Mechanical Regulation of Striated Muscle Nitric Oxide Signaling in Muscular Dystrophy

by

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ABSTRACT

The dystrophin-glycoprotein complex (DGC) is a transmembrane structure that links the cytoskeleton of muscle cells to the extracellular matrix. Genetic disruption of this complex in muscular dystrophies causes sarcolemmal instability that results in injury and death of the muscle cells and causes altered activation of mechanosensitive signaling pathways. These features suggest dual structural and signaling roles for the dystrophin-glycoprotein complex. While much research has focused on protein-protein interactions that enable the DGC's structural function, less is known about how the complex regulates signaling within muscle cells. Therefore, the goal of this thesis was to investigate the mechanisms whereby the dystrophin-glycoprotein complex regulates muscle nitric oxide (NO) production, a phenomenon that is crucial to normal muscle function and is disrupted in several forms of muscular dystrophy. A novel live cell imaging assay was developed to measure the mechanical activation of NO production in isolated muscle cells and investigate the biochemical signaling pathways involved in this process. This investigation identified dystrophin-dependent mechanoregulation of AMP-activated protein kinase (AMPK) as a key component of mechanosensitive NO production in striated muscle. Since defective muscle NO production contributes to diminished exercise tolerance in muscular dystrophy, subsequent studies investigated the therapeutic potential for acute pharmacologic AMPK activation to restore striated muscle NO production and improve exercise tolerance in a mouse model of dystrophin-deficient muscular dystrophy. Acute AMPK activation stimulated NO production in isolated dystrophin-deficient striated muscle cells *in vitro*, and increased the exercise capacity of dystrophin-deficient mice *in vivo*. These results suggest that acute AMPK activation may be a viable therapeutic strategy to improve exercise tolerance in muscular dystrophy patients. Finally, a novel transgenic mouse model was generated in order to test the contribution of asymmetric dimethylarginine (ADMA),

an endogenous nitric oxide synthase inhibitor, to poor exercise tolerance in muscular dystrophy. These experiments suggested that ADMA contributes to exercise-induced fatigue in female dystrophin-heterozygous mice, a model for female carriers of Duchenne muscular dystrophy mutations. They also indicated that ADMA may affect exercise tolerance via effects to promote hypertrophy and impair the contractile function of the dystrophin-heterozygous heart. Considered together, the findings of this thesis support the idea that nitric oxide production is impaired in dystrophin-deficient muscle due to combined effects of disrupted intracellular signaling cascades and increased release of endogenous nitric oxide synthase inhibitors from damaged cells. Thus, this research provides evidence for the hypothesis that both the structural and signaling functions of the dystrophin-glycoprotein complex are critical for the appropriate regulation of striated muscle nitric oxide production.

CHAPTER 1

The dystrophin-glycoprotein complex in muscular dystrophy and the role of nitric oxide signaling in striated muscle.

Muscle is a mechanically-active tissue that converts the chemical potential energy of adenosine triphosphate (ATP) into mechanical work via muscle contraction, allowing for movement of the skeleton and the circulation of blood. In order for this process to occur and to continue over a lifetime, muscle must have a robust capacity for metabolism to maintain an adequate supply of ATP, as well as an ability to withstand both internal and applied external forces, while at the same time transmitting forces generated by actin-myosin cross-bridge cycling between sarcomeres and between neighboring cells. Meeting the demand for increased work output by both the heart and skeletal muscle during exercise requires a capacity for striated muscle to dramatically increase metabolic flux, adapt to increased mechanical stress imposed on the plasma membrane, and fine-tune the local delivery of oxygen and nutrients to match increased metabolic needs. In muscular dystrophies, primary mutations in components of the sarcolemmal dystrophin-glycoprotein complex (DGC) and membrane repair proteins impair the ability for striated muscle cells to prevent or heal disruptions in the plasma membrane incurred during periods of elevated mechanical stress. Such defects directly contribute to death of the muscle cells and the progressive muscle degeneration and wasting seen in these diseases. Secondary disruptions in DGC-dependent signaling pathways are also observed in muscular dystrophies caused by mutations in DGC components. In particular, loss of dystrophin expression in Duchenne muscular dystrophy (DMD) is correlated with disruptions in nitric oxide (NO) production by neuronal nitric oxide synthase in both skeletal and cardiac muscle, and a consequent disruption of NO-dependent regulation of blood flow within the muscle tissue(1).

This observation suggests a critical role for dystrophin and the DGC in the regulation of striated muscle NO production. However, the precise mechanisms by which dystrophin affects muscle NO signaling, and the contribution of disrupted muscle NO signaling to the pathophysiology of the muscular dystrophies, is poorly understood and is the foundation for the research of this dissertation. The goal of this work is to understand how striated muscle NO production is normally regulated by physiological mechanical stresses imposed on the muscle, and how genetic disruption of the DGC disrupts this process. Because mechanical stimulation is a primary driver for NO production in striated muscle, initial experiments sought to understand the role of dystrophin in the cell-intrinsic molecular signaling pathways coupling cell length changes to increased cellular nitric oxide production. These experiments were followed by an *in vivo* study testing whether acute pharmacologic activation of the same signaling pathways could bypass the defect caused by loss of dystrophin and improve exercise tolerance in a mouse model of Duchenne muscular dystrophy. Finally, a novel genetic mouse model was generated to explore the potential impact of endogenous cell-extrinsic factors released following mechanical injury of dystrophic muscle cells on NO signaling and exercise performance in DMD. In order to understand how dystrophin may participate in the regulation of striated muscle nitric oxide production, and the potential contribution of disrupted NO signaling to impaired muscle function in DMD, this introduction will first review the clinical phenotype of DMD, then summarize the literature supporting a signaling function of the DGC as well as recent evidence for a role of reduced striated muscle NO production in the impaired ability to perform and withstand exercise observed in dystrophinopathies.

Muscular dystrophy and the dystrophin-glycoprotein complex.

The group of muscle wasting diseases known as muscular dystrophies were first described in the mid-1800s by physicians reporting case studies of patients presenting with progressive muscle weakness, muscle atrophy, and eventual loss of ambulation(2, 3). The most common of these disorders was first characterized in depth by Meryon(4) and Duchenne de Boulogne(5). Their reports describe young boys, and in some cases multiple boys within a given family, who demonstrated an early difficulty and excessive fatigue with walking or standing,

that eventually degenerated into an apparent paralysis with near-complete loss of voluntary muscle movement. Meryon noted a sex-specific predisposition to this disorder, citing one family consisting of four affected sons and six unaffected daughters(4). While the limb muscles of affected boys generally underwent atrophy, Duchenne and Meryon described a peculiar hypertrophy of the calf muscles, leading Duchenne to term the disease “pseudo-hypertrophic paralysis.” Microscopic examination of the muscle tