MOLECULAR PATHOGENESIS OF GENETIC AND INHERITED DISEASES

BGP-15 Improves Aspects of the Dystrophic Pathology in mdx and dko Mice with Differing Efficacies in Heart and Skeletal Muscle

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Duchenne muscular dystrophy is a severe and progressive striated muscle wasting disorder that leads to premature death from respiratory and/or cardiac failure. We have previously shown that treatment of young dystrophic mdx and dystrophin/utrophin null (dko) mice with BGP-15, a coinducer of heat shock protein 72, ameliorated the dystrophic pathology. We therefore tested the hypothesis that later-stage BGP-15 treatment would similarly benefit older mdx and dko mice when the dystrophic pathology was already well established. Later stage treatment of mdx or dko mice with BGP-15 did not improve maximal force of tibialis anterior (TA) muscles (in situ) or diaphragm muscle strips (in vitro). However, collagen deposition (fibrosis) was reduced in TA muscles of BGP-15 treated dko mice but unchanged in TA muscles of treated mdx mice and diaphragm of treated mdx and dko mice. We also examined whether BGP-15 treatment could ameliorate aspects of the cardiac pathology, and in young dko mice it reduced collagen deposition and improved both membrane integrity and systolic function. These results confirm BGP-15’s ability to improve aspects of the dystrophic pathology but with differing efficacies in heart and skeletal muscles at different stages of the disease progression. These findings support a role for BGP-15 among a suite of pharmacological therapies for Duchenne muscular dystrophy and related disorders. (Am J Pathol 2016, 186: 3246–3260; http://dx.doi.org/10.1016/j.ajpath.2016.08.008)

Duchenne muscular dystrophy (DMD) is a severe and progressive muscle wasting disorder caused by mutations in the dystrophin gene, resulting in the absence of the membrane-stabilizing protein, dystrophin.1 DMD affects approximately 1 in 3500 to 6000 live male births,3 with patients becoming wheelchair dependent usually before their teenage years and eventually succumbing to respiratory and/or cardiac failure.1–5 Although a cure for DMD will eventually come from corrective gene therapy, limitations of delivery systems, gene-carrying capacity, dissemination efficiency, expression persistence, and immunological tolerance pose significant challenges for immediate clinical application.6

The heat shock protein (Hsp) family is a group of proteins induced by cellular stress that are implicated in cellular protection.7 During periods of stress, Hsp family members bind denatured proteins, preventing further breakdown and protein aggregation.8,9 Hsp70/72 is the most widely studied and well-characterized member of the Hsp family, and its induction shown beneficial for several muscle conditions.10–13 Up-regulation of the inducible form (Hsp72), through transgenic and pharmacological approaches, enhanced muscle strength and improved histopathological

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features in the severely affected diaphragm muscles of young dystrophic mdx and dystrophin/utrophin double-knockout (dko) mice. Most important for clinical application, Hsp72 induction reduced kyphosis and increased lifespan of dko mice. BGP-15 is a pharmacological coinducer of Hsp72, shown safe and well tolerated in phase 2 clinical trials for diabetes and insulin resistance. BGP-15 is a hydroxylamine derivative that amplifies the endogenous stress response by altering the organization of cholesterol-rich membrane domains to specifically target stressed cells. This is a desirable trait for therapeutics administered systemically and chronically.

We investigated the effect of BGP-15 administration to young (3- to 4-week-old) mdx and dko mice and showed a dramatically improved pathophysiology and lifespan. However, although DMD is diagnosed early in life, significant muscle damage will have already occurred to cause functional deficits. Early-stage treatment of dko mice does not correlate with the pathology of DMD patients at the time of diagnosis and so the clinical translation of these potentially beneficial effects may not be realized. This is a limitation for other potential treatments for DMD, including growth-promoting agents like myostatin inhibitory antibodies, which were beneficial only when administered early in the disease progression but not later. In addition, it is unclear whether BGP-15 is similarly effective only as a preventive intervention or whether it can improve an advanced pathology. Therefore, to fully elucidate the therapeutic potential of BGP-15 for treating muscular dystrophy, we investigated whether BGP-15 treatment was similarly beneficial in older mdx and dko mice with an established pathology.

Cardiomyopathy develops throughout adolescence and is evident in most DMD patients by 18 years of age. Because of improved clinical management of respiratory symptoms, cardiac problems have become a critical aspect of the disease. Targeting only the skeletal musculature can exacerbate cardiomyopathy in mdx mice, suggesting increased movement and skeletal muscle activity may cause stress to the myocardium and accelerate cardiac damage. In addition, long-term treatment with the tumor necrosis factor-α blocking drug infliximab (Remicade), which can benefit the skeletal muscle pathology, had significant negative effects on cardiac function in mdx mice. These findings indicate that treating both skeletal and cardiac muscle pathology is necessary for DMD. Cardiac dysfunction is evident in dystrophic mdx and dko mice, but its severity in dko mice more closely resembles that in DMD. Therefore, therapies are needed that can preserve muscle mass, promote muscle growth, and maintain structure and function of both skeletal muscle and the myocardium without deleterious effects on the dystrophic heart.

