two sensor electrodes by converting the resistance to voltage. The system was calibrated as described (Yang *et al.*, 1999). The pressure-volume loop data were obtained with PVAN software (Conductance Technologies, San Antonio, TX) following data acquisition with a Powerlab DAQ system (ADInstruments, Colorado Springs, CO). Regression analyses of multiple isochronal pressure-volume loops were produced by compression of the Inferior Vena Cava in order to decrease the preload volume for calculation of the End Systolic

Pressure-Volume Relationship index (slope) and the End Diastolic pressure-volume Relationship index (slope).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) from ground myocardial tissues. cDNAs were synthesized with Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen, Carlsbad, CA) at 37°C for 1.5 hours from 2 µg of total RNA in a 35 µl reaction mixture. Following reverse transcription, 3 µl products were used for 25 µl PCR with Taq polymerase (Takara Bio, Japan) and primer sets as listed in Table 1. The PCR products were detected by 1% agarose gel electrophoresis and ethidium bromide staining.

Light and Electronic Microscopy

After anesthesia, the hearts were fixed *in situ* by vascular perfusion of saline for 10 mins followed by 10 mins perfusion of 2% glutaraldehyde plus 2% paraformaldehyde (pH 7.4, Karnovsky solution). For histology analyses, the hearts were excised and the left ventricles were embedded in paraffin for thin (5 micron) transversal sectioning and staining with Hematoxylin and Eosin. For electron microscopy, the left ventricular tissues were cut into 3 mm blocks and fixed 3 days in Karnovsky solution before fixation in osmium, dehydration in ethanol and resin embedment for standard procedures of electron microscopy.

Immunoblotting

An equal amount (20-40 µg) of proteins from each sample was separated by SDS polyacrylamide gel electrophoresis for overnight transfer at 30 volts onto a PVDF membrane as described (Chen *et al.*, 2005). The primary monoclonal antibodies bound to p21, α -Smooth Muscle Actin or Actin were recognized by Horseradish Peroxidase conjugated-secondary antibody (Zymed, South San Francisco, CA) for detection by enhanced chemiluminescence.

Enzymatic Assay

Superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) activity assays were performed using assay kits from Cayman Chemicals. Samples were prepared according to the manufacture protocol for detection by a plate reader (Alexa, CA) of SOD at 450 nm, catalase at 540 nm and GPx at 340 nm.

Flow Cytometry

Mouse Inflammation Kit was used for quantifications of multiple soluble cytokines using serum samples per instruction (BD Biosciences). The calibrators were six cytokines ranging in concentration from 0 to 5 ng/ml for the assay system containing capture antibody beads and PE detection reagent. Thus six standard curves were obtained from one set of calibrators and the levels of serum cytokines were calculate based on these standard curve using BD Cytometric Bead Array software.

Quantification and Statistic Analysis

The intensities of bands from RT-PCR or Western blots were quantified using NIH Image J 1.32. All values were expressed as means $\pm$ standard deviations. Statistical significances were determined using Stata 8.2 software (Statacorp, Texas, USA). Comparisons were made by Student's *t* test for two samples or by ANOVA with post hoc Bonferroni test for multiple samples.

RESULTS

p21 Knockout Mice Are Resistant to Dox Induced Cardiomyopathy

Dox is known to induce p21 gene expression in various cell types. To address whether Dox induced p21 in the myocardium, we measured levels of p21 mRNA or protein. Dox treatment resulted in elevated levels of p21 mRNA and protein in the myocardia of WT animals (Fig 1A&B). Towards the end of Dox administration, an average 16% or 13% decrease of body weight was observed for WT or p21KO animals (Fig 1C). WT mice treated with Dox were highly sensitive to handling, anesthesia and the surgical procedure necessary for measurement of cardiac function, with a combined procedural mortality rate of 52% (Table 2). In contrast, p21KO animals treated with Dox had 19% procedural mortality rate (Table 2). The ratios of heart to body weight, a sign of cardiac hypertrophy, showed over 10% increase in WT but no increase in p21KO animals treated with Dox (Table 2). The protocol of Dox administration has been shown to induce cardiac specific injury (Sunet *al.*, 2001). Blood glucose and serum alanine aminotransferase and aspartate aminotransferase were measured to exclude a role of Dox in disturbance of general metabolism or causing liver injury (Table 2).

