Utrophin deficiency worsens cardiac contractile dysfunction present in dystrophin-deficient mdx mice

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Janssen, Paul M. L., Nitisha Hiranandani, Tessily A. Mays, and Jill A. Rafael-Fortney. Utrophin deficiency worsens cardiac contractile dysfunction present in dystrophin-deficient mdx mice. Am J Physiol Heart Circ Physiol 289: H2373–H2378, 2005.—The loss of utrophin in patients with Duchenne muscular dystrophy (DMD) causes devastating skeletal muscle degeneration and cardiomyopathy. Dystrophin-deficient (mdx) mice have a much milder phenotype, whereas double knockout (DKO) mice lacking both dystrophin and its homolog, utrophin, exhibit the clinical signs observed in DMD patients. We have previously shown that DKO and mdx mice have similar severities of histological features of cardiomyopathy, but no contractile functional measurements of DKO heart have ever been carried out. To investigate whether DKO mice display cardiac dysfunction at the tissue level, contractile response of the myocardium was tested in small, unbranched, ultrathin, right ventricular muscles. Under near physiological conditions, peak isometric active developed tension (F_{dev} in mN/mm²) at a stimulation frequency of 4 Hz was depressed in DKO mice (15.3 ± 3.7, n = 8) compared with mdx mice (24.2 ± 5.4, n = 7), which in turn were depressed compared with wild-type (WT) control mice (33.2 ± 4.5, n = 7). This reduced F_{dev} was also observed at frequencies within the murine physiological range; at 12 Hz, F_{dev} was (in mN/mm²) 11.4 ± 1.8 in DKO, 14.5 ± 4.2 in mdx, and 28.8 ± 5.4 in WT mice. The depression of F_{dev} was observed over the entire frequency range of 4–14 Hz and was significant between DKO versus mdx mice, as well as between DKO or mdx mice versus WT mice. Under β-adrenergic stimulation (1 μmol/l isoproterenol), F_{dev} in DKO preparations was only (in mN/mm²) 14.7 ± 5.1 compared with 30.9 ± 8.9 in mdx and 41.0 ± 4.9 in WT mice. These data show that cardiac contractile dysfunction of mdx mice is generally worsened in mice also lacking utrophin.

trabeculae; papillary muscle; mouse; muscular dystrophy; β-adrenergic stimulation; force frequency; heart

Duchenne muscular dystrophy (DMD) is a progressive muscle-wasting and inevitably fatal disease caused by the absence of the dystrophin protein. Although patients with DMD exhibit severe skeletal muscle pathology, they also have distinct ECGs, conduction abnormalities, arrhythmias, and cardiomyopathy (26, 34). At least 25% of DMD patients die from cardiac failure (14). Patients with the milder allelic Becker muscular dystrophy (BMD) all show a severe cardiomyopathy, and the majority of patients succumb to cardiac failure (29). Because DMD patients have much less skeletal muscle deterioration, they may go on to develop this severe cardiomyopathy due to an increased workload on the heart, compared with DMD patients whose mobility is compromised in their early teens (5).

The genotypic murine model for DMD is the dystrophin-deficient (mdx) mouse. Although these mice do not exhibit the gross clinical signs of DMD and have an almost normal life span, histological studies reveal the underlying skeletal muscle degeneration and cardiomyopathic features (3). The mdx mice have served as a very useful model for studying skeletal muscle, and despite differences in severity and distribution of fibrosis, they have also been shown to share important clinical features with DMD cardiomyopathy (30). ECGs of conscious young mdx mice (10–12 wk) show similar abnormalities as seen in DMD patients (4). Although the ECGs of younger mice are nearly normal, by 42 wk of age, mdx mice exhibit dilated cardiomyopathy; the hearts are hypertrophied, dilated, and have poor contraction and fewer beats per minute (30). Several more invasive studies have examined cardiac contractile function in these mice. Contraction speed and force are significantly altered in atria, even in young mdx mice, before any observed necrosis (35). This suggests that the impaired function is not a result of fibrosis or cell death. Cardiac receptors also have been suggested to play a role in the contractile dysfunction of these mice (23). When compared with control animals, mdx cardiomyocytes are also more susceptible to injury under mechanical stress and suffer contractile failure due to the loss of sarcolemmal integrity (6). This study suggests that dystrophin protects the myocardium by forming a link to redistribute mechanical stress from the sarcolemma to the cytoskeleton. We have previously shown that dystrophin does play a mechanical role in cardiomyocytes similar to its role in skeletal muscle, where it links the cytoskeleton and the extracellular matrix through a direct interaction with the dystrophin-associated membrane protein complex (DAPC) (16, 27, 37).

