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PRE-CLINICAL RESEARCH

Doxycycline Attenuates Protein Aggregation in Cardiomyocytes and Improves Survival of a Mouse Model of Cardiac Proteinopathy

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Objectives The goal of this pre-clinical study was to assess the therapeutic efficacy of doxycycline (Doxy) for desmin-related cardiomyopathy (DRC) and to elucidate the potential mechanisms involved.

Background DRC, exemplifying cardiac proteinopathy, is characterized by intrasarcoplasmic protein aggregation and cardiac insufficiency. No effective treatment for DRC is available presently. Doxy was shown to attenuate aberrant intranuclear aggregation and toxicity of misfolded proteins in noncardiac cells and animal models of other proteinopathies.

Methods Mice and cultured neonatal rat cardiomyocytes with transgenic (TG) expression of a human DRC-linked missense mutation R120G of αB-crystallin (CryAB^{R120G}) were used for testing the effect of Doxy. Doxy was administered via drinking water (6 mg/ml) initiated at 8 or 16 weeks of age.

Results Doxy treatment initiated at 16 weeks of age significantly delayed the premature death of CryAB^{R120G} TG mice, with a median lifespan of 30.4 weeks (placebo group, 25 weeks; $p < 0.01$). In another cohort of CryAB^{R120G} TG mice, Doxy treatment initiated at 8 weeks of age significantly attenuated cardiac hypertrophy in 1 month. Further investigation revealed that Doxy significantly reduced the abundance of CryAB-positive microscopic aggregates, detergent-resistant CryAB oligomers, and total ubiquitinated proteins in CryAB^{R120G} TG hearts. In cell culture, Doxy treatment dose-dependently suppressed the formation of both microscopic protein aggregates and detergent-resistant soluble CryAB^{R120G} oligomers and reversed the up-regulation of p62 protein induced by adenovirus-mediated CryAB^{R120G} expression.

Conclusions Doxy suppresses CryAB^{R120G}-induced aberrant protein aggregation in cardiomyocytes and prolongs CryAB^{R120G}-based DRC mouse survival. (J Am Coll Cardiol 2010;56:1418–26) © 2010 by the American College of Cardiology Foundation

Desmin-related myopathy, a well-characterized example of proteinopathy, features the presence of desmin-positive protein aggregates in myocytes. Genetic studies linked this disease to mutations in desmin, αB-crystallin (CryAB), and myotilin genes (1). Among these mutations, missense mutation R120G of CryAB (CryAB^{R120G}) is the best studied. Transgenic (TG) overexpression of either mouse or human CryAB^{R120G} in mouse hearts causes aberrant protein aggregation and cardiomyopathy, recapitulating key features of human desmin-related cardiomyopathy (DRC) (2,3). Recent studies show that intrasarcoplasmic amyloidosis, a major type of aberrant protein aggregation in DRC and associated cardiomyopathy, are reversible on suppression of CryAB^{R120G} expression and, more remarkably, significantly attenuated by voluntary exercise (4,5). Proteasome proteolytic function is severely impaired in CryAB^{R120G} TG mouse hearts, and aberrant protein aggregation seems to be both responsible for and further exacerbated by proteasome functional insufficiency, forming a vicious cycle (1,6).

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It is believed that abnormal protein aggregation and accumulation are deleterious in all proteinopathy, regardless of the primary cause. Notably, aberrant protein aggregation in the form of pre-amyloid oligomers has been observed in most failing human hearts, resulting from either dilated or hypertrophic cardiomyopathy (4). Moreover, aberrant protein aggregation recently was shown to trigger autophagic activation in pressure-overloaded hearts (7). To this end, the well-documented CryAB^{R120G} DRC mice represent a useful animal model for the investigation into the pathogenic role of cardiac aberrant protein aggregation as well as for therapeutic targeting of aberrant protein aggregation in congestive heart failure.

Doxycycline (Doxy) is a Food and Drug Administration-approved second-generation antibiotic of the tetracycline family. It is suitable for long-term use because of its favorable safety profile. It was demonstrated that Doxy has other important pharmacologic actions besides its antibiotic properties. For example, Doxy has been shown to be an inhibitor for matrix metalloproteinases (MMPs) (8). It also was reported that Doxy inhibits the formation of amyloid aggregates both in vitro and in vivo (9). Furthermore, some investigators (9–11) have reported that Doxy attenuated and delayed toxicity of oculopharyngeal muscular dystrophy possibly by reducing aggregation and inhibiting cell death pathways. These important recent discoveries prompted us to test whether Doxy has therapeutic value in cardiac proteinopathies. Our study revealed that Doxy significantly attenuates CryAB^{R120G}-induced aberrant protein aggregation in cardiomyocytes and prolongs the survival of CryAB^{R120G} DRC mice, providing compelling evidence that Doxy is a promising candidate for a clinical trial to treat cardiac proteinopathies.

Methods

Animals. The FVB/N inbred strain stable TG mice with cardiomyocyte-restricted overexpression of the mouse CryAB^{R120G} were used in this study (2). Animal use and care protocols used in this study were approved by the Institutional Committee for the Use and Care of Animals of the University of South Dakota.

Administration of Doxy and echocardiography. Doxy (Sigma-Aldrich Corp., St. Louis, Missouri) was given in drinking water (6 mg/ml) containing 5% sucrose, starting at 8 or 16 weeks of age. The control group was given drinking water containing 5% sucrose without Doxy. Echocardiography was performed as described (12).