Figure 2 shows the results from histological and ultrastructural analyses of the left ventricles of WT or p21KO mice. Cardiomyocytes from Dox treated WT animals exhibited elongated nuclei, indicating cell size enlargement (Fig 2A). Electron microscopy images showed mitochondrial swelling, disarray of myofilaments and vacuolar structures in cardiomyocytes of WT animals treated with Dox (Fig 2B). In contrast, the p21KO animals did not show these morphological changes (Fig 2A&B).

Hemodynamic parameters provide quantitative measurements of cardiomyopathy. Dox treated WT mice presented an impaired contractile function indicated by a decrease in the maximum rate of change in systolic pressure over time (dP/dt max), and reduced cardiac output or ejection fraction (Table 3). In contrast, p21KO mice did not show significant alterations in cardiac contractility and efficiency due to Dox administration (Table 3). Figure 3A shows representative pressure-volume loops of one animal in each group. Left ventricular performance parameters were evaluated by occlusion of the Inferior Vena Cava. The preload recruitable stroke work (PRSW), obtained by regression plot of the stroke work versus end diastolic volume, indicated a decrease in left ventricular contractility for WT but not p21KO animals after Dox treatment (Fig 3B). The end diastolic pressure volume relationship (EDPVR) was significantly altered with Dox treatment in WT but not p21KO mice (Fig 3C). A decrease in the end systolic pressure-volume relationship (ESPVR) was noticeable in the WT but not p21KO animals following Dox treatment (Fig 3D).

Increased expression of ANF in the ventricles serves as a biomarker of heart failure. An increase of α -smooth muscle actin (α -SMA) positive cells also correlates with heart failure. Since ANF is a secreted protein, its expression was measured by levels of mRNA. Measurements of ANF mRNA or α -SMA protein showed elevations in the myocardium of WT but not p21KO animals treated with Dox (Fig 4A&B). These findings were consistent with the histological and hemodynamic data showing p21KO animals were resistant against Dox induced cardiomyopathy.

Investigating the Mechanism of Resistance in p21 Knockout Mice

Since it is commonly believed that Dox induces cardiomyopathy via oxidative stress, we questioned whether p21KO animals had an elevated antioxidant reservoir. Glutathione Peroxidase-1 (GPx1) and catalase are protective against Dox toxicity and inducible upon oxidative stress (Kang *et al.*, 1996; Gao *et al.*, 2008). With Dox treatment, WT animals showed elevation of mRNA or activities of both GPx and catalase mRNA (Fig 5A-B). SOD activity from myocardial tissue was not significantly altered by Dox treatment in WT or p21KO mice (data not shown).

Dox and quinones can be metabolized by NAD(P)H: quinone oxidoreductase (NQO1). A higher basal level of NQO1 in p21KO animals would indicate faster detoxification rate (Gutierrez, 2000). In contrast, p21KO animals expressed lower basal levels of NQO1 gene (data not shown). Nrf2 is a transcription factor that controls the expression of a number of antioxidant and detoxification genes. p21KO mice did not show elevated level of Nrf2 protein in the myocardium (data not shown). All these negative data lead us to direct our focus on pathways outside conventional antioxidant enzymes in an effort to understand the resistance of p21KO mice against Dox induced cardiomyopathy.

To explore the difference between WT and p21KO mice with or without Dox treatment, we turned to Affymetrix microarray technique and found that Dox increased expression of 21 genes related to immune response in the myocardium of WT mice but only 4 genes in this category of p21KO mice. To test that p21 participated in an inflammatory reaction leading to heart failure, we used Multiplex Mouse Inflammation Kit to measure levels of 6 cytokines in one experiment. Dox treatment caused significant elevation of IL-6, IL-12, IFN γ and TNF α in the blood of WT animals (Fig 6). In contrast, p21KO mice did not show such changes (Fig 6). When mRNA levels of these cytokines were measured using myocardial tissues, among those detected, i.e., IL-6, IL-10, IFN γ and TNF α only IL-6 showed significant elevation in Dox treated WT but not p21KO animals (Fig 7).

