Early right ventricular fibrosis and reduction in biventricular cardiac reserve in the dystrophin-deficient mdx heart

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Meyers TA, Townsend D. Early right ventricular fibrosis and reduction in biventricular cardiac reserve in the dystrophin-deficient mdx heart. Am J Physiol Heart Circ Physiol 308: H303–H315, 2015. First published December 5, 2014; doi:10.1152/ajpheart.00485.2014.—Duchenne muscular dystrophy (DMD) is a progressive disease of striated muscle deterioration. Respiratory and cardiac muscle dysfunction are particularly clinically relevant because they result in the leading causes of death in DMD patients. Despite the clinical and physiological significance of these systems, little has been done to understand the cardiorespiratory interaction in DMD. We show here that prior to the onset of global cardiac dysfunction, dystrophin-deficient mdx mice have increased cardiac fibrosis with the right ventricle being particularly affected. Using a novel biventricular cardiac catheterization technique coupled with cardiac stress testing, we demonstrate that both the right and left ventricles have significant reductions in both systolic and diastolic function in response to dobutamine. Unstimulated cardiac function is relatively normal except for a significant reduction in the ventricular pressure transient duration compared with controls. These biventricular analyses also reveal the absence of a dobutamine-induced increase in isovolumic relaxation in the right ventricle of control hearts. Simultaneous assessment of biventricular pressure demonstrates a dobutamine-dependent enhancement of coupling between the ventricles in control mice, which is absent in mdx mice. Furthermore, studies probing the passive-extension properties of the left ventricle demonstrate that the mdx heart is significantly more compliant compared with age-matched C57BL/10 hearts, which have an age-dependent stiffening that is completely absent from dystrophic hearts. These new results indicate that right ventricular fibrosis is an early indicator of the development of dystrophic cardiomyopathy, suggesting a mechanism by which respiratory insufficiency may accelerate the development of heart failure in DMD.

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Duchenne muscular dystrophy (DMD) is an X-linked disease characterized by progressive skeletal and cardiac muscle degeneration (14, 21), resulting from the loss of the protein dystrophin (29). Dystrophin is a large multidomain cytoskeletal protein that has many cellular functions, including forming a link between the actin cytoskeleton and the sarcolemmal membrane of the striated muscle cell (24, 25). From this subsarcolemmal location it has been demonstrated that dystrophin provides mechanical support to the membrane (47, 73), contributes to the transmission of force from the sarcomeres to the extracellular matrix (52), and serves as a signaling scaffold (8, 27). Clinically, DMD presents initially with progressive skeletal muscle weakness during the first decade of life. The initial stages of the disease are primarily the result of degeneration of skeletal muscles which are, over time, replaced with a mixture of adipose tissue and fibrosis such that most patients are nonambulatory by their teenage years (11). As the disease progresses, the muscles of respiration are increasingly affected resulting in significant respiratory insufficiency (4, 31, 42). This respiratory failure is a leading cause of death in DMD and therapies that improve ventilation have resulted in significant increases in life expectancy for DMD patients (22, 23).

Around the time that respiratory dysfunction becomes apparent, so too does cardiac function begin to falter. Careful examination of cardiac function reveals that subclinical dysfunction is often present even earlier in the disease process (33, 54). As in the skeletal muscle, the cardiac manifestation of DMD is characterized by a relentless downward trajectory (15, 53). This decline in cardiac function can be slowed using steroids and standard heart failure treatments (40, 56), but there remains no effective specific therapy for DMD, and inevitably these patients die of respiratory and/or cardiac complications.

Our understanding of the molecular pathogenesis of DMD has been greatly assisted by the dystrophin-deficient mdx mouse (10). This mouse has a relatively mild phenotype, with subclinical myopathic disease evident at baseline. However, it is clear that the mdx mouse is susceptible to muscle injury as either stress (18, 20, 34, 47, 73) or age (16, 37, 51, 68) result in significant myopathic changes becoming evident. In some sense the mdx mouse provides insights into the earlier aspects of the disease, offering the opportunity to understand the mechanisms underlying the initiating aspects of the dystrophic disease process. Of all the skeletal muscles in the mdx mouse, the diaphragm shows the most significant levels of fibrosis and degeneration (61). This pathology translates into a progressive reduction in respiratory function in the mdx mouse, with dysfunction present as early as 3 mo of age (30, 32, 44). This chronic respiratory insufficiency of the mdx mouse makes this model particularly valuable for examining how respiratory dysfunction interacts with the dystrophic heart.

Alveolar hypoxia secondary to hypoventilation by weakened respiratory muscles presents a unique hemodynamic challenge to the dystrophic heart. Specifically, decreases in alveolar oxygen levels induce constriction of the pulmonary arterial tree. This increase in pulmonary resistance places an additional afterload upon the right ventricle and decreases the preload filling the left ventricle. Evidence of increased pulmonary vascular resistance has been observed in mdx mice where studies using cardiac magnetic resonance imaging show right ventricular dilation (17, 74). These changes are consistent with an increase in right ventricular afterload. Other studies using catheter-based hemodynamic approaches have shown a reduction in left ventricular preload, also consistent with an increase in pulmonary vascular resistance decreasing the loading of the left ventricle (66). Interestingly, transgenic replacement of dystrophin in skeletal muscle, including the diaphragm, cor-
METHODS

Clinical management of DMD patients. The consequences of correcting dystrophic skeletal muscle without the correction of the heart are not clear. Studies in mice give mixed results, with some studies suggesting improvements in cardiac function with the selective correction of skeletal muscle (17), some showing no effect (69), and others demonstrating increased myocardial damage (66). Importantly, human patients with selective disruption of dystrophin expression in the myocardium display a severe cardiac phenotype with earlier onset and more rapid progress of heart failure compared with DMD patients (3, 63).

In this study a novel biventricular cardiac catheterization procedure is used to examine both right and left ventricular function of the dystrophic mdx heart. The simultaneous recording of both right and left ventricular function permits a detailed characterization of how the dystrophic disease process affects the two pumping chambers of the heart. This analysis is essential for assessing the role that alterations in pulmonary circulation have on the development of dysfunction in both the right and left ventricles of the dystrophic heart. These investigations are significant because a detailed understanding of the hemodynamic consequences of respiratory insufficiency and its potential to accelerate cardiac dysfunction could impact the clinical management of DMD patients.

METHODS

Animals. Initial studies defining the procedures and measures of the biventricular recordings were performed in young 3- to 4-mo-old C57BL/6J mice obtained directly from Jackson Labs (Bar Harbor, ME). Both mdx (C57BL/10ScSn-Dmdmdx) and the background control (C57BL/10 SnJ) mice were obtained from a colony maintained at the University of Minnesota. The genetics of these colonies were maintained by purchasing new breeders from Jackson Labs every four generations. Subanalysis of previously published hemodynamic data demonstrated that there was no significant difference between male and female mdx mice in the 4- to 7-mo-old age range, furthermore no significant sex differences were observed in the 9- to 10-mo-old cohort in this study, although very old female mdx mice do display decreased cardiac function (7). The lack of evidence for sex-dependent hemodynamic changes in our target age group led us to use both sexes in these studies. All studies were reviewed and approved by the University of Minnesota’s Institutional Animal Care and Use Committee.

Cardiac catheterization. Biventricular catheterization was developed from the techniques required for left ventricular catheterization described elsewhere (64, 73). Briefly, animals were anesthetized with 2% isoflurane and ventilated with 4 cmH2O positive end-expiratory pressure (PEEP). The apex of the heart was exposed through a thoracotomy and removal of the pericardium. A 1.2-Fr pressure transducer (Transonic, Ithaca, NY) was inserted into the right ventricle through an apical stab incision made with a 27-gauge needle. The course of this incision included a small part of the septum (see Fig. 3) to provide sufficient myocardial tissue to prevent leakage through the right ventricular wall. This was particularly important for smaller mice with right ventricular walls too thin to seal around the catheter. Next, the 1.2-Fr pressure-volume catheter (Transonic) was inserted through a second stab incision into the left ventricular cavity. Several parameters used to calculate the volume were obtained in preliminary pilot studies including blood resistivity (1.2 ohm-m) and epsilon: sigma ratio (800,000). Left ventricular catheter placement was optimized to obtain low-amplitude oscillations of the degree parameter, indicating that the catheter was centered in the ventricular chamber. End-systolic and end-diastolic parallel conductances were determined prior to the beginning of the experimental protocol.