Neonatal rat cardiomyocyte cultures and adenovirus infection. Neonatal rat cardiomyocytes (NRCMs) were isolated and cultured as described (6,12). Recombinant adenoviruses expressing hemagglutinin (HA)-tagged CryAB^{R102G} (Ad-HA-CryAB^{R120G}) or recombinant adenoviruses expressing β-galactosidase (Ad-β-Gal) were created as described (6). The viruses were used at a multiplicity of infection of 10 to infect the cultured NRCMs.

Immunofluorescence confocal microscopy and Western blot analyses. Sample preparation, immunofluorescence staining, and Western blot analyses were performed as described (6,12). The antibodies used include the rabbit polyclonal antibodies against CryAB (Stressgen, Victoria, British Columbia, Canada), Atg5 (Novus Biologicals, Littleton, Colorado), ubiquitin, Atg7 (Sigma-Aldrich Corp.), and the mouse antibodies against HA-tag (Santa Cruz Biotechnology, Santa Cruz, California) and sarcomeric a-actinin (Sigma-Aldrich Corp.), LC3 (MBL International, Woburn, Massachusetts), beclin-1 (Santa Cruz Biotechnology), Alexa Fluor 488 antirabbit Ig, Alex-Fluor 568 antimouse Ig (Invitrogen, Eugene, Oregon), and horseradish peroxidase-conjugated antimouse or antirabbit secondary antibodies (Santa Cruz Biotechnology). Alexa Fluor 568 conjugated phalloidin (Invitrogen, Eugene, Oregon) was used to stain F-actin.

Filter-trap assay. This assay was performed as previously described (4,6).

Statistical methods. The log-rank test was used for the Kaplan-Meier survival analysis. All quantitative data are presented as mean ± SD and were analyzed by 1-factor or multiple-factor analyses of variance using SigmaStat software version 3.0 (Systat, Point Richmond, California),

Abbreviations and Acronyms

- **Ad-β-Gal** = recombinant adenoviruses expressing β-galactosidase
- **Ad-HA-CryAB^{R120G}** = adenoviruses expressing hemagglutinin-tagged CryAB^{R102G}
- **CryAB** = αB-crystallin
- **CryAB^{R120G}** = missense mutation R120G of αB-crystallin
- **CTL** = control
- **Doxy** = doxycycline
- **DRC** = desmin-related cardiomyopathy
- **Hsp** = heat shock protein
- **LC3** = microtubule associated protein light chain 3
- **MMP** = matrix metalloproteinase
- **NRCM** = neonatal rat cardiomyocyte
- **NTG** = nontransgenic
- **TG** = transgenic

Table 1 Baseline Echocardiography Analyses of Mice Used for Kaplan-Meier Survival Analysis

	NTG	TG-CTL	TG-Doxy
n	23	18	19
Body weight (g)	26 ± 3.7	27 ± 4.2	27 ± 3.6
Heart rate (beats/min)	509 ± 55	414 ± 39*	413 ± 31*
LVPW-d (mm)	0.72 ± 0.09	0.94 ± 0.12*	0.96 ± 0.10*
LVID-d (mm)	3.8 ± 0.34	3.6 ± 0.26†	3.6 ± 0.32†
LVPW-s (mm)	1.12 ± 0.11	1.41 ± 0.22*	1.46 ± 0.18*
LVID-s (mm)	2.23 ± 0.33	1.92 ± 0.26*	1.90 ± 0.31*
FS (%)	41.7 ± 4.5	47.2 ± 4.8*	46.9 ± 5.4*
EF (%)	73.2 ± 5.1	79.1 ± 4.8*	78.8 ± 5.3*
SV (μl)	46 ± 7.6	44 ± 6.6	42 ± 7.4
CO (μl)	23,094 ± 4,199	18,037 ± 3,093*	17,256 ± 3,439*

For all parameters, there is no statistically significant difference between the TG CTL group and the TG Doxy group immediately before Doxy treatment. Compared with NTG littermates, *$p < 0.01$, †$p < 0.05$.

CO = cardiac output; CTL = control; -d = end-diastole; Doxy = doxycycline; EF = ejection fraction; FS = fractional shortening; LVID = left ventricle internal diameter; LVPW = left ventricle posterior wall; NTG = nontransgenic; -s = end-systole; SV = stroke volume; TG = transgenic.

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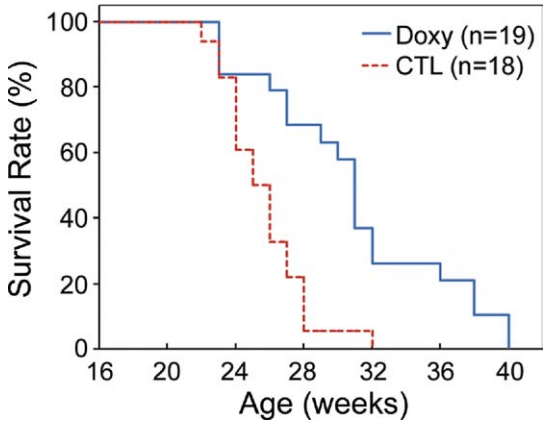

Figure 1 Kaplan-Meier Survival Curves of Missense Mutation R120G of αB-Crystallin (CryAB^{R120G}) TG Mice Treated With Doxy

Treatment of line 134 littermate CryAB^{R120G} transgenic (TG) and nontransgenic (NTG) mice with doxycycline (Doxy) or vehicle control (CTL) was initiated at 16 weeks of age. No premature death was observed for Doxy- or CTL-treated NTG mice (data not shown). The lifespan of CryAB^{R120G} TG mice was prolonged significantly by Doxy treatment ($p < 0.01$).