Where utrophin knockout (KO) mice show no dysfunctional phenotype (8, 10), double KO (DKO) mice, lacking both dystrophin and the dystrophin homolog, utrophin, have a much more severe phenotype than do mdx mice. They show all the clinical signs of DMD, including short stature, kyphosis, hind-limb weakness, labored breathing, and premature death by 20 wk of age (9). Although we have previously shown that DKO mice are not dying from heart failure at this young age, and heart weight-to-body weight ratios (HW/BW) are not different from their wild-type (WT) littermates, our laboratory (16, 31) has shown that cardiac muscles of both mdx and DKO mice at 10 wk of age show inflammation, cardiomyocyte degeneration,

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and fibrosis. Although the severity of histological abnormalities is the same in DKO and mdx hearts, DKO mice exhibit earlier onset and more severe functional deficits than mdx mice. At 2 mo of age, sedated DKO mice exhibit abnormal ECG patterns with drastically decreased S- to R-wave ratios and faster heart rates. These differences do not appear until 6 mo of age in sedated mdx mice, and they do not equal the severity seen in DKO mice until 12 mo of age. These ECG abnormalities, along with the polyphasic R wave seen in 6- and 12-mo-old mdx mice, have also been observed in 70–80% of DMD patients (1, 34). These studies show that both DKO and mdx mice prove useful in further examining the role of dystrophin in skeletal muscle and heart. However, in 10-wk-old DKO and mdx mice, HW/BW indicates no severe gross anatomical changes, and cardiac contractile function has not been investigated. Thus, in the present study, we will test the hypothesis that utrophin compensates, to some extent, for a lack of dystrophin in cardiac muscles of mdx mice by examining the functional impairment of DKO hearts relative to mdx and WT controls. We show that DKO mice exhibit more severe deficits in contractile strength of the myocardium than mdx mice.

MATERIALS AND METHODS

Murine models. Utrophin KO mice: mdx mice were bred to generate utrophin/dystrophin-deficient (DKO) and mdx littermates. Genotypes were determined as previously described (9). C57BL/10 mice, which are the background strain of the mdx and DKO mice, were maintained as a separate breeding stock and used as WT controls for all experiments. Eight-week-old sex-matched mice from all three genotypes were used for isolation of cardiac muscle preparations. At this age, HW/BW ratios of both mdx and DKO mice are not different from WT mice. The investigation conforms with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996).

Muscle preparation and experimental setup. Mice were euthanized by cervical dislocation. After unilateral thoracotomy and intraventricular (via the apex) heparin administration, hearts were rapidly excised and placed in Krebs-Henseleit buffer containing (in mmol/l) 120 NaCl, 5 KCl, 1.2 MgSO₄, 1.2 NaH₂PO₄, 20 NaHCO₃, 0.25 Ca²⁺, and 10 glucose (pH 7.4), equilibrated with 95% O₂-5% CO₂. Additionally, 20 mmol/l 2,3-butanediol monoxime (BDM) was added to the dissection buffer to prevent cutting injury. The effects of BDM after brief exposure have been found to be reversible (20, 24). Hearts were cannulated via the ascending aorta and retrograde perfused with the same buffer for several minutes. Blood was thoroughly washed out, and from the right ventricle, thin, uniform, nonbranched trabeculae or small papillary-like muscles (running from the septal wall very close to the right ventricular free wall to the tricuspid valve) were carefully dissected, leaving a block of tissue at one end from the right ventricular free wall or septum and a small part of the valve at the other end to facilitate mounting. Thus all muscles came from a small well-defined region, and variation due to location in the ventricle was thus minimized. The normal papillary muscles were not used as their dimensions well exceed the ~200 μm cutoff size above which core hypoxia develops under the experimental baseline conditions of the protocol (37°C, 4 Hz). The dimensions of the muscles were measured by using a calibration reticule in the ocular of the dissection microscope (×40, resolution ~10 μm). The cross-sectional area was calculated, assuming an ellipsoid cross-sectional shape. Average dimensions were 0.22 × 0.18 × 1.5 mm and not different among the groups. From each heart, one or two muscles were selected to be included in the analysis of contractile properties. All experimental protocols conformed to institutional guidelines regarding the use and care of animals.