Once the animal was instrumented, 6–8 ml/kg of 10% albumin was infused through a jugular catheter over a 10-min period to replace both insensible and blood volume loss during surgery. Anesthesia was decreased to 1% isoflurane and the temperature stabilized to 37°C. Following this initial stabilization period, baseline hemodynamic data were collected. Hemodynamic data include occlusion of the inferior vena cava within the thoracic cavity and compression of the abdominal viscera. Stepwise dobutamine doses were given by constant-rate infusions with a maximal volume of 0.3 ml kg⁻¹ min⁻¹ delivered by a syringe pump (Kent Scientific, Torrington, CT). Analog data were collected by a Ponemah DAQ16 (DSI, St. Paul, MN).

Imaging studies. Hearts were fixed overnight in 10% formalin and stored in 70% ethanol prior to paraffin embedding and sectioning. Short-axis cross-sections of the hearts were sectioned at 4 µm and stained with hematoxylin and eosin and Sirius red/fast green using standard histochernical procedures. Images were collected on a Nikon E200 microscope fitted with polarizing filters. Individual images were merged using an image stitching plug-in in ImageJ (49) to form a single large high-resolution image of the entire section. These montaged images were used for the subsequent quantification. All quantification of the fibrosis was performed in a blinded fashion on images de-identified using randomly generated numerical file names. For assessment of the regional distribution of lesions, the montaged heart images were divided into three domains: the right ventricular free wall, the left ventricular free wall, and the interventricular septum. Fibrosis-positive areas of these sections were determined by splitting the color channels of the bright-field sirius red/fast green images. The blue channel was thresholded to provide the total number of pixels within the heart. Fibrotic areas were determined by setting a negative threshold in the green channel, as the lesions contain very low levels of green. Two sections from most hearts were analyzed, having been taken from distinct regions along the long axis of the heart. The raw quantification results from both sections were combined to produce the final measure of fibrosis for each heart.

Data analysis and statistics. Hemodynamic data were analyzed by the Ponehma Physiological suite. Additional analysis of beat-to-beat interactions was performed by custom Excel (Microsoft, Redan, WA) macros that allowed for the efficient characterization of changes associated with various loading conditions. Representative transients are the average of 15–30 beats, aligned to the peak pressure and averaged (MatLab, Mathworks, Natick, MA). Statistical analyses were performed using Prism (GraphPad, La Jolla, CA) and R (version 3.0.2). Student’s t-test was only used for the comparison of two data sets; all other analyses used a two-way ANOVA with a Tukey post hoc test to assess the statistical significance of multiple comparisons.

RESULTS

Dystrophic cardiomyopathy is a progressive disease that is characterized by the accumulation of fibrosis throughout the myocardium. We examined histological sections of hearts from 9-mo-old mdx and control C57BL/10 mice. In agreement with previous work, the mdx hearts display significantly more overall fibrosis than age-matched C57BL/10 controls (Fig. 1). This increased level of fibrosis is evident in young (4–6 mo old) mdx mice and continues to increase with age. Further morphological analysis reveals that the right ventricle of the mdx mouse heart accumulates significantly more fibrosis than either the septum or left ventricular free wall (Fig. 2). In contrast, at 9 mo of age, C57BL/10 mice have only small differences in the levels of fibrosis between regions of the
heart: 2.7 ± 0.3%, 1.8 ± 0.2%, and 2.3 ± 0.2% for the right ventricle, interventricular septum, and left ventricle, respectively. In addition to elevated levels of fibrosis, the rate of accumulation of right ventricular fibrosis in the dystrophic heart is nearly 50% greater than that observed in either the left ventricular free wall or interventricular septum: 0.53 ± 0.13, 0.34 ± 0.06, and 0.37 ± 0.07%/mo for the right ventricle, interventricular septum, and left ventricle, respectively. The morphology of the fibrosis consists of areas of replacement fibrosis and areas with significant increases in interstitial fibrosis. The birefringence signal observed in C57BL/10 sections is uniformly green to yellow in color in contrast to the orange to red birefringence associated with fibrosis in the mdx heart sections. This shift to longer wavelengths of birefringence has been documented to occur with larger, more tightly packed collagen bundles (19). This suggests that the fibrosis present in the dystrophic heart is both more widely distributed and consists of thicker strands of collagen.

To assess global function of the dystrophic heart, both right and left ventricular function needs to be assessed. To address this need, a murine biventricular catheterization methodology was developed to allow simultaneous measurements of both right and left ventricular function (Fig. 3A). This approach allows for the detection of alterations in both ventricular coupling and the contractile function of each ventricle. The left ventricle is assessed using pressure-volume loop analysis (Fig. 3, B and C). This methodology is well suited for the left ventricle where the symmetry of its cavity is well approximated by a cylinder, the geometry assumed by the single segmented conductance catheter. In contrast, the asymmetrical geometry of the right ventricle makes this methodology more difficult to interpret, so in these studies only a pressure catheter was used to assess right ventricular function. An important strength of pressure-volume loop analysis is the ability to transiently vary the loading conditions on the heart while collecting pressure and volume data throughout. In these studies two procedures are used to transiently alter the loading of the heart. First is the occlusion of the inferior (caudal) vena cava, a procedure which blocks venous return from the caudal portion of the mouse. As expected, this decrease in preload is first evident in the right ventricle, where peak systolic pressure begins to decline 3–4 beats before the beginning of the reduction in left ventricular systolic pressure. In young C57BL/6 mice, which were used to validate these procedures, it took this reduction in preload 3.7 ± 0.6 beats to transit the lungs and reach the left ventricle. With occlusion of venous return, systolic pressures of both ventricles drop to ~60% of baseline systolic pressure. The second procedure used to alter loading on the heart was abdominal compression. In contrast to occlusion of venous return, abdominal compression affects both ventricles simultaneously. This indicates that this procedure increases both venous return to the right ventricle, by compression of the mesenteric circulation, and afterload on the left ventricle, by compression of the abdominal aorta. The effect is much larger in the left ventricle where systolic pressure increases to 135 ± 3% of baseline compared with 115 ± 2% in the right ventricle. This likely results from the low compliance of the abdominal aorta, where small changes in volume result in large changes in pressure vs. the high compliance of the abdominal venous compartment.

A central aim of this study is to assess the role of right ventricular dysfunction in the pathophysiology of dystrophic cardiomyopathy. The methodologies described above were applied to a population of 9-mo-old mdx and C57BL/10 mice. As seen with the younger C57BL/6, with the occlusion of venous return, the decline in the peak systolic pressure of the right ventricle precedes the decline in the left ventricle (Fig. 3E). The time between reductions in right ventricular pressure and left ventricular pressures is modulated by the capacitance of the pulmonary circulation, which is controlled by changes in pulmonary arterial resistance and pulmonary venous compliance. In C57BL/10 mice, but not mdx, dobutamine induces a significant reduction in the transit time of this pressure wave through the pulmonary circulation (Fig. 3E), likely through a reduction in pulmonary venous compliance. Importantly, hemoglobin saturation levels are not different between genotypes or dobutamine dose (all conditions average ~96%); indicating that hypoxia is not a confounding variable altering pulmonary circulation in this preparation. In the left ventricle the magnitude of the pressure decline resulting from the reduction of...
venous return is significantly lower in mdx mice with pressure drops of $30.5 \pm 2.0$ mmHg compared with a $39.3 \pm 1.9$ mmHg drop in wild-type hearts. A similar difference is present in the right ventricle where pressures drop $11.9 \pm 0.5$ mmHg in C57BL/10 compared with $9.5 \pm 0.8$ mmHg in mdx. Interestingly, abdominal compression results in a significantly greater increase in left ventricular systolic pressure in mdx mice (25.5 $\pm 1.7$ mmHg) compared with C57BL/10 (20.8 $\pm 1.1$ mmHg). There is no significant difference between the genotypes in the right ventricular response to abdominal compression.

The 9-mo-old mdx mice used in this study have relatively normal cardiac function at baseline, but significant deficits are revealed with stimulation by dobutamine. Two-way ANOVA revealed significant decreases in left and right ventricular systolic function in mdx mice compared with C57BL/10 mice (Fig. 4, A–E). The differences between dystrophic and wild-type hearts are most evident with dobutamine stimulation where the systolic function of the left ventricle of mdx mice is significantly attenuated compared with control hearts at both doses of dobutamine. Despite the increased fibrosis, the right ventricle has a relatively normal response to dobutamine, although higher doses are required to observe statistically significant increases in systolic function. Interestingly, both strains demonstrate that the load-independent measure of contractility, preload recruitable stroke work (PRSW), is significantly increased by dobutamine at both doses examined here.
Two-way ANOVA demonstrates that the mdx hearts have lower PRSW but respond to dobutamine to the same degree as C57BL/10 hearts. These data indicate that alterations in the loading of the heart play an important role in the control of the systolic response to dobutamine in the dystrophic heart.

Evaluation of parameters of global cardiac function also reveal significant reductions in the cardiac reserve of 9-mo-old mdx mice. Compared with C57BL/10 mice, mdx mice have lower left ventricular cardiac output and stroke work, a difference that is increased at both doses of dobutamine used in this study (Fig. 5, A and B). Interestingly, there is no difference between the strains in the chronotropic response to dobutamine (Fig. 5D). Two-way ANOVA demonstrates a significant genotype effect indicating a lower stroke volume in mdx hearts compared with that of C57BL/10 hearts (Fig. 5C), suggesting that the significant reductions in cardiac output and stroke work are driven primarily by reductions in stroke volume. These increases are driven by the significant dobutamine-induced reductions in end-systolic volume observed in C57BL/10 hearts, which are not significantly evident in dobutamine-stimulated mdx hearts (Fig. 5E). No detectable changes in the end-diastolic volume in C57BL/10 or mdx hearts are observed [50.0 ± 2.5 (C57BL/10) vs. 47.1 ± 1.8 μl (mdx) at baseline and 51.9 ± 3.0 vs. 51.1 ± 3.7 μl with 15 μg·kg⁻¹·min⁻¹ dobutamine]. While right ventricular volumes are not assessed in these studies, it is likely that the changes in stroke volume observed in the left ventricle are mirrored in the right ventricle as any significant mismatch in cardiac output between the ventricles would be expected to alter diastolic pressures, which was not observed in these studies.

Both at baseline and with dobutamine stimulation, the integral of the left ventricular pressure transient is significantly lower in the mdx heart (Fig. 6). At baseline this reduction is due to a combination of slightly lower systolic pressures and a significant reduction in the duration of the pressure transient (Fig. 6, A and E). For these analyses the pressure transient...
duration is defined as the period between beginning of the pressure increase and the minimum dP/dt. Dobutamine stimulation significantly shortens the transient duration in both C57BL/10 and mdx hearts, such that the pressure transient duration becomes equal between both genotypes. These changes in pressure transient duration are also observed in the right ventricle of mdx and C57BL/10 hearts (Fig. 6, F–J). This reduction in pressure transient duration is also evident in 4- to 6-mo-old mdx and C57BL/10, with left ventricular pressure transient durations of 47.6 ± 1.0 and 51.5 ± 1.4 ms in mdx and C57BL/10, respectively, and right ventricular pressure transient durations of 47.5 ± 1.0 and 52.2 ± 1.3 ms for mdx and C57BL/10, respectively.

Along with these changes in contraction, the dystrophic heart displays significant differences in the dobutamine-induced lusitropic function. In control animals dobutamine causes significant acceleration of isometric relaxation in the left ventricle (Fig. 7, A, C, and E); however, dobutamine has no effect on the isometric relaxation of the mdx heart. The minimum dP/dt in the right ventricle is significantly increased with dobutamine treatment in C57BL/10 mice and, to a lesser degree, in mdx mice (Fig. 7B). In contrast, tau, the time constant of the exponential curve characterizing the decline of the right ventricular pressure transient, is not altered by dobutamine (Fig. 7, D and F). Minimum dP/dt displays significant load dependence (Table 1), which may explain the discrepancy in the right ventricle, favoring tau as a more load-independent measure of relaxation.

There is a significant difference in the end-diastolic pressure-volume relationship between 9-mo-old C57BL/10 and mdx mice. Alterations in left ventricular loading provide the opportunity to examine the passive compliance of the left ventricle. In the mouse, over the volumes examined in these studies, this relationship is well approximated by a linear model ($r^2$ values of 0.83 ± 0.01 for C57BL/10 and 0.83 ± 0.02 for mdx). Surprisingly, these studies demonstrate that older mdx mice have increased compliance compared with C57BL/10, despite increased levels of fibrosis (Fig. 8, B and C). Similar studies performed in 4- to 6-mo-old animals reveal an age-dependent decrease in compliance in C57BL/10 (young 0.31 ± 0.03 vs. old 0.40 ± 0.04 mmHg/µl) that is absent in mdx hearts (young 0.28 ± 0.04 vs. old 0.26 ± 0.03 mmHg/µl).

**DISCUSSION**

Real-time biventricular hemodynamics by direct catheterization in mice, as shown here, provides a unique physiological window into the emerging cardiomyopathy of the dystrophin-deficient heart in vivo. We demonstrate that right ventricular fibrosis precedes left ventricular fibrosis and significant sys-
BIVENTRICULAR DYSFUNCTION IN THE MDX MOUSE

Systolic and diastolic dysfunction is present in both ventricles, including apparent increased organ-level compliance. These findings are evidence that the well-known respiratory dysfunction in DMD results in right ventricular damage. Systolic dysfunction is evident in both ventricles of the mdx heart; however, despite the increased fibrosis, the right ventricular function is slightly better compared with the left ventricle. Our new findings suggest that right ventricular damage precedes the onset of significant cardiac complications. This observation places additional focus on the cardiorespiratory interaction in muscular dystrophy and may have implications for the clinical management of DMD patients.

In the present study we demonstrate that the right ventricle is particularly severely affected throughout the life of the mdx mouse with a larger percentage of fibrosis present in all age groups examined (Fig. 2). The mechanism underlying the increased right ventricular fibrosis is not clear; the size and shapes of the lesions suggest the fibrotic replacement of damaged cardiac myocytes. This is consistent with a model where the respiratory dysfunction of the mdx mouse (30, 32, 44) results in hypoventilation and increased constriction of the pulmonary vessels. This increase in afterload on the right ventricle has the potential to increase membrane damage and myocyte loss within this ventricle, as has been documented in the left ventricle where increases in afterload result in substantial increases in myocyte damage (18, 34). These results support the hypothesis that respiratory dysfunction in the mdx mouse contributes to the progression of dystrophic cardiac disease; however, the possibility that the right ventricle is somehow more susceptible to injury or fibrosis cannot be definitively ruled out. The presence of early right ventricular lesions in DMD patients is difficult to assess. Lesions within the left ventricle are readily assessed using late gadolinium enhancement (41, 50). This method is well suited for picking up relatively large regions of fibrosis, which are present where there is significant muscle mass. The right ventricle, with its small mass and potentially smaller lesion sizes may be underrepresented in these studies. It is important to note that there remains little difference in hemodynamic function between the right and left ventricles of the mdx mouse; similar results are seen in DMD patients (6, 58). The failure to observe interventricular hemodynamic differences may result from the tight coupling of the ventricles, in that dysfunction of the right ventricle will lead to dysfunction of the left ventricle. It should also be noted that we have not documented right ventricular volumes. It is possible that right ventricular dilation may be present, although stroke volume and pressures remain intact.

Fig. 5. Significant reductions in cardiac reserve of aged mdx hearts. In a variety of measures of global cardiac function the 9-mo-old mdx show limited response to dobutamine stimulation. In C57BL/10 mice there are significant changes in cardiac output (A), stroke work (B), stroke volume (C), heart rate (D), end-systolic volume (E), and ejection fraction (F). The hearts of mdx mice show either no change or only with the highest dose of dobutamine. Specifically, mdx hearts have significantly lower cardiac output and stroke work following dobutamine stimulation. *P < 0.05, difference from baseline C57BL/10. †P < 0.05, difference from baseline mdx. ‡P < 0.05, difference from C57BL/10 at that dose.
Despite increased fibrosis and the decreased compliance of isolated dystrophic myocytes (65, 67, 73), the mdx hearts in this study display significantly greater left ventricular compliance compared with C57BL/10 hearts (Fig. 8). Furthermore, our data indicate that the passive extension of the left ventricle is subject to dynamic regulation by β-adrenergic stimulation, with infusion of dobutamine significantly increasing the compliance of the left ventricle in both C57BL/10 and mdx hearts. A potential mechanism underlying this observation is alterations in titin, an important determinant of sarcomere passive properties (28). In hearts with dilated cardiomyopathy there is a change in titin isoforms that increases the compliance of the left ventricle (1). In this elegant study, Langendorff perfused hearts were used to assess the passive properties of the left ventricle in mdx and C57BL/10. These changes in passive properties were independent of myocyte contractile function or extracellular Ca$^{2+}$. The studies reported here further support this observation in the intact heart. The increased compliance of the dystrophic heart is only observed in models where the extracellular matrix is present, supporting a hypothesis that the interface between myocytes and the extracellular matrix may be weakened without dystrophin. Recent data demonstrate that α-dystroglycan glycosylation is altered in the dystrophic heart (38, 59) offering a possible mechanism underlying this weakened interaction with the extracellular matrix. Additional studies will be required to further understand the molecular basis underlying the regulation of the passive properties of the dystrophic heart.

In both the right and left ventricle under baseline conditions the duration of the pressure transient was significantly truncated in the mdx heart relative to C57BL/10 (Fig. 6). Interest-
ingly, a similar shortening of the ejection period has been observed using tissue Doppler echocardiography in DMD patients (58). This shortened pressure transient is also present in younger mdx mice, suggesting that it is not related to the progression of dystrophic cardiac disease. The duration of the pressure transient is determined by the timing of the repolarization of the ventricular myocytes. The repolarization occurs more rapidly in the mdx heart is unknown. A possible explanation is directly associated with the loss of dystrophin; it is well documented that mdx cardiac myocytes have increases in cytoplasmic calcium (71, 73). This calcium influx is localized to the membrane compartment and would be ideally positioned to increase the Ca$^{2+}$-dependent inactivation of the L-type Ca$^{2+}$ channel current (35). This increased Ca$^{2+}$ influx is localized to the membrane compartment and would be ideally positioned to increase the Ca$^{2+}$-dependent inactivation of the L-type Ca$^{2+}$ channel current. The closing of the L-type Ca$^{2+}$ channel results in the cessation of Ca$^{2+}$-induced Ca$^{2+}$ release and is followed shortly by declines in intracellular Ca$^{2+}$ and force generation. β-Adrenergic receptor stimulation has been shown to invoke a similar Ca$^{2+}$-dependent acceleration of inactivation of the L-type channel (26), which is consistent with the observations in the present study.

Table 1. Data summarizing the load dependence of measures of diastolic function

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<th>C57BL/10</th>
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<tr>
<td></td>
<td>Baseline</td>
<td>Dobutamine (5 μg·kg$^{-1}$·min$^{-1}$)</td>
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<tr>
<td>dP/dEDV slope</td>
<td>97.0 ± 6.6*</td>
<td>152.0 ± 13.6*</td>
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<tr>
<td>Tau-EDV slope</td>
<td>-0.013 ± 0.013</td>
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Values are mean ± SE; n = 17–19. EDV, end-diastolic volume. Data derived from changes in both preload and afterload in the left ventricle. *Significantly different from zero.
The reduced cardiac function of the mdx heart is clearly revealed in the presence of dobutamine where deficiencies in both systolic and diastolic function become apparent. The reduced systolic cardiac reserve is roughly the same between the right and left ventricles, with both being reduced relative to C57BL/10 hearts (Fig. 4). Other studies have shown that 9- to 12-mo-old and 22-mo-old mdx mice have significantly decreased levels of Ser16-phosphorylated phospholamban, an important subcellular target of β-adrenergic signaling (36, 71). These studies are consistent with the presence of chronic adrenergic receptor stimulation in these older mdx mice, which may, in part, explain the reduced cardiac reserve. The retention of the chronotropic response to dobutamine indicates that the pacemaking cells in the sinoatrial node retain their responsiveness to β-adrenergic receptor activation, indicating that β-adrenergic signaling remains partially intact (Fig. 5).

There are some interesting differences in diastolic function between the right and left ventricle of wild-type hearts (Fig. 7). In the left ventricle of C57BL/10, dobutamine stimulation results in large increases in the maximal rate of relaxation and a marked shortening of tau, the time constant of isovolumetric relaxation. This is consistent with the well-described lusitropic function of β-adrenergic receptor stimulation in cardiac myocytes. These responses are absent from the mdx left ventricle. In the right ventricle both C57BL/10 and mdx hearts have significant increases in the minimum dP/dt induced by dobutamine; however, dobutamine does not affect tau in either genotype. This apparent contradiction in relaxation properties likely stems from the differential load dependence of the minimum dP/dt documented by this study (Table 1) and others (60, 70). In contrast, tau appears independent of changes in loading conditions (Table 1) (60, 70). In the left ventricle, the minimum dP/dt occurs shortly after the closure of the mitral valve and is driven, in part, by the filling of the coronary arteries. The rate at which the coronary arteries fill is dependent largely on the pressure within the aorta; thus the minimum dP/dt is highly dependent upon left ventricular afterload (9). It is not clear if the dependence on load seen in the left ventricle is applicable to the right ventricle; however the filling of the coronary vessels would be expected to have similar effects on the pressure change within the right ventricle. These data indicate that tau is a purer, load-independent measure of myocardial isometric relaxation. The failure of the right ventricle to accelerate its relaxation in the presence of dobutamine observed here and elsewhere (62) highlights the differences in the relaxation properties of these two chambers. The sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) and phospholamban are present at roughly the same levels in both ventricles (5, 55), although the Ca\(^{2+}\) reuptake is slower in right ventricular myocytes (55). The slower kinetics of Ca\(^{2+}\) uptake...
are consistent with the relatively prolonged relaxation of the right ventricular pressure transient compared with that in the left ventricle observed in this study. Chamber geometry is another important difference between the right and left ventricles; it is possible that the increased systolic function induced by dobutamine is stored in the twisted structure of the contracted left ventricle and its release contributes to the acceleration of the isometric relaxation of the left ventricle. The geometry and mechanics of right ventricular contraction would preclude this form of energy storage in the contracted ventricle, which is then unavailable to contribute to the subsequent relaxation.

The biventricular recording methods used in this study provide unique insight into the coupling of the right and left ventricle through the pulmonary circulation. The transit of a pressure wave through the pulmonary circulation in the mouse takes ~4 cardiac cycles or ~400 ms (Fig. 3). Similar studies performed in the dog have shown that it takes 2–3 cardiac cycles, 1.5–2 s, for a similar pressure wave to cross the canine lung (46). Furthermore, in wild-type C57BL/10 mice this transit time varies with dobutamine treatment. This is consistent with the presence of β-adrenergic receptor-mediated vasocostriction in both pulmonary arteries and veins (2, 48), as decreasing the compliance of the pulmonary circulation would increase the velocity of the pressure wave. Interestingly, this effect of dobutamine is lost in dystrophic mice, suggesting abnormalities in the neurohormonal regulation of the mdx pulmonary circulation.

In summary this study demonstrates the presence of significant fibrosis throughout the mdx heart, with the right ventricle being particularly affected from an early age. The increased right ventricular fibrosis may result from increased afterload following either sympathetic activation or hypoxia-induced constriction of the pulmonary artery secondary to hyperventilation. The latter of these two is supported by the severely dystrophic diaphragm in old mdx mice (12) and the already compromised respiratory function of the young mdx mouse (30, 32, 43, 44). Together these observations indicate that respiratory insufficiency occurs in the mdx mouse and is progressive in nature. This respiratory dysfunction and resulting hypoxia will result in the constriction of the pulmonary arteries. We propose a model by which this constriction of the pulmonary vasculature contributes to the increased fibrosis observed in the right ventricle. This has potential clinical significance suggesting that earlier initiation of supportive respiratory therapy could limit right ventricular damage and delay the onset of cardiomyopathy in DMD patients.

GRANTS

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: T.A.M. and D.T. performed experiments; T.A.M. and D.T. analyzed data; T.A.M. and D.T. interpreted results of experiments; T.A.M. and D.T. prepared figures; T.A.M. and D.T. edited and revised manuscript; T.A.M. and D.T. approved final version of manuscript; D.T. conception and design of research; D.T. drafted manuscript.

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