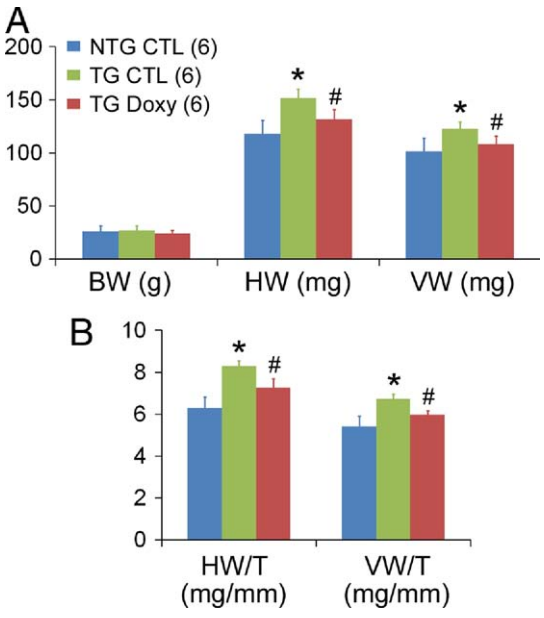

Figure 2 Doxy Reduces Cardiac Hypertrophy in CryAB^{R120G} TG Mice

Initiated at 8 weeks of age, a cohort of line 134 TG and NTG mice were treated with Doxy or CTL for 4 weeks and were used for gravimetric analyses. Body weight (BW), heart weight (HW), and ventricular weight (VW) are shown in **A**. The HW to tibial length ratio (HW/T) and the VW to tibial length ratio are presented in **B**. Shown are mean ± SD. *$p < 0.05$ versus the NTG CTL group. #$p < 0.05$ versus the TG CTL group. Abbreviations as in Figure 1.

where applicable. The Holm-Sidak test was used for post-hoc pairwise comparisons. A p value <0.05 was considered statistically significant.

Results

Doxy treatment significantly prolongs survival of mice with CryAB^{R120G} DRC. CryAB^{R120G} TG mice (line 134) develop concentric cardiac hypertrophy and diastolic malfunction at 3 months, display overt congestive heart failure between 5 and 6 months, and die shortly afterward (2). Because of the expedited course of disease progression in this TG line, we used it to perform a Kaplan-Meier survival analysis on chronic Doxy treatment.

A cohort of 37 CryAB^{R120G} TG mice was divided randomly into 2 groups: the Doxy (TG Doxy group; 19 mice) and the placebo (TG control [CTL] group; 18 mice) groups. A parallel cohort of nontransgenic (NTG) littermates was treated similarly to detect any potential adverse effects of Doxy or placebo on normal animals. Echocardiography was performed the day before the initiation of treatment. The treatment was initiated at 16 weeks of age when concentric cardiac hypertrophy and significantly decreased cardiac output were evident (Table 1). Mice of the TG CTL group showed a median lifespan of 25 weeks, similar to what was observed previously in the untreated TG mice (2). However, the premature death was significantly delayed in the TG Doxy group, with 60% of them still alive by the time all TG CTL mice had died. Their median lifespan was 30.4 weeks, 20.16% longer than that of the TG CTL group ($p < 0.01$) (Fig. 1).

The treatment to the NTG cohort was terminated when all mice in the TG cohort died. No difference in animal

Table 2 Echocardiography Assessments After 4 Weeks of Doxy Treatment

Parameters	NTG CTL	NTG Doxy	TG CTL	TG Doxy
Heart rate (beats/min)	479 ± 61	496 ± 57	460 ± 31	440 ± 36
LVPW-d (mm)	0.74 ± 0.07	0.69 ± 0.14	0.93 ± 0.05*	0.82 ± 0.06†‡
LVID-d (mm)	3.78 ± 0.15	3.73 ± 0.18	3.59 ± 0.19†	3.52 ± 0.23†
LVPW-s (mm)	1.15 ± 0.11	1.02 ± 0.20	1.62 ± 0.19*	1.36 ± 0.14†§
LVID-s (mm)	2.25 ± 0.11	2.34 ± 0.14	1.75 ± 0.31*	1.79 ± 0.30†
FS (%)	40.37 ± 1.80	37.41 ± 2.59	51.37 ± 6.73*	49.33 ± 5.98*
EF (%)	71.82 ± 2.13	68.18 ± 3.19	82.87 ± 6.22*	81.18 ± 5.70*

Treatment with Doxy or vehicle was initiated at 8 weeks of age. n = 6 for each group. Compared with the NTG CTL group, *$p < 0.01$, †$p < 0.05$. Compared with the TG CTL group, ‡$p < 0.01$, §$p < 0.05$.
Abbreviations as in Table 1.

death was observed between the Doxy- and placebo-treated NTG mice (data not shown).

Doxy treatment attenuates cardiac hypertrophy in DRC mice. To evaluate the effects of Doxy on cardiac hypertrophy and left ventricular function in DRC mice at an earlier time, another cohort of NTG and TG mice was subjected to 4 weeks of Doxy treatment starting at 8 weeks of age. Previous characterization of this mouse line revealed that cardiac hypertrophy started between 1 and 3 months of age and the down-regulation of α-myosin heavy chain and CryAB^{R120G} expression was observed at 6 months (2). Hence, choosing the period between 2 and 3 months avoids the potential impact from the down-regulation of TG expression. At 4 weeks after Doxy treatment, echocardiography assessments revealed significantly increased thickness in the left ventricle posterior wall at the end of both diastole and systole in the TG CTL mice, but the increases were prevented or were attenuated significantly in the TG Doxy group (Table 2). These indicate that a 4-week Doxy treatment is sufficient to suppress cardiac hypertrophy. The increase in ventricular wall thickness in CryAB^{R120G} TG CTL mice was accompanied by decreased internal diameter at end-diastole and -systole and elevated ejection fraction and fractional shortening. Doxy treatment did not significantly alter ejection fraction, fractional shortening, or the end-diastolic left ventricle internal diameter in the TG mice (Table 2). The changes in cardiac mass assessed by echocardiography were confirmed by gravimetric measurements at terminal experiments. The heart weight-to-tibial length ratio and the ventricular weight-to-tibial length ratio were significantly lower in the TG Doxy group than in the TG CTL group (Fig. 2).

Doxy treatment reduces aberrant protein aggregation in DRC mouse hearts. Because protein aggregation is an important pathogenic process in DRC and Doxy has been shown to suppress intranuclear protein aggregation in nonmyocytes, we further tested whether Doxy's protection against DRC was associated with any effect on aberrant protein aggregation in the heart.

Compared with the TG CTL group, TG Doxy hearts showed substantially less CryAB-positive protein aggregates by immunofluorescence confocal microscopy (Fig. 3), sig-

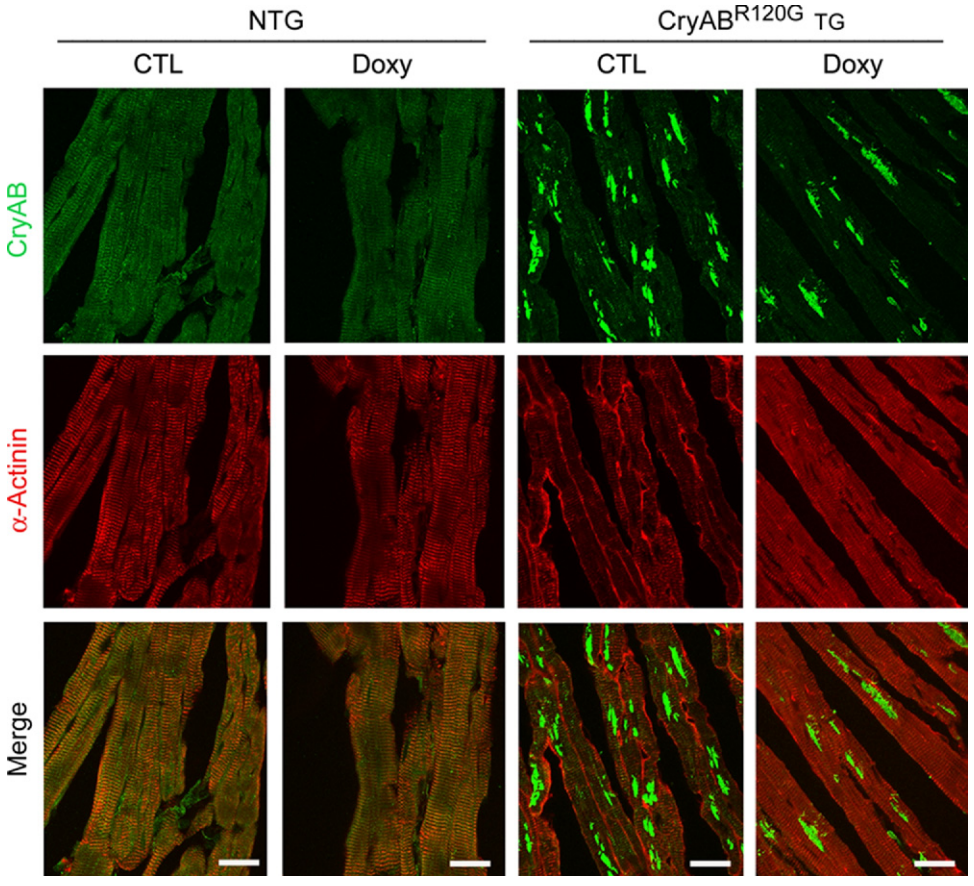

Figure 3 Doxy Reduces CryAB-Immunopositive Aggregates in CryAB^{R120G} TG Hearts

Line 708 CryAB^{R120G} TG and NTG mice were treated with Doxy or CTL for 2 months, starting at 8 weeks of age. Paraformaldehyde perfusion-fixed ventricular myocardium was immunostained for CryAB **(green)** and α-actinin **(red)**. Scale bar = 30 μm. Abbreviations as in Figure 1.

nificantly less sodium dodecyl sulfate (SDS)-resistant CryAB oligomers by filter-trap assays (Fig. 4B), and markedly less ubiquitinated proteins (Fig. 4C). Doxy treatment did not change total CryAB protein levels (Fig. 4A).

Doxy dose-dependently inhibits aberrant protein aggregation induced by CryAB^{R120G} in cultured NRCMs. To determine whether Doxy-induced suppression of aberrant protein aggregation in the heart is cardiomyocyte-autonomous, we further tested the effect of Doxy on CryAB^{R120G}-induced aberrant protein aggregation in cultured NRCMs. One day after the Ad-HA-CryAB^{R120G} infection, different doses (0.5 to 10 mM) of Doxy were administered daily to the culture media, and the cells were collected at 3 or 11 days after treatment. As revealed previously (4,6,13), CryAB-positive protein aggregates were formed in the cytoplasm of cardiomyocytes infected by Ad-HA-CryAB^{R120G}, but not by Ad-β-Gal. The extent of the aggregates was reduced markedly by Doxy treatment (Fig. 5). Western blot analyses showed that Doxy treatments did not alter the HA-CryAB^{R120G} protein level discernibly in either the soluble or the insoluble fractions (Fig. 6A), but the filter-trap assays revealed that the SDS-resistant oligomeric forms of HA-CryAB^{R120G} significantly reduced by Doxy in a dose-dependent manner (Figs. 6B and 6C), indicating inhibition of aberrant protein aggregation by Doxy. Moreover, this inhibitory effect seems to be more pronounced at 11 days than at 3 days.

To determine the potential mechanism of the inhibition of protein aggregation by Doxy, we examined the protein expression of heat shock protein 25 (Hsp25) and p62/SQSTM1. Hsps play a critical role in preventing protein aggregation (13,14). p62 was shown to promote the formation of ubiquitinated protein inclusion bodies (15,16). As shown in Figures 6D and 6E, p62 in both the soluble and the insoluble fractions and Hsp25 in the insoluble fraction were significantly up-regulated by CryAB^{R120G} expression, and the up-regulation of p62 but not Hsp25 was significantly less in Doxy-treated cells versus vehicle-treated cells.

Activation of autophagy in DRC hearts is not enhanced by Doxy. Autophagy plays an important role in protein quality control (17). Autophagic activation was shown to protect against DRC in mice (18). Hence, we examined the conversion of the native form of microtubule-associated protein light chain 3 (LC3-I) to the lipidated form of LC3 (LC3-II) (a commonly used marker of autophagic activation) and several other autophagy-related proteins (19). The protein level of LC3-II, the LC3-II-to-LC3-I ratio, and a cleaved form of Atg5 (autophagy-related gene 5) in TG CTL hearts were significantly greater than those in the NTG CTL group, but no statistically significant difference in these parameters was detected between TG CTL and TG Doxy groups (Figs. 7A and 7B) (data not shown). In cultured NRCMs overexpressing HA-CryAB^{R120G}, Doxy at 1

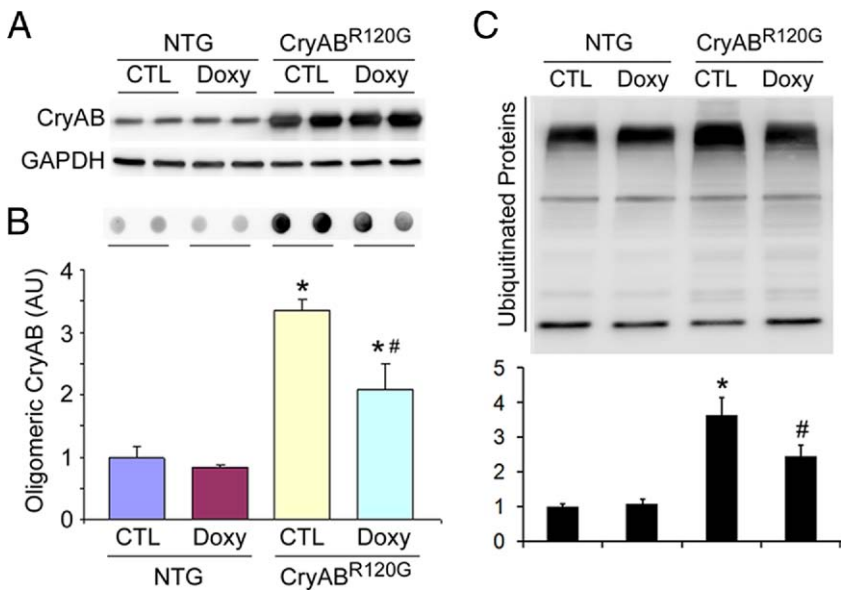

Figure 4 Doxy Inhibits CryAB^{R120G}-Induced Protein Aggregation in Mouse Hearts

The total and the soluble fractions of myocardial proteins were extracted from mice of the same cohort, and treatment was as described in Figure 3. **(A)** Total ventricular myocardial proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, and CryAB levels were analyzed by Western blots. **(B)** SDS-resistant oligomeric CryAB in myocardial proteins was measured by the filter-trap assay. A representative image **(upper panel)** and the densitometry data **(bottom panel)** are shown. **(C)** Quantitative Western blot analyses of ubiquitinated proteins in the total myocardial protein extract. A representative image **(upper panel)** and a summary of densitometry data from 4 repeats **(lower panel)** are shown. *$p < 0.05$ versus NTG mice. #$p < 0.05$ versus TG CTL mice. AU = arbitrary unit; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; other abbreviations as in Figure 1.