Discussion

This study indicates that p21KO mice were resistant to Dox induced cardiomyopathy. In WT animals, induction of p21 gene by Dox treatment correlated with morphological, hemodynamic and biochemical evidence of cardiomyopathy. In p21KO mice, most of the parameters measured were not significantly altered by Dox treatment. In addition, WT animals treated with Dox had a significantly higher mortality rate during various measurement procedures compared to p21KO mice. Although we did not observe elevations of SOD, GPx and catalase in p21KO mice, cells derived from p21KO mouse hearts have

been shown to be less vulnerable to ROS generation (Roy *et al.*, 2003). Therefore p21KO mice may have non-conventional manners of reduction of oxidative stress.

Inhibition of inflammatory response may contribute to the observed resistance of p21KO mice to Dox cardiotoxicity. Measurements of 6 cytokines in the blood confirmed the absence of systematic immune response in p21KO mice (Fig 6). In humans, inflammation is a well known risk factor for cardiovascular disease. Inflammatory response is often detected in failing hearts (Mehta and Li, 1999; Yndestad *et al.*, 2006; Heymans *et al.*, 2009). Heart failure patients show elevated levels of circulating IL-6 and TNF α (MacGowan *et al.*, 1997; Plenz *et al.*, 2001; Bradham *et al.*, 2002; Boffa *et al.*, 2009). Our observation of increased serum levels of IL-6 and TNF α in WT mice dosed with Dox is consistent with the literature regarding heart failure.

A few reports indicate a role of p21 in the inflammatory response. The p21 gene contributes to differentiation of monocytes to macrophage and inflammatory response in the liver, lung, vasculature and central nervous system (Wagayama *et al.*, 2002; Ring *et al.*, 2003; Merched and Chan, 2004). Similar to what we have observed in the myocardium, lack of p21 appears to be protective against atherosclerosis in apoE(-/-) mice or lung injury induced by cigarette smoke in correlation with reduced inflammatory response (Merched and Chan, 2004; Yao *et al.*, 2008). Inflammation often contributes to fibrosis, and interstitial fibrosis has been observed in the myocardium in association with Dox induced cardiomyopathy (Chatterjee *et al.*, 2010). Our observed elevation of α -SMA indicates fibrosis and proliferation of interstitial cells in the myocardium of Dox treated WT animals. Such fibrotic change was absent in p21KO mice, again supporting that lack of inflammatory response may contribute to the observed resistance of p21KO mice against Dox induced cardiomyopathy.

P21KO mice may exhibit enhanced capacity of repair and regeneration. Ray, *et al.* reported that p21 deficiency resulted in augmented conversion of cardiac fibroblasts to myofibroblasts, which exhibit characteristics of smooth muscle cells and are capable of contracting thus contributing to tissue repair (Roy *et al.*, 2007; van den Borne *et al.*, 2010). An increasing number of reports suggest that cell cycle proteins play a role in repair and regeneration of the myocardium (Ahuja *et al.*, 2007). Down regulation of cell cycle kinases occurs during the process of human heart failure (Qiu *et al.*, 2008), whereas stimulating cell cycle reentry such as by elevating cyclin D2 in transgenic mice improves cardiac function following cardiac injury (Hassink *et al.*, 2008). Additional repair mechanisms involve cardiac progenitor cells, which can migrate to damaged areas of the heart, proliferate and differentiate into fully functional cardiomyocytes (Orlic *et al.*, 2001; Anversa *et al.*, 2006a; Anversa *et al.*, 2006b; Kajstura *et al.*, 2008). We have preliminary evidence that p21KO mice have a higher reservoir of cardiac progenitor cells compared to WT mice, supporting an enhanced capacity of repair and regeneration.

Clinical manifestation of cardiac injury varies tremendously among individuals. For patients treated with Dox during chemotherapy, some developed cardiomyopathy months or years later, whereas others did not experience cardiotoxicity. Currently there is no method to predict individual susceptibility to cardiotoxicity of Dox. Polymorphisms of p21 gene exist in humans (www.ncbi.nlm.nih.gov). Polymorphisms of R149G, S31R, and G215A of p21

have been found in association with an increased risk of oral, esophageal, or advanced breast cancer (Bahl *et al.*, 2000; Ralhan *et al.*, 2000; Staalesen *et al.*, 2006). There is evidence that certain p21 polymorphisms affect the efficiency of p21 function and the outcome of human myocardial infarction (Rodriguez *et al.*, 2007). In addition to polymorphism, levels of p21 protein differ considerably between individuals (Xie *et al.*, 2004). If p21 is a central controller of the heart to chemical stress, polymorphism and differences in the expression level may contribute to individual variations in cardiotoxicity of Dox.

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Abbreviation

ANF	Atrial Natriuretic Factor
α-SMA	alpha smooth muscle actin
Dox	Doxorubicin
EDPVR	end diastolic pressure volume relationship
ESPVR	end systolic pressure-volume relationship
GPx	glutathione peroxidase
IL	interleukin
IFN	Interferon
NQO1	NAD(P)H: quinone oxidoreductase
p21 KO	p21 knockout
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PRSW	preload recruitable stroke work
PVDF	Polyvinylidene Fluoride
ROS	reactive oxygen species
SOD	Superoxide Dismutase
RT	reverse transcription
TNFα	Tumor Necrosis Factor alpha
WT	wild type

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Highlights

- >We addressed the functional significance of p21 elevation by doxorubicin in the myocardium.
- >Doxorubicin causes dilated cardiomyopathy in wild type mice.
- >Knocking out p21 gene results in resistance against doxorubicin induced cardiomyopathy.
- >Lack of inflammatory response correlates with the resistance in p21 knockout mice.

Figure 1. Dox Induced p21 mRNA and Protein in the Myocardium of Wild Type Mice
Mice were divided into 4 groups: wild type control (WT, n=15), WT treated with Dox (n=12), p21KO control (n=13) and p21KO treated with Dox (n=13). Larger p21KO mice were chosen for Dox injection so the dose could be kept the same as with WT mice. Myocardial tissues were collected at the end of 10 weeks of treatment for measurements of p21 mRNA by RT-PCR (A) or p21 protein by Western blot (B). Animals were weighed at the time points indicated (C). The data represents means $\pm$ standard deviations. An asterisk indicates significance difference ($p < 0.05$) between the means of treated group versus control.

Figure 2. Histology of Dox Induced Dilated Cardiomyopathy in Wild Type Mice

Wild type (WT) or p21KO mice were treated with Dox over a course of 10 weeks as described in the Methods. The longitudinal sections from the left ventricles were used for histology with H&E staining (A) and electron microscopy analyses (B). Images of the tissue section of left ventricles were shown under a microscope with 4x lens (A). Arrows indicate elongated nuclei (A). Electron microscopy images of tissue blocks from left ventricles were obtained at x3500 fold magnification (B).

Figure 3. Hemodynamic Function Measurements Indicate p21KO Mice Were Resistant to Dox Mice at the end of 10 weeks of Dox treatment were used to measure hemodynamic function using a Millar 1.4 Fr catheter system. Representative pressure-volume loops were shown for one animal from each group (A). Preload Recrutable Stroke Work (PRSW, B), End Diastolic Pressure-Volume Relationship (EDPVR, C) and End Systolic Pressure-Volume Relationship (ESPVR, D) were obtained by decreasing the preload during transient Inferior Vena Cava occlusion from WT control (n=6), WT treated with Dox (n=6), p21KO control (n=6), and p21KO treated with Dox (n=6). An asterisk indicates significance difference ($p < 0.05$) between the means of treated group versus control.

Figure 4. Measurements of Heart Failure Biomarkers Indicate p21KO Mice Were Resistant to Dox

The myocardial tissues from WT or p21KO mice following saline or Dox treatment were collected at the end of 10 weeks and were analyzed for ANF by RT-PCR (A,B) or α-smooth muscle actin (α-SMA) by Western blot (C). GAPDH (A, B) or actin (C) was included as a loading control. The intensities of the bands were quantified by NIH Image J program and were presented as means ± standard deviations (B). An asterisk indicates significance difference (p < 0.05) between the means of treated group versus control.

Figure 5. p21KO Mice Did Not Contain Higher Levels of Antioxidant Enzymes

The myocardial tissues were collected at the end of the 10 weeks of Dox treatment from WT or P21KO mice for measurements of glutathione peroxidase (GPX1) mRNA or total GPX activity (A), Catalase mRNA or catalase activity (B). The data represent means $\pm$ standard deviations from measurements of 3- animals or as indicated. An asterisk indicates significance difference ($p < 0.05$) between the means of treated group versus control.

Figure 6. Evaluation of Circulating Cytokines Following Dox Treatment

Sera were collected from WT or p21KO mice at the end of 10 weeks treatment of Dox for measurements of 6 cytokines simultaneously using Mouse Inflammation Kit (BD, Bioscience) and flow cytometry. The profile of 6 cytokines from one mouse in each group was shown (A). The results from WT (n=6), WT treated with Dox (n=8), p21KO (n=6) or p21KO treated with Dox (n=7) were presented as means $\pm$ standard deviations. An asterisk indicates significance difference (p<0.05) between the means of treated group versus control.

Figure 7. Expression of Inflammatory Cytokines in the Myocardium

The myocardial tissues were collected at the end of 10 weeks of Dox treatment from WT or P21KO mice for isolation of total RNA, and RT-PCR using primers as indicated in Table 1. The intensities of the bands were quantified by NIH Image J program and were presented as means $\pm$ standard deviations (B). An asterisk indicates significance difference (p < 0.05) between the means of treated group versus control.

Table 1

PCR Primers and Conditions

Gene	Primer	PCR condition
p21	5' AGTGTGCCGTTGTCTCTCG 5' ACACCAGAGTGCAAGACAAGC	94°C 30', 60°C 30', 72°C 30', 30 cycles
EC-SOD	5' CTGAGGACTTCCCAGTGAGC 5' GGTGAGGGTGTCTCAGAGTGGT	94°C 30', 60°C 30', 72°C 30', 30 cycles
catalase	5' GCAGATACCTGTGAACTGTC 5' GTAGAATGTCCGCACCTGAG	94°C 30', 60°C 30', 72°C 30', 30 cycles
GPx1	5' CCTCAAGTACGTCCGACCTG 5' CAATGTCGTTGCGGACACC	94°C 30', 60°C 30', 72°C 30', 30 cycles
NQO1	5' CATTCTGAAAGGCTGGTTTGA 5' CTAGCTTTGATCTGGTTGTCAG	94°C 30', 60°C 30', 72°C 30', 30 cycles
ANF	5' GTGTACAGTGCGGTGTCCAA 5' ACCTCATCTTCTACCGGCATC	94°C 30', 55°C 60', 72°C 60', 40 cycles
GAPDH	5' ACCCCTTCATTGACCTCAACTACA 5' AGTGATGGCATGGACTGTGGTCAT	94°C 30', 60°C 30', 72°C 30', 30 cycles
IL-6	5' ATGAAGTTCCTCTCTGCAAGAGACT 5' CACTAGGTTTGCCGAGTAGATCTC	94°C 30', 60°C 30', 72°C 60', 30 cycles
IL-10	5' CACTACCAAAGCCACAAAGC 5' CATGGCCTTGAGACACCTT	94°C 30', 55°C 60', 72°C 60', 30 cycles
INF γ	5' CACACTGCATCTTGGCTTTGC 5' CCTTGCTGTTGCTGAAGAAGGTAG	94°C 30', 60°C 30', 72°C 60', 30 cycles
TNF α	5' CCA GAC CCT CAC ACT CAG AT 5' AAC ACC CAT TCC CTT ACA AG	94°C 30', 60°C 30', 72°C 60', 30 cycles

Table 2**Mortality Rate and Liver Function of Mice Treated with Dox**

	WT	WT Dox	p21 KO	p21 KO Dox
Procedural Mortality	16% (n= 18)	*52% (n=25)	7% (n=14)	19% (n=16)
heart/body weight ratio	5.22± 0.63 (n=15)	*5.93±1.04 (n=12)	5.50±1.12 (n=13)	5.77±1.12 (n=13)
Heart weight (mg)	170.83± 30.40	157.68±34.78	152.93±16.21	142.55±23.36
Body weight (g)	32.59±3.57	27.40±5.68	28.85±6.18	24.99±2.87
ALT (U/L)	8.5 + 3.4	6.5 + 1.1	11.0 + 3.0	9.7 + 3.2
AST (U/L)	30.9 + 12.2	37.3 + 7.4	41.7 + 13.9	39.9 + 11
Glucose (mg/dL)	199.0 + 50.4	211.4 + 24.2	196.7 + 59.2	211.8 + 34.6

WT or p21KO mice were treated with saline or Dox. The mortality rate was recorded as a result of anesthesia or catheterization, since Dox treatment alone did not cause loss of mortality. The blood was collected for measurements of alanine transaminase (ALT), aspartate aminotransferase (AST) or glucose at the end of 10 weeks Dox treatment by the Pathological Service Core at University of Arizona animal care facility. The data are presented as means ± SD (n=3 unless indicated). An asterisk indicates significance difference (p<0.05) between the means of treated group versus control using Student's t test.

Table 3

Hemodynamic Parameters of Wild Type or p21KO Mice Following Dox Treatment

Parameters	WT	WT Dox	p21KO	p21KO Dox
Heart rate (bpm)	507.59±43.25	544.41±39.44	486.17±23.63	503.89±35.12
Maximum Volume (μL)	15.71±2.01	13.29±5.10	13.03±5.32	17.49±6.13
Minimum Volume (μL)	5.14±1.47	5.37±3.03	3.96±2.46	5.3±2.01
End-systolic Volume (μL)	5.51±1.59	5.72±3.01	4.8±3.14	5.65±2.06
End-diastolic Volume (μL)	14.48±2.19	12.35±5.24	11.87±4.87	15.90±6.38
Maximum Pressure (mmHg)	98.18±14.74	86.42±28.51	98.54±19.54	92.05±15.19
Minimum Pressure (mmHg)	1.46±1.21	4.38±2.53 **	3.00±1.23	2.12±1.27
End-systolic Pressure (mmHg)	86.72±11.80	82.60±27.50	87.94±19.98	82.25±14.41
End-diastolic Pressure (mmHg)	4.64±1.59	8.64±6.88	5.94±1.33	5.32±0.97
Stroke Volume (μL)	10.57±1.17	7.99±3.59*	9.07±4.45	12.19±4.91
Ejection Fraction (%)	67.66±6.33	52.48±10.41 **	64.38±10.27	68.67±8.32
Cardiac Output (μL/min)	5481.59±966.24	3920.31±1730.19*	5153.52±1470.19	6305.82±2348.45
Stroke Work (mmHg * μL)	829.20±164.19	525.93±321.18*	675.80±294.33	912.40±548.12
Arterial Elastance (Ea)	8.33±1.64	7.04±2.32	12.75±9.45	7.26±2.57
dP/dt max (mmHg/sec)	10423.97±2431.07	7176.26±2777.26 *	6944.75±1141.39	9069.41±2718.05
dP/dt min (mmHg/sec)	-6497.46±1665.83	-5266.88±1661.75	-5669.31±690.31	-6878.06±2343.20
dV/dt max (μL/sec)	401.43±123.86	337.12±143.20	403.80±301.14	483.33±195.02
dV/dt min (μL/sec)	-427.34±146.91	-310.40±124.49 *	-333.69±98.89	-447.31±158.41
P@dVdt max (mmHg)	10.62±19.43	8.94±13.84	16.09±12.19	15.44±15.43
P@dPdt max (mmHg)	49.13±10.14	44.57±17.99	39.05±6.25	43.02±10.4
V@dPdt max (μL)	14.87±2.03	13.93±6.52	12.69±5.11	15.55±5.91
V@dPdt min (μL)	5.51±1.61	5.74±2.30	4.55±2.68	5.80±1.27
Tau_w (msec)	6.40±1.27	8.22±1.41 **	7.48±0.78	6.45±1.52
Maximal Power (mWatts)	5.47±1.93	3.46±2.14*	4.21±1.14	5.13±2.54

Hemodynamics parameters were recorded from Wild Type mice treated with Saline (WT, n=9) or with Dox (WT Dox, n=10) and p21KO mice treated with saline (p21KO, n=8) or with Dox (p21KO Dox, n=9) by Millar catheters. P=pressure, V=volume. dP/dt reflects maximum or minimum pressure change in the left ventricle, whereas dV/dt indicates maximum or minimum volume change of the left ventricle. Tau is isovolumic relaxation constant. The numbers represents means ± SD with

* indicates $p < 0.05$

** indicates $p < 0.01$ when treated group was compared to control group by Student's t test.