With the use of the dissection microscope, muscles were mounted between a platinum-iridium, basket-shaped extension of a force transducer (KG7, Scientific Instruments, Heidelberg, Germany) and a hook (valve end) connected to a micromanipulator. This method has been shown (7, 19, 21, 22, 40) to minimize end-damage compliance of the preparation and to prevent excessive loss of force throughout the experimental protocols. Muscles were superfused with the same buffer at 37.5°C as above (with the exception that BDM was omitted) and stimulated at 4 Hz. Extracellular Ca²⁺ concentration was raised to 1.5 mmol/l, and muscles were allowed to stabilize for at least 30 min before the experimental protocol was initiated. The 4-Hz baseline was selected rather than a more physiological 12 Hz, because isometric contractions at 12 Hz cause a superphysiological stress on the muscle and impose an energetic demand that likely exceeds the capacity of the muscle to generate sufficient amounts of ATP. Isometric contractions at 12 Hz can only be sustained for 1–2 min with minimal loss of force, whereas at 4 Hz, contractions can be maintained for several hours. However, to study more physiological contractions, 12-Hz contractions were assessed, but only for brief periods. Generally, muscles were stretched to an optimal length where a small increase in length resulted in nearly equal increases in resting tension and active developed tension. This length was selected to be comparable to the maximally attained length in vivo at the end of diastole (around 2.2 μm sarcomere length) (33).

Determination of contractile properties. To obtain a broad scope of contractile function and dysfunction, the three main mechanisms utilized in vivo to physiologically modify force of contraction, length-dependent activation, frequency-dependent activation, and adrenergic stimulation were assessed in the mice. To assess the effect of muscle length on developed force (Fdev) and relaxation time, the following protocol was performed. First, slack length (length of muscle without any preload) and optimal length were determined. The difference was divided into three equal steps, and the muscle was stretched sequentially with each step increasing the length of the muscle until the baseline length was achieved. Parameters were recorded when the muscle had stabilized at each length. We assessed the effects of increasing stimulation frequencies between 2 and 12 Hz (and in selected trabeculae up to 16 Hz). At each frequency, forces were allowed to reach steady state before data were recorded. The effects of β-adrenergic stimulation were assessed by a concentration-response curve with isoproterenol (10⁻⁹–10⁻⁶ mol/l) at a baseline stimulation frequency of 4 Hz.

Data analysis and statistics. In all the experiments performed, the parameters of Fdev and diastolic force (Fdia) were determined and normalized to the cross-sectional area of the muscle. Additionally, as a model-independent parameter of force decay kinetics, time to peak force (TTP), as well as time from peak force to 50% relaxation (RT₅₀), was determined. Parameters were calculated offline and also online to facilitate immediate judgment of the preparation quality. Preparations that did not exceed 10 min/mm² sometime during the protocol or muscles that displayed excessive rundown of Fdev (>10%/h) were excluded.

Multiple ANOVA was used to determine significant differences between the interventions, with post hoc t-test when appropriate. A two-sided P value of <0.05 was considered significant. Experiments (n = 6–12) are included in each protocol, and all values are means ± SE unless stated otherwise.

RESULTS

Physiological changes in cardiac contractile strength are governed via three main mechanisms: length-dependent activation (Frank-Starling mechanism) (12, 36), frequency-dependent activation (Bowditch effect) (2), and adrenergic stimulation (fight/flight response). To characterize potential deficien-
cies in cardiac contractile strength, we tested the contractile performance on trabeculae isolated from 8-wk-old DKO, mdx, and WT mice while varying the above three mechanisms, thereby encompassing their entire physiological range. The main finding of our studies is that in the bulk of the contractile parameters measured, DKO muscles perform significantly worse than both mdx and WT muscles, whereas mdx muscles exhibit a milder, but also significant, dysfunction compared with WT muscles.

Under near physiological conditions of temperature, and at a preload resulting in sarcomere length around the end-diastolic values of 2.2 μm (33), peak isometric active $F_{\text{dev}}$ at a stimulation frequency of 4 Hz was significantly depressed in DKO mice compared with mdx mice (Fig. 1). In addition, $F_{\text{dev}}$ was depressed in both DKO and mdx mice compared with WT controls. As evidenced by the TTP and TTP to RT50, DKO muscles also contracted and relaxed more slowly compared with those in WT mice (Fig. 1), but the difference between mdx versus WT and DKO versus mdx did not reach statistical significance. However, mdx and DKO muscles generated less force; thus, when looking at the maximal and minimal rates of force development ($dF/dt_{\text{max}}$ and $dF/dt_{\text{min}}$, respectively), the impaired relaxation becomes very clear; under baseline conditions, in WT muscles $dF/dt_{\text{max}}$ and $dF/dt_{\text{min}}$ were $1,077 \pm 152$ and $-921 \pm 127$ mN/mm², respectively, significantly higher than those of mdx ($760 \pm 182$ and $-614 \pm 154$ mN/mm², respectively; $P < 0.05$ vs. WT mice), which in turn were significantly higher than those of DKO mice ($473 \pm 124$ and $-366 \pm 110$ mN/mm², respectively; $P < 0.05$ vs. mdx). $F_{\text{dev}}$ was assessed for WT, mdx, and DKO muscles at 85%, 90%, and 95% of the optimal muscle length. A nearly linear and highly positive relationship between length and $F_{\text{dev}}$ was observed in all three groups, indicating that the length-dependent activation mechanism was not significantly affected in DKO or mdx cardiac muscle (Fig. 2). Passive tension rose more steeply in mdx ($P < 0.05$) and DKO ($P < 0.05$) muscles compared with WT muscles. In addition, this force-length relationship was steeper in DKO versus mdx ($P < 0.05$) mice.

The observed reduction of $F_{\text{dev}}$ in DKO, and to a lesser extent in mdx muscles, was not only observed at the low frequency of stimulation of 4 Hz but was likewise observed at all frequencies (Fig. 3) within and in excess of the murine physiological range (8–12 Hz). The shape of the force-frequency relation in WT controls is very similar to those observed under similar experimental conditions previously. Between 4 and 8 Hz, a normal increase in developed force is observed. Therewith, force declines somewhat with further increases in frequency, possibly due to development of core hypoxia. In none of the murine strains examined in this study were we able to find muscles with diameters of <50 μm (as they frequently occur in some other strains). Thus data in excess of 8 Hz may possibly be influenced by a mild hypoxia. However, all mus-
Negative force-frequency relationship than mice (Fig. 4).

Lengthening contractions, and damage after eccentric contractions of skeletal muscle has normalized peak force, force drop after eccentric contractions of skeletal muscle damage (8). In addition, utrophin KO mutants had no histopathological abnormalities in cardiac or skeletal muscles (8).

We observed that DKO mice exhibited a significant decrease in cardiac function in dystrophin-deficient DKO mice and whether the possible alterations differ from cardiac function in dystrophin-deficient mdx mice. The main objective of this study was to determine whether cardiac contractile function is altered in utrophin/dystrophin-deficient DKO mice and whether the possible alterations differ from cardiac function in dystrophin-deficient mdx mice. The results clearly show not only that DKO mice exhibit a significant contractile impairment but also that this impairment is worse than that in the mdx murine model. At only 10 wk of age, we observed that DKO mice exhibited a significant decrease in contractile function, accompanied by an impaired relaxation, and depressed β-adrenergic response. This was observed in the absence of an increased HW/BW, as has been reported for old (12 mo) mdx mice. DKO mice typically die between 10 and 20 wk of age due to their skeletal muscle abnormalities (9, 31).

It is important to note that mice lacking only utrophin show no histopathological abnormalities in cardiac or skeletal muscle and no increase in serum creatine kinase levels compared with WT controls, suggesting that they do not have any cardiac or skeletal muscle damage (8). In addition, utrophin KO skeletal muscle has normalized peak force, force drop after lengthening contractions, and damage after eccentric contractions that is not different from WT controls (10). Histopathological analysis on all muscle and nonmuscle tissues from 2-yr-old utrophin KO mice likewise show no differences from age-matched WT controls (J. Rafael-Fortney, unpublished observations). Thus it appears that dystrophin fully compensates for utrophin in utrophin KO mice, but not vice versa, because mdx mice show a significant dysfunction compared with WT mice. These prior studies thus indicate that dystrophin is functionally more important than utrophin in the heart. The current data show that the absence of both utrophin and dystrophin causes a more severe cardiac dysfunction compared with dystrophin-only deficiency as shown in RESULTS. Although the lack of dystrophin leads to the skeletal and cardiac muscle deficiencies of DMD, utrophin deficiency has not been shown to be associated with a human disease.

The impaired contractile strength is independent of preload; a significant depression of active force development is also noted at shorter lengths. In addition, the force-length experiments revealed that the passive stiffness of the muscles was greater in the DKO versus mdx group, whereas it was also greater in the mdx versus WT groups. This likely reflects a stiffer extracellular matrix, because this explanation seems to be in line with the observed histopathological findings reported earlier in these mice (16, 31). However, a separate effect on increased stiffness of myofilaments or cytoskeleton cannot be altogether discarded as partially underlying the increased passive force observed in DKO and mdx mice. Active force-length relation assessments revealed that the active developed force depressed in the DKO group, as well as in the mdx group (but to a lesser extent) compared with the WT group. Combining the findings of increased passive tension with depressed active tension, we conclude that at equal total preload, active force development in DKO and mdx mice would be even more depressed in both DKO and mdx muscles at all frequencies compared with those in control mice (*P < 0.05). Multiple ANOVA (MANOVA) indicated that intervention strain (denoting mdx, DKO, or WT muscles) was a significant modulator of position of force frequency, indicating that force development during force frequency was strain dependent. **P < 0.05, mdx < WT; ***P < 0.005, DKO < mdx.

DISCUSSION

The main objective of this study was to determine whether cardiac contractile function is altered in utrophin/dystrophin-deficient DKO mice and whether the possible alterations differ from cardiac function in dystrophin-deficient mdx mice. The results clearly show not only that DKO mice exhibit a significant contractile impairment but also that this impairment is worse than that in the mdx murine model. At only 10 wk of age, we observed that DKO mice exhibited a significant decrease in contractile function, accompanied by an impaired relaxation, and depressed β-adrenergic response. This was observed in the absence of an increased HW/BW, as has been reported for old (12 mo) mdx mice. DKO mice typically die between 10 and 20 wk of age due to their skeletal muscle abnormalities (9, 31).

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depressed compared with WT mice, likely exaggerating the already present depressed contractile function.

In addition to the depressed force development, relaxation was impaired in DKO and mdx muscles as well. Under all condition, dF/dt max and dF/dt min were significantly lower in DKO muscles compared with mdx muscles, which in turn were significantly lower than WT muscles. Although the cellular basis for this impaired relaxation was not investigated in this initial functional assessment of DKO mice, it fits the overall aspect of the data in regard to DKO hearts, which more so than in mdx hearts mimic contractile parameters observed in end-stage failing tissue.

The impaired contractile strength was exhibited at all frequencies spanning the in vivo range for the mouse. Function was compared directly with age-matched WT controls, and cardiac contraction parameters in WT muscles were found to be nearly identical to that reported for very similar conditions in control groups of previous studies (28, 38, 39). Likewise, the force-frequency relation of the control mice was virtually identical to those in a previous study. To date, all studies on isolated murine myocardium at body temperature show that the force-frequency relationship is positive between 2 and 8–10 Hz and then flattens or slightly decreases. Clearly, the force-frequency relationship (18), and a negative force-frequency relationship (15), a negative frequency dependence and impairment of relaxation (15), a negative force-frequency relationship (15), and a reduced β-adrenergic response (17). When compared with the mdx model, the DKO model likely represents a more complete model to study the underlying mechanism that shift the hearts from patients with DMD into the downward spiral of heart failure.

Although utrophin is also upregulated in patients with DMD and BMD (11), this upregulation is not adequate because patients eventually develop cardiomyopathy. Restoring the vital link between the cytoskeleton and DAPC by expressing full-length dystrophin in even 50% of mdx myocytes prevents a stress-induced cardiomyopathy and allows normal left ventricle function (42). This gives hope for a possible gene therapy strategy where either dystrophin, or possibly utrophin, is delivered at high levels in half of the cardiomyocytes in the heart.

In summary, this study provides the first functional evidence that a significant contractile dysfunction in DKO mice exists at the tissue level, and this dysfunction is greater than that observed in mdx mice. The dysfunction of DKO trabeculae, more so than in mdx trabeculae, closely mimics many major hallmarks of end-stage cardiac failure: a reduction in force development and impairment of relaxation (15), a negative force-frequency relationship (18), and a reduced β-adrenergic response (17). When compared with the mdx model, the DKO model likely represents a more complete model to study the underlying mechanism that shift the hearts from patients with DMD into the downward spiral of heart failure.

REFERENCES