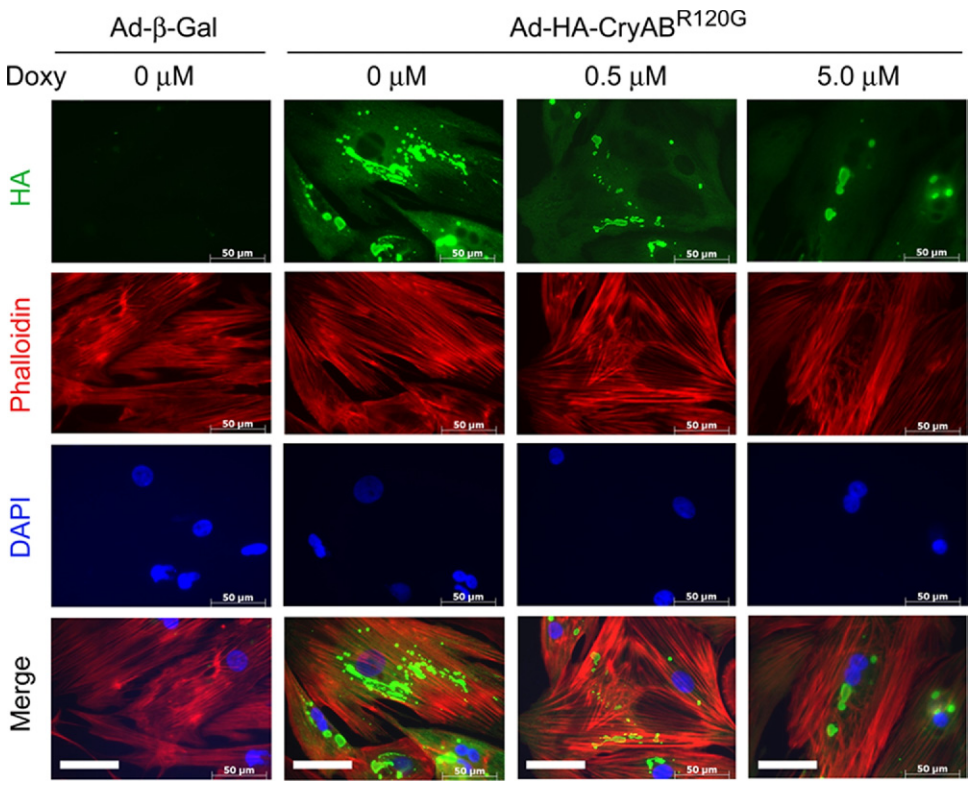

Figure 5 **Fluorescent Confocal Micrographs of Immunostained NRCMs Overexpressing CryAB^{R120G}**

Neonatal rat cardiomyocytes (NRCMs) were infected with recombinant adenoviruses expressing β-galactosidase (Ad-β-Gal) or adenoviruses expressing hemagglutinin-tagged CryAB^{R102G} (Ad-HA-CryAB^{R120G}) for 24 h, followed by the addition of various concentrations of Doxy as indicated. After 11 days of Doxy treatment, the cells were fixed and immunostained for HA-CryAB^{R120G} **(green)**. F-actin was stained by phalloidin **(red)** and nuclei with 4′,6-diamidino-2-phenylindole (DAPI) **(blue)**. Scale bar = 50 μm. Abbreviations as in Figure 1.

and 5 mM, but not 0.5 mM, significantly reduced LC3-II protein levels, but did not alter the LC3-II-to-LC3-I ratio. Doxy treatment did not change Atg7 and beclin1 protein expression (Figs. 7C and 7D). These results indicate that autophagy is activated in the TG heart, but that Doxy-elicited cardioprotection is independent of autophagy.

Collectively, our in vivo and in vitro experiments demonstrate that Doxy can effectively inhibit aberrant protein aggregation induced by CryAB^{R120G}, which likely contributes to its protection against DRC.

Discussion

Despite recent advances in understanding the genetic basis of DRC (1,3), no effective therapy is available to treat this devastating disease. Using both cell culture and a DRC mouse model, the present study revealed for the first time that Doxy can inhibit aberrant protein aggregation in cardiomyocytes, can attenuate significantly a DRC-linked misfolded protein-induced adverse cardiac remodeling, and effectively can prolong the lifespan of a well-documented TG mouse model of DRC. These results provide compelling evidence that Doxy is a promising drug candidate to treat DRC.

The dosage and route chosen here for Doxy administration were previously proven effective in treating a mouse model of oculopharyngeal muscular dystrophy (10). It should be noted that Doxy concentration used in the drinking water (6 mg/ml) for this study was 6-fold higher than what is used commonly to manipulate transgene expression in the tetracycline-inducible transgenic system. We tested only Doxy here, but other tetracycline derivatives, especially those with better tissue permeability (e.g., minocycline), could be as effective or even more effective, as demonstrated in neural proteinopathies (20).

Notably, Doxy treatment in our survival study was initiated at a relatively late stage when DRC pathologic features and clinical signs are readily detectable (Table 1). The rationale behind this experimental design is to maximize its clinical relevance. It remains to be tested, but it is very likely that the survival improvement by Doxy would be much greater should the treatment be started earlier. Supporting this prediction, we have observed that a significant attenuation of cardiac hypertrophy without deteriorating cardiac

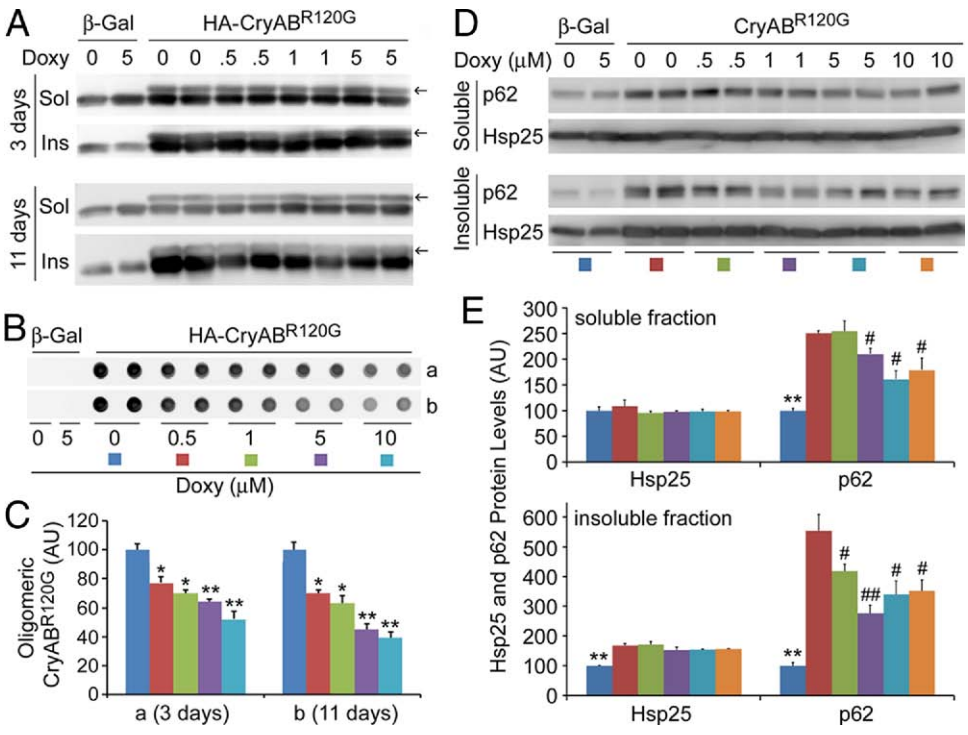

Figure 6 **Doxy Dose-Dependently Inhibits CryAB^{R120G}-Induced Protein Aggregation in Cultured NRCMs**

Cultured NRCMs were infected with Ad-HA-CryAB^{R120G} or Ad-β-Gal for 24 h before Doxy treatment initiation. **(A)** Three or 11 days after Doxy treatment, the soluble (Sol) and the insoluble (Ins) fractions of proteins were extracted from NRCMs and subjected to Western blot analysis for CryAB. HA-CryAB^{R120G} **(arrows)** runs slower than endogenous CryAB. **(B)** Representative images of the filter-trap assays for SDS-resistant HA-CryAB^{R120G} oligomers in cells at 3 **(a)** or 11 **(b)** days of Doxy treatments using antihemagglutinin antibodies. **(C)** Changes in the oligomers detected in 4 repeats. *$p < 0.05$. **$p < 0.01$ versus the 0 μM Doxy group. **(D and E)** Western blot analyses of Hsp25 and p62 in cells that underwent 11 days of Doxy treatment. **$p < 0.01$ versus all HA-CryAB^{R120G} groups. #$p < 0.05$. ##$p < 0.01$ versus Doxy-untreated HA-CryAB^{R120G}-expressing cells. Abbreviations as in Figures 1, 4, and 5.

function was detected 1 month after Doxy treatment was initiated at 8 weeks of age (Fig. 2, Table 2), 8 weeks earlier than the starting point of the survival study.

The mechanisms underlying Doxy's beneficial effects on DRC are potentially quite complex because of Doxy's versatile pharmacologic actions. Besides its antimicrobial action, Doxy is also known to inhibit MMPs. By breaking down the extracellular matrix, MMPs play important roles in tissue remodeling, cell migration, angiogenesis, and interstitial remodeling (21). Hence, Doxy's MMP inhibition property is believed to contribute to a wide range of its biological effects. Timed administration of Doxy seems to protect cardiac function by modulating post–myocardial infarction remodeling (22–25). We cannot rule out the possibility that Doxy's MMP inhibition property may contribute to its beneficial effects on DRC, but 2 lines of evidence stand against this possibility. First, previous characterization showed no significant interstitial fibrosis in the heart of the DRC mice used here (2). Second, compared with NTG, myocardial activities of MMPs were not increased in TG mice (data not shown). Notably, it was reported recently that Doxy mitigated cardiac remodeling without significantly affecting myocardial MMP activities (26).

Misfolded proteins, when failed to be repaired, are escorted by the chaperones to degradation by the ubiquitin–proteasome system (1). When chaperones and/or the ubiquitin–proteasome system are overwhelmed, misfolded proteins undergo aberrant aggregation, which produces initially soluble oligomers. If not removed in time, the oligomers will fuse to form large insoluble aggregates. The soluble oligomers generally are believed to be toxic, whereas the insoluble aggregates perhaps are not (1). Cardiac toxicity of aberrant protein aggregation was demonstrated directly by the sufficiency of expressing a mutant prion protein or polyglutamine preamyloid oligomers in cardiomyocytes to induce heart failure in mice (27,28). In the present study, we observed that not only insoluble aggregates (Figs. 3 and 5), but also oligomeric CryAB^{R120G} (Figs. 4 and 6), were decreased significantly by Doxy treatment in vivo and in vitro. These data suggest that Doxy may act as a pharmacologic chaperone that prevents CryAB^{R120G} from inducing aberrant oligomerization of endogenous and TG CryAB, allowing the formation of normal CryAB polymers that can exert the normal chaperoning function. Indeed, both tetracycline and Doxy have been shown to interact with prion proteins and to reduce in vitro prion protein aggregation