A contributing mechanism to the improved skeletal muscle pathology we observed in young dystrophic mice after Hsp72 induction was the preservation of maximal sarcoplasmic/endoplasmic reticulum Ca2+-ATPase (SERCA) activity, the main protein responsible for removal of intracellular Ca2+ from the cytosol. Because DMD is characterized by muscle fibers with chronically elevated intracellular Ca2+ ([Ca2+]i) levels that can activate inflammatory and muscle degradative pathways, and because SERCA dysfunction is implicated in the skeletal muscle and cardiac pathologies, we also tested whether BGP-15 treatment preserved SERCA activity in the heart.

Materials and Methods

Experimental Animals

All experiments were approved by the Animal Ethics Committee of The University of Melbourne (Melbourne, VIC, Australia) and conducted in accordance with the Australian code of practice for the care and use of animals for scientific purposes, as stipulated by the National Health and Medical Research Council (Australia). C57BL/10 mice were obtained from the Animal Resources Centre (Canning Vale, WA) and dko mice [originally provided through collaboration with Prof. Dame Kay Davies (University of Oxford, Oxford, UK)] were bred in the Biological Research Facility at The University of Melbourne. Genotypes from tail biopsy specimens were determined using real-time PCR with specific probes designed for each gene (Transnetyx Inc., Cordova, TN). All experimental mice were male and were housed under a 12-hour light/dark cycle with food and standard chow provided ad libitum.

To assess whether BGP-15 administration could confer effects on an already established dystrophic pathology, 20-week-old mdx and 8-week-old dko mice were administered BGP-15 (15 mg/kg in 0.9% sterile saline; N-Gen Research Laboratories Inc., New York, NY) daily via oral gavage for 4 (dko) or 5 (mdx) weeks. Age-matched vehicle-treated dystrophic and healthy wild-type control (C57BL/10) mice received an equivalent volume of 0.9% sterile saline via daily oral gavage. Because of the severity of the dko phenotype, a shorter treatment period was used with a significant number of mice reaching humane end point criteria (ie, kyphosis score of 5 and sustained 15% loss of body mass) after 12 weeks of age. The average lifespan of mice in our dko colony was approximately 14 to 15 weeks, with the severity of the dystrophic pathology at 8 weeks of age (when treatment commenced) indicated by an average kyphosis score of 2.5. The kyphosis score indicates the severity of spinal curvature on palpation of conscious mice and ranked 1 to 5, with 1 indicating no spinal deformity and 5 being the most severe. To assess the effect of BGP-15 administration as a preventive treatment for the dystrophic cardiomyopathy and to confirm previous findings on skeletal muscles of young mice, 4-week-old dko mice were administered BGP-15 (15 mg/kg in 0.9% sterile saline daily via oral gavage) for 5 to 6 weeks, with other groups of aged-matched dko and C57BL/10 mice treated similarly with vehicle only. Because BGP-15 is a hydroxylamine derivative that affects only stressed cells, a group of C57BL/10 mice treated with BGP-15 was not included.
To assess Hsp72 induction via BGP-15, 4- and 10-week-old dko mice and age-matched C57BL/10, mdx, and dko mice. All young mice assessed were 4 weeks of age across all genotypes. The older adult C57BL/10 and mdx mice were 20 weeks of age, but the dko mice were assessed at 10 weeks of age because of their truncated lifespan.

Assessment of Skeletal Muscle Contractile Properties

At the conclusion of the treatment period, mice were anesthetized with sodium pentobarbital (Nembutal; 60 mg/kg; Sigma-Aldrich, Castle Hill, NSW, Australia) via i.p. injection and contractile properties of the mouse TA muscles assessed in situ, as described previously. At the conclusion of these measurements, the TA, extensor digitorum longus, soleus, and quadriceps muscles were carefully excised, blotted on filter paper, and weighed on an analytical balance. The TA muscle was mounted in embedding medium and frozen in thawing isopentane for later histochemical analyses. TA muscle cross-sectional area was determined from the equation: Cross-Sectional Area = Muscle Mass/(L_f × 1.06), where L_f represents optimal fiber length and 1.06 represents the density of mammalian skeletal muscle. The entire diaphragm and rib cage were surgically excised, and costal diaphragm muscle strips composed of longitudinally arranged full-length muscle fibers were isolated and prepared for functional assessment in vitro, as described previously. On completion of the functional analyses, the diaphragm muscle strip was trimmed of tendon and any nonmuscle tissue, blotted once on filter paper, weighed on an analytical balance, mounted in embedding medium, and frozen in thawing isopentane for later histochemical analyses. Mice were sacrificed as a consequence of diaphragm and heart excision while anesthetized deeply. Hearts were trimmed of atria, the midsections removed and mounted in embedding medium, frozen in liquid nitrogen—cooled isopentane, and stored at −80°C for histochemical analyses. The remaining two-thirds of the ventricles were frozen in liquid nitrogen and stored at −80°C for subsequent biochemical analyses.

Echocardiographic Analysis of Cardiac Structure and Function

At the end of treatment, cardiac structure and function were evaluated by transthoracic two-dimensional B- and M-mode echocardiography (GE Vivid 9 15-mHz i13L linear array transducer; General Electric, Fairfield, CT) performed under light anesthesia (inhalation of isoflurane at 1.5%). Acquisition and offline analysis was performed with GE EchoPac software version 110, revision 1.8 (General Electric). The parasternal short axis was used for systolic parameters (interventricular septum, left ventricular posterior wall, left ventricular internal dimension, fractional shortening, ejection fraction, heart rate, stroke volume, end diastolic volume, and end systolic volume). Mitral valve (MV) blood flow Doppler (early filling MV E, active filling MV A) and tissue Doppler (early filling MV E', active filling MV A') were measured in apical four-chamber view for diastolic parameters. For each measurement, at least three consecutive cycles were sampled. Because exercise can exacerbate the pathology in dystrophic mice, mice used for cardiac analysis underwent voluntary wheel running (using Activity Wheel Monitor software model 86065; Lafayette Instruments, Lafayette, IN) overnight, either 48 or 72 hours before echocardiography. There were no differences in parameters between mice that were run 48 or 72 hours before analysis (data not shown).

Evans Blue Dye Uptake and Histology

To quantify cardiomyocyte damage and local areas of necrosis, Evans Blue Dye (Sigma, St. Louis, MO) was injected i.p. (1% w/v; 10 μL per gram body mass), as described previously, and hearts excised and frozen 24 or 48 hours later. Sections (5 μm thick) were cut on a cryostat and Evans Blue Dye detected as red autofluorescence using a fluorescence microscope (Axio Imager D1; Carl Zeiss, Göttingen, Germany). There was no difference in Evans Blue Dye infiltration or echocardiographic parameters between mice injected at 24 or 48 hours before analysis (data not shown). Serial sections (5 μm thick) were cut transversely through the TA, diaphragm, and heart using a refrigerated (−20°C) cryostat (CTI Cryostat; IEC, Needham Heights, MA) and stained with Masson trichrome or Van Gieson stain to assess collagen infiltration. Digital images were obtained using an upright microscope with camera (Axio Imager D1), controlled and quantified using AxioVision AC software release 4.8.2 (Carl Zeiss).

Antibodies

The following primary antibodies were used: Hsp70 (ADI-SPA-812; Enzo Life Sciences, Farmingdale, NY), SERCA1 ATPase (ab2812; Abcam, Cambridge, UK), SERCA2 ATPase (MA3-919; Thermo Fisher Scientific, Scoresby, VIC, Australia), Ser16 phospholamban (PLN; 07-052; Upstate, Lake Placid, NY), Thr17 PLN (AO10-13; Badrilla, Leeds, UK), total PLN (05-205; Upstate), cyclophilin F (D) (ab110324; Abcam), P-p65 (30335; Cell Signaling, Beverly, MA), T-p65 (47645; Cell Signaling), P-Jun N-terminal kinase (JNK) 1 and 2 (ab4821; Abcam), T-JNK 1 and 2.
(Sc-7345; Santa Cruz Biotechnology, Dallas, TX), Mito-Profile (MS604; MitoSciences, Eugene, OR), P-IGF-R1β (3024S; Cell Signaling), and total IGF-R1 (F0805; Santa Cruz Biotechnology). All primary antibodies were diluted in 5% bovine serum albumin/Tris-buffered saline—Tweed-20 at 1:1000 [Hsp70, SERCA1 ATPase, SERCA2 ATPase, P- and T-p65, P- and T-JNK 1, cyclophilin D, MitoProfile, P-insulin-like growth factor-1 receptor (IGFR1β), and T-IGFR1β], 1:3000 (Ser16 PLN, total PLN), or 1:5000 (Thr17 PLN). It was necessary to use a positive control for the detection of phosphorylated IGFR, and C2C12 treated with insulin and anisomycin (Cell Signaling; 21101S) was used for this purpose.

Western Blotting

Skeletal muscles and hearts were immediately snap frozen in liquid nitrogen. Muscle samples (20 to 30 mg) were homogenized (Polytron 2100; Kinematica, Lucerne, Switzerland) for 3 to 15 seconds on ice in homogenizing buffer [10 mmol/L Tris-HCl, pH 7.5; 100 mmol/L sodium chloride; 1 mmol/L EDTA; 1 mmol/L EGTA; 10% glycerol; 1% Triton X-100; 0.1% SDS; 1 mmol/L sodium fluoride; 20 mmol/L sodium pyrophosphate; 2 mmol/L sodium orthovanadate; 0.5% sodium deoxycholate; 1 mmol/L phenylmethanesulphonyl fluoride; 0.1% protease inhibitor cocktail (P8340; Sigma-Aldrich), and approximately 20 μL of whole muscle were added to 1 mL of reaction buffer in a plastic cuvette. Cuvettes were loaded into a spectrophotometer, and A340 was measured at 37°C (Multiscan Spectrum; Thermo Electron, Waltham, MA). Maximal SERCA activity was determined by progressively adding 100 mmol/L CaCl2 until a plateau or maximal activity was reached. The specific SERCA inhibitor 29, 59-di(tert-butyl)-1, 4-benzohydroquinone, was added to a final concentration of 40 mmol/L to determine basal activity.

Superoxide Indicator Dihydroethidium Intensity

Fresh frozen heart cross sections (5 μm thick) were incubated in 2 mmol/L dihydroethidium (Life Technologies Australia, Scoresby, VIC) (0.1% dimethyl sulfoxide) in phosphate-buffered saline at 37°C for 30 minutes. Sections were rinsed in phosphate-buffered saline before air drying and application of cover with fluorescent mounting medium. Dihydroethidium intensity was detected as red fluorescence using a fluorescence microscope (Axio Imager D1).

Statistical Analysis

Data were analyzed with GraphPad Prism software version 7 (GraphPad Software Inc., La Jolla, CA). Unpaired t-tests were used for comparisons between two groups. For comparisons between more than two groups, a one- or two-way analysis of variance was used, as appropriate, with Tukey’s post hoc multiple comparison test when significance was detected. The level of significance was set at P < 0.05 for all comparisons. All values are presented as means ± SEM.

Results

Later-Stage BGP-15 Treatment Does Not Improve Skeletal Muscle Pathology in Older mdx Mice

The mdx mouse is the most widely used animal model of DMD. To test the efficacy of later-stage treatment on the dystrophic muscle pathology, 20-week-old mdx mice were administered BGP-15 (15 mg/kg per day) via oral gavage for 5 weeks. Treatment did not change body mass or muscle mass normalized to body mass (Figure 1, A and B) and did not improve maximal force output or force output over a range of stimulation frequencies in either diaphragm muscle strips (in vitro) or TA muscles (in situ) (Figure 1, C–F). Fibrotic infiltration was not different in the diaphragm...
(Figure 1, G and I) or TA muscles (Figure 1, H and J) of treated compared with untreated older mdx mice. Because the most significant pathological alterations occur in mdx mice between 2 and 8 weeks of age and the disease progresses relatively slowly until approximately 18 months of age, the lack of an effect of BGP-15 treatment in older mdx mice is likely attributed to the lack of disease progression during this period.

BGP-15 Induces Hsp72 Expression in the TA Muscles and Heart But Not in the Diaphragm of dko Mice

The dko mouse, which lacks dystrophin and the homologous protein utrophin, exhibits a severe phenotype that more closely resembles the disease progression in DMD. Although BGP-15 can induce Hsp72 in the diaphragm muscles of mdx mice, its capacity to induce
Hsp72 expression in dko mice had not been determined. Because BGP-15 indirectly induces Hsp72 expression by activating heat shock factor 1 (HSF-1), the basal expression of which is reduced in other models of myopathy,\textsuperscript{10} it is possible that Hsp72 induction could be diminished if the pathology is well progressed. The effect of BGP-15 on Hsp72 and HSF-1 protein expression was investigated in TA muscles (Figure 2, A and D), diaphragm (Figure 2, B and E), and hearts (Figure 2, C and F) of young 4-week-old dko mice and more severely affected 10-week-old dko mice. Muscles were excised 6 hours after administration of a single bolus of BGP-15 (15 mg/kg) or saline, which has previously been shown to be the optimal time point for Hsp72 induction.\textsuperscript{12} No BGP-15–treated C57BL/10 mice were included because BGP-15 induces Hsp72 expression only in stressed cells and has no effect on otherwise healthy wild-type mice.\textsuperscript{10,14,19,34} We verified this in 4- and 10-week-old C57BL/10 mice administered a single bolus of BGP-15 (15 mg/kg) or saline via oral gavage, where Western blot analysis revealed no induction of Hsp72 in TA muscles, diaphragm, or hearts of wild-type mice at either age after BGP-15 administration (Supplemental Figure S1).

Hsp72 expression was induced significantly in the TA muscles and hearts of 4- and 10-week-old dko mice (Figure 2, A, C, D, and F), but not in the diaphragm of 4- (\(P = 0.1\)) or 10- (\(P = 0.98\)) week-old dko mice (Figure 2, B and E). HSF-1 expression was not different between 4- and 10-week-old dko mice and not altered after BGP-15 treatment (Figure 2, A–F). Comparison of Hsp72 induction levels between 4- and 10-week-old mice revealed no differences in TA, diaphragm, and heart muscles from dko mice (Supplemental Figure S2). In addition, no differences were observed in HSF-1 protein expression between 4- and 10-week-old dko mice (Supplemental Figure S3).

The basal expression levels of HSF-1 and Hsp72 were also assessed to see if there was an impact on...
BGP-15–mediated induction. Hsp72 protein expression was elevated in TA muscles from 10-week-old dko mice compared with C57BL/10 and mdx mice (Supplemental Figure S4A). HSF-1 protein expression was elevated in the TA muscles of 4-week-old mdx mice compared with 10-week-old mdx mice, respectively (Supplemental Figure S6A). However, no significant differences in HSF-1 protein expression were observed between groups in the heart (Supplemental Figure S6B).

Later-Stage BGP-15 Treatment Reduces Fibrosis in TA Muscles of Older dko Mice

Daily BGP-15 treatment of young dko mice delayed progression of the dystrophic pathology in skeletal muscles.14
To determine the efficacy of BGP-15 in mice with established muscle pathology, 8-week-old *dko* mice were administered BGP-15 (15 mg/kg per day) via oral gavage for 4 weeks. This shorter treatment period (compared to 5 weeks in *mdx* mice) was used because the phenotype of *dko* mice is severe and a large number of mice meet the humane end point criteria (kyphosis score of 5 and sustained 15% loss of body mass) by 12 weeks of age. BGP-15 treatment did not alter body mass (Figure 3A), muscle mass (Figure 3B), or kyphosis score (Figure 3C) in older *dko* mice with the established dystrophic pathology. No differences in maximal force of diaphragm muscle strips (Figure 3, D and E) or TA muscles (Figure 3, F and G) were observed between treated and untreated older *dko* mice. Collagen infiltration in the diaphragm was not altered with treatment (Figure 3, H and K) but was approximately 50% lower in TA muscles of treated *dko* mice compared with untreated *dko* mice (Figure 3, I and L). Collagen infiltration in ventricular cross sections was also lower in BGP-15-treated *dko* mice compared with saline-treated *dko* mice (Figure 3, J and M). Together, these data indicate more limited beneficial effects of BGP-15 when administered to older dystrophic mice with established disease pathology.

**Later-Stage BGP-15 Treatment Does Not Improve SERCA Function in Muscles of Older *dko* Mice**

Preserved SERCA function contributed to the improved dystrophic muscle pathophysiology in young *mdx* mice receiving BGP-15 early in life. We therefore investigated whether BGP-15 treatment to older *dko* mice affected SERCA1 and SERCA2 protein expression and maximal SERCA activity in diaphragm and TA muscles. In the diaphragm, SERCA1 but not SERCA2 protein expression was lower in saline-treated *dko* mice compared with C57BL/10 controls (Figure 4, A and C). However, BGP-15 treatment did not significantly alter SERCA1 or SERCA2 protein expression compared with C57BL/10 controls or saline-treated *dko* mice (Figure 4, A and C). Maximal SERCA activity was

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**Figure 4** Sarcoplasmic/endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) protein expression and maximal activity in preparations from diaphragm and tibialis anterior (TA) muscles from older *dko* mice treated with BGP-15 as a later-stage treatment compared with saline-treated *dko* and C57BL/10 mice. Representative blots and quantification of SERCA1 protein expression in the diaphragm (A) and TA (B) muscles, SERCA2 protein expression in the diaphragm (C) and TA (D) muscles and maximal SERCA activity in the diaphragm (E) and TA (F) muscles from older BGP-15–treated *dko* mice compared with saline-treated *dko* and C57BL/10 mice. Protein expression was normalized to total protein expression. A straight line through representative membranes indicates where they have been spliced (C), *n* = 8 (A–D); *n* = 10 to 12 (E and F). *P* < 0.05 versus C57BL/10. a.u., arbitrary unit.
decreased in diaphragm muscles from dko mice compared with C57BL/10 mice but was not improved with BGP-15 treatment (Figure 4E). In TA muscles, no differences in SERCA1 or SERCA2 protein expression or maximal SERCA activity were evident between groups (Figure 4, B, D, and F).

**Early-Stage BGP-15 Treatment Improved Cardiac Pathology in Young dko Mice**

In older dko mice, there was reduced collagen infiltration in ventricular cross sections in BGP-15–treated compared with untreated mice (Figure 3, J and M). Because BGP-15 induced the greatest improvements in skeletal muscle function when administered as an early intervention,14 we also investigated whether treatment (15 mg/kg per day for 6 weeks) improved cardiac pathology in young 4-week-old dko mice. Because the phenotype of different dko mouse colonies can vary over time, we first confirmed the efficacy of BGP-15 to improve the skeletal muscle pathology in these mice. Consistent with our previous observations,14 BGP-15–treated mice showed a significant reduction of collagen infiltration in the diaphragm (Supplemental Figure S7, A and B). Assessment of some hallmarks of the cardiac pathology revealed approximately 50% less collagen infiltration in ventricular cross sections of BGP-15–treated young dko mice (Figure 5, A and C). Increased permeability of the sarcolemma, as evident from Evans Blue Dye infiltration, was reduced by approximately 75% in the hearts of BGP-15–treated dko mice (Figure 5, B and D). These improvements occurred without any effect on heart mass normalized to tibial length (Figure 5E). Furthermore, tibial length did not vary between groups (C57BL/10 + saline, 12.86 ± 0.25 mm; dko + saline, 12.55 ± 0.12 mm; dko + BGP-15, 12.96 ± 0.19 mm; P = 0.29, n = 8 to 9).

Echocardiography at the end of the treatment period revealed left ventricular remodeling in the hearts of saline-treated dko mice (Figure 5F), with thinner posterior wall thickness (H), lower normalized heart mass (E), and lower ejection fraction (I). These effects were observed in dko mice treated with BGP-15, with similar changes seen in saline-treated C57BL/10 controls (Figure 5A and G). Original magnification: ×126 (A); ×63 (B).

![Collagen infiltration, membrane integrity, and echocardiographic analyses of the structure and function of hearts from young dko mice administered BGP-15 as an early-stage treatment compared with saline-treated dko and C57BL/10 control mice. Representative images of cardiac muscle cross sections stained with Van Gieson (A) and Evans Blue Dye (EBD; B) indicating collagen infiltration (pink, highlighted by arrows) and reductions in membrane integrity (red), respectively. Quantification of Van Gieson stain showing the percentage collagen infiltration in heart cross sections (C) and of EBD infiltration relative to total muscle area (D). E: Heart mass was normalized to tibial length. F: Representative images from M-mode echocardiogram showing interventricular septal wall, left ventricular (LV) posterior wall, and left ventricle chamber diameter during cardiac cycling. Quantification of posterior wall thickness (G) and septal wall thickness (H) at the end of diastole. I: Diastolic function was determined by deceleration time (MVDec T) of blood flow during early ventricular filling. J: Systolic function was represented by ejection fraction (%) assessed by B-mode echocardiogram. n = 6 to 8 (C–E); n = 10 to 12 (G–J). *P < 0.05 versus C57BL/10 + saline; †P < 0.05 versus dko + saline. Scale bars: 100 μm (A and B). Original magnification: ×126 (A); ×63 (B).
Systolic function

Diastolic function

(Blood pressure) (Figure 5G) and septal walls compared with C57BL/10 mice (Figure 5H). These parameters were not improved with BGP-15 treatment. Diastolic function was reduced significantly in saline-treated dko mice compared with C57BL/10 control mice (Figure 5I). Diastolic function of BGP-15-treated dko mice was not significantly different compared with saline-treated dko mice or C57BL/10 mice (Figure 5I). Systolic function was not different between saline-treated dko mice and C57BL/10 mice, but was 20% higher in BGP-15-treated dko mice (Figure 5I). No changes were observed in left ventricular chamber diameter between BGP-15-treated and saline-treated dko mice (Table 1). End diastolic volume was reduced in both dko groups, and end systolic volume was reduced only in the BGP-15-treated dko mice (Table 1). Active filling of the left ventricle, assessed by blood flow Doppler (MV A), was increased in both groups of dko mice (Table 1).

Early BGP-15 Treatment Does Not Alter SERCA Activity, Mitochondrial Proteins, or Inflammatory Markers in Hearts of Dystrophic Mice

To assess whether SERCA regulation contributes to the improved cardiac pathology in BGP-15-treated dko mice, SERCA2 protein expression and PLN phosphorylation were examined. PLN is a regulatory SERCA accessory protein that can be activated by phosphorylation at Ser16 and Thr17, and so both sites were assessed via Western blot and normalized to total PLN. Neither SERCA2 protein expression (Figure 6A) nor PLN phosphorylation at Ser16 (Figure 6B) or Thr17 (Figure 6C) was altered after BGP-15 treatment.

Previous studies identified mitochondrial dysfunction and JNK phosphorylation as potential mechanisms in myopathies other than muscular dystrophy. We found no changes in phosphorylation of p65 (a component of the nuclear factor κB inflammatory pathway) (Figure 6D), JNK (Figure 6, E and F), cyclophilin D (Figure 6G), superoxide indicator dihydroethidium (Figure 6H), or mitochondrial electron transport chain subunits (I-V) (Figure 6, I–M) in hearts from BGP-15-treated dko mice compared with untreated dko mice and C57BL/10 mice. However, some of these findings may be complicated by insufficient power. These parameters were also assessed in hearts from older dko mice treated with BGP-15, but no improvements were evident (Supplemental Figure S8).

BGP-15 Treatment Does Not Alter IGFR Phosphorylation in the Hearts of dko Mice

A recent study investigating the effect of BGP-15 on mice with an atrial fibrillation/heart failure phenotype showed improvements were independent of Hsp72 and corresponded with phosphorylation of the IGFR. Phosphorylated IGFR and total IGFR were therefore assessed in hearts from young and old dko mice treated with BGP-15.
However, unlike this previous study, the phosphorylation levels detected via Western blot were too low for reproducible, quantifiable results. Phosphorylated and total IGFR were run on separate gels as the protein levels of IGFR did not allow for membrane stripping (Supplemental Figure S9, A and B). Although there were no apparent differences between groups, a contribution of IGFR phosphorylation remains possible.

**Discussion**

Consistent with our previous finding that BGP-15 improved the dystrophic pathology of skeletal muscles from young mdx and dko mice, we confirmed that BGP-15 treatment of 4-week-old dko mice reduced skeletal muscle collagen infiltration and now reveal an improved cardiac pathology compared with untreated mice. Although BGP-15 treatment improved some aspects of the dystrophic pathology, such as fibrosis, it did not improve skeletal muscle function in older mdx or dko mice. Our findings identify a therapeutic window for BGP-15 treatment of muscular dystrophy in dko mice. For improvements in skeletal muscle parameters, BGP-15 should be administered as early as possible to slow the disease progression, whereas cardiac benefits were evident even when treating older mice. These results have therapeutic implications for when treatments should be administered clinically to different stages of the DMD pathology.

Although we had previously identified the therapeutic potential of Hsp72 induction and BGP-15 treatment for
slowing the progression of the dystrophic pathology in the limb muscles and diaphragm of young mdx and dko mice. These benefits of early treatment in dystrophic mice may not translate easily to the clinical setting given that DMD is not usually diagnosed until a significant muscle pathology is apparent. The lack of improvement in the skeletal muscle pathology of older mdx mice with BGP-15 treatment is not surprising. Given that the pathology is relatively stable at this age, and consistent with our previous findings showing that early- but not later-stage intervention with a myostatin inhibitory antibody improved the dystrophic pathology in mdx mice, BGP-15 treatment is likely able to slow the disease progression but not reverse the pathology.

Basal expression levels of Hsp72 are higher in muscles of DMD patients and young mdx mice. We assessed the basal expression of Hsp72 in the TA, diaphragm, and hearts of dko mice compared with C57BL/10 to determine whether this affected BGP-15-mediated induction. Although there were significant differences observed between groups, Hsp72 levels did not decrease with age and did not account for the pattern of Hsp72 induction observed after acute BGP-15 treatment. Therefore, differences in basal Hsp72 expression are unlikely to explain our observations that Hsp72 was not significantly induced in the diaphragm muscle after BGP-15 administration. It is possible that excessive collagen accumulation delays Hsp72 induction in the diaphragm muscle and we simply missed the increased expression because of the timing of sample collection (6 hours after BGP-15 administration). In addition, the lack of a clear relationship between changes in HSF-1 and HSP72 protein expression is not surprising as changes to HSF-1 expression occur earlier than with Hsp72. Thus, the absence of altered HSF-1 and Hsp72 expression after BGP-15 treatment does not definitively refute this mechanism. Sapra et al demonstrated phosphorylation of IGFR to be an alternative mechanism for BGP-15, improving cardiac function in mice with an atrial fibrillation/heart failure phenotype. We similarly assessed this mechanism but could not confirm phosphorylation of IGFR in the hearts of dystrophic mice after BGP-15 administration. If IGFR phosphorylation contributes to improvements in the hearts of dko mice, then its involvement is less apparent than in previous studies.

We have shown that early-stage treatment of BGP-15 to more severely affected young dko mice reduced fibrosis in the diaphragm, increased lifespan, and improved skeletal muscle function. Treating older dko mice with BGP-15 did not improve force-producing capacity of TA muscles and diaphragm muscle strips but reduced fibrosis in the TA muscles. Together, these data show reduced therapeutic efficacy of BGP-15 for dystrophic skeletal muscles when the disease is already well advanced. This is consistent with gene and pharmacological interventions that have had limited therapeutic efficacy as later-stage interventions.

Although respiratory insufficiencies have previously accounted for approximately 90% of deaths in DMD, cardiomyopathy has become more prevalent and a critical aspect of the disease, presumably attributed to the increased use of corticosteroids and benefits of ventilator assist devices. Because specific therapeutic targeting of the skeletal musculature (independent of the heart) can exacerbate the cardiomyopathy in mdx mice and prolonged ambulation in patients has been hypothesized to worsen the heart pathology, targeting both skeletal and cardiac muscles is important so as to avoid accelerating the cardiac pathology. Our previous study did not assess the effect of BGP-15 on the dystrophic heart, and because we observed the most pronounced improvements in the skeletal muscle pathology when mice were treated from a young age, we examined the effects of early-stage BGP-15 treatment on the hearts of young dko mice. BGP-15 reduced fibrosis, improved membrane integrity, and enhanced systolic function in dko mice. This is the second study to demonstrate the efficacy of

![Diagram of disease progression of dko mice](image-url)
BGP-15 for cardiac pathologies, with Sapra et al showing similarly beneficial effects in mice with an atrial fibrillation/heart failure phenotype. Although one study observed improved cardiac pathology with specific therapeutic targeting of skeletal muscle in dystrophic mice and hypothesized that improvements in diaphragm function could drive improvements in heart function, it has also been shown that specific rescue of skeletal muscles in mdx mice induced cardiac dysfunction. This effect is also observed in patients experiencing inherited X-linked dilated cardiomyopathy, where loss of dystrophin in the heart with little or no skeletal muscle pathology leads to an early-onset cardiac phenotype. Therefore, if BGP-15 treatment was not exerting a direct effect on the heart, an exacerbation of cardiac pathology would be likely. Our observation of a clear and significant induction of Hsp72 in the hearts of dko mice means that BGP-15 treatment is having a direct effect on this tissue. As systolic function was not reduced in saline-treated dko mice relative to control, it was surprising to observe increased systolic function in the hearts of dko mice after BGP-15 administration. This effect is likely specific to the dystrophic condition because previous studies have shown no change in systolic function after BGP-15 administration to control mice. We show herein that BGP-15 can improve aspects of the cardiac pathology and that treatment is not detrimental to the dystrophic heart. Although there is scope for the primary mechanism of cardiac improvement to derive from effects on the diaphragm or right ventricle, our findings show that BGP-15 treatment can improve pathology in dystrophic hearts and has clinical merit for treating the cardiac pathology in DMD patients.

BGP-15 treatment has been shown to increase mitochondrial number and oxidative capacity and alter JNK signaling in other models of myopathy, but we found mitochondrial dysfunction and inflammatory signaling were not different between dystrophic and wild-type mice and were unaltered with treatment. Despite BGP-15 having beneficial effects on the hearts of dko mice, the mechanisms underlying these improvements are unclear and further studies are required to elucidate these processes.

Based on findings from this study and our previous study, we can conclude that in dystrophic mice, BGP-15 treatment improved diaphragm muscle pathology only when administered early in life and that the TA muscles and heart showed structural and functional benefits with early treatment, but also conferred structural improvements in older mice with a later-stage intervention. BGP-15’s efficacy to improve the dystrophic pathology did not correlate with basal expression levels of Hsp72 in the muscles tested but was related to the stage when treatment commenced relative to the severity of disease, highlighting an optimal therapeutic window where each muscle can be targeted most effectively (Figure 7). A similar therapeutic window exists for antibody-mediated myostatin inhibition showing benefits in young mdx mice but not older mice, and peptide-conjugated phosphorodiamidate morpholino oligomers—mediated exon skipping approaches being unable to prevent disease progression when administered to dko mice with an advanced pathology, despite a near complete restoration of dystrophin. Clearly, different therapeutic approaches for DMD face similar limitations for optimal efficacy based on when treatment commences relative to the disease progression.

This notion is also supported by our finding that BGP-15 treatment in dko mice exerted muscle-specific effects. Previous analyses in dko mice revealed pathological features evident in the diaphragm within the first weeks of life and in TA muscles 3 to 4 weeks later, but effects in the heart were not observed until later in life. An early intervention with BGP-15 improved the pathology in all muscles examined, whereas efficacy was diminished when treatment commenced later in older mice. The findings also suggest that muscles with a slower dystrophic progression (eg, the heart) can still benefit from a later intervention.

Fibrosis can be an irreversible feature of the DMD pathology and is likely to be a major factor defining the therapeutic window for interventions like BGP-15. Fibrosis also impairs the efficacy of gene- and cell-based therapies by acting as a physical barrier for cell and viral delivery, replacing muscle fibers that could be targeted and restricting the spaces in which myofibers can regenerate. Thus, early prevention of collagen infiltration, like with BGP-15, may improve treatment efficacy and expand the therapeutic window of opportunity for gene- and cell-based approaches when they eventually become viable for clinical implementation.

Although these findings support the efficacy of BGP-15 for treating the dystrophic pathology, further studies are required to clarify the mechanism by which these benefits occur to highlight alternative clinical applications for BGP-15 and clarify potential limitations. In addition, understanding how BGP-15 elicits different induction patterns of the Hsps across tissue types will help optimize its application. Our findings that BGP-15 can improve the cardiac pathology even in older dko mice help define the therapeutic window where it is likely to confer benefits for the different aspects of the dystrophic pathology and support its potential as a promising therapy for DMD.

**Supplemental Data**

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2016.08.008.

**References**


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15. Ermlerova NV, Martinez L, Vetrone SA, Jordan MC, Roos KP, Sweeney HL, Spencer MJ: Long-term administration of the TNF blocking drug Remicade (cV1q) to mdx mice reduces skeletal and cardiac muscle fibrosis, but negatively impacts cardiac function. Neuromusc Disord 2014, 24:583–595