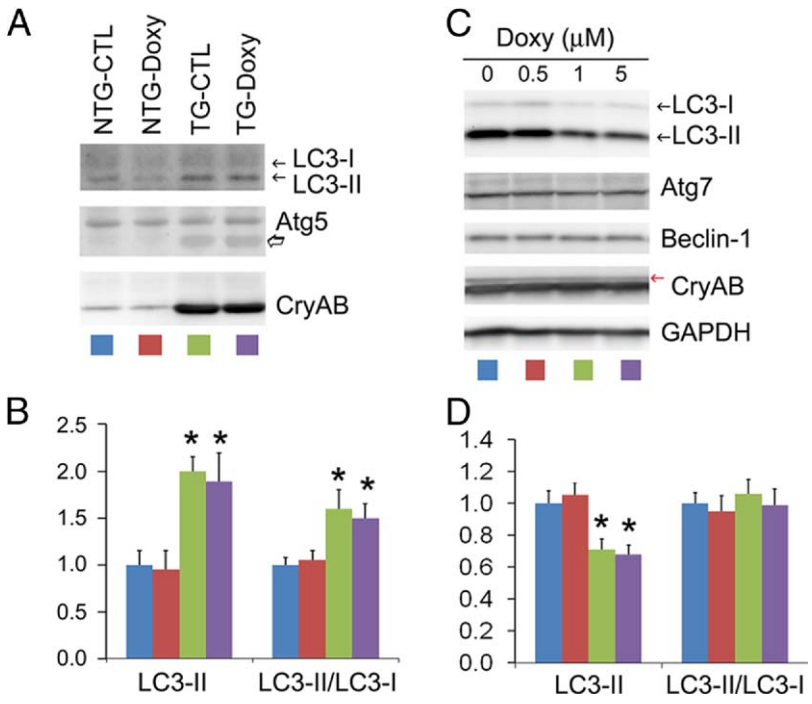

Figure 7 **Western Blot Analyses for Autophagy-Related Proteins**

(A and B) Total myocardial proteins from mice of the same cohort and treatment as described in Figure 3 were used. Representative immunoblot images are shown in **A**. The **open arrow** marks a short (or cleaved) form of autophagy-related gene 5 (Atg5). Densitometry results from microtubule associated protein light chain 3 (LC3) immunoblots are summarized in **B**. **(C and D)** Cultured NRCMs were treated with Doxy for 72 h, starting at 24 h after Ad-HA-CryAB^{R120G} infection. Cell lysates were used for the immunoblots. Representative images are shown in **C**. The **red arrow** marks HA-CryAB^{R120G}. Densitometry results from the LC3 immunoblots are summarized in **D**. *$p < 0.05$ versus the **blue bar** and **red bar** groups; $n = 4$ biological repeats. Abbreviations as in Figures 1, 4, and 5.

and in vivo infectivity (29). Supporting this notion, the same extent of CryAB^{R120G} protein overexpression, in the presence of Doxy, caused significantly less up-regulation of p62 (Fig. 6) and less accumulation of ubiquitinated proteins (Fig. 4C). Our data suggest that Doxy's inhibition of aberrant protein aggregation of misfolded CryAB^{R120G} may contribute to its protection against DRC.

Consistent with its presence in the protein aggregates-associated desminopathy (30), p62 was up-regulated significantly in both the soluble and insoluble fractions of cultured cardiomyocytes overexpressing CryAB^{R120G}. Interestingly, the increases in p62 protein levels were attenuated significantly by Doxy (Figs. 6D and 6E). p62 is known to interact with both ubiquitinated proteins in the aggregates and autophagosomes and target protein aggregates for selective autophagic degradation (31). p62 also mediates the formation of ubiquitin-positive inclusion bodies in hepatocytes and neurons when autophagy is impaired, with varying consequences (16). The role of p62 up-regulation on aberrant protein aggregation in cardiomyocytes has not been defined, but our results indicate that attenuation of p62 up-regulation likely is beneficial to the heart and may be an underlying mechanism of Doxy. Consistent with this postulate, the down-regulation of p62 by Doxy treatment did not seem to be caused by autophagic activation, because Doxy did not enhance autophagic activation in either the TG heart or in NRCMs expressing CryAB^{R120G} (Fig. 7).

It was reported in a TG mouse model of human CryAB^{R120G} that stress-inducible Hsp's were up-regulated differentially in DRC hearts, with a major increase in Hsp25 expression associated with progression to heart failure and increased mortality (3). However, Hsp's such as Hsp22, Hsp70, and HspB8 disrupt oligomer formation induced by CryAB^{R120G} under certain conditions (13,14). In both CryAB^{R120G} TG mouse hearts (data not shown) and CryAB^{R120G} expressing cultured cardiomyocytes, we observed a significant Hsp25 up-regulation, but Doxy treatment failed to alter it (Figs. 6D and 6E). Hence, the suppression of protein aggregation by Doxy is unlikely through an effect on Hsp25.

DRC, by itself, is not a common disease, but it exemplifies cardiac proteinopathies featured by intrasarcoplasmic aberrant protein aggregation. Hence, the significance of this study could be far beyond DRC because preamyloid oligomers were observed in the heart tissue of a large subset of human congestive heart failure cases resulting from hypertrophic/dilated cardiomyopathies (4). Moreover, aberrant protein aggregates also were observed in pressure-overloaded mouse hearts (7).

Interestingly, although chronic Doxy treatment was shown to attenuate isoproterenol and transaortic constriction-induced cardiac hypertrophy (32), Vinet et al. (26) reported that 1 month, but not 2 months, of low-dose Doxy enhanced transaortic constriction-induced hypertrophy. Protective actions of Doxy on rat diabetic cardiomyopathy also were reported recently (25).

Conclusion

The present study provides compelling pre-clinical evidence that Doxy is a promising drug candidate to treat DRC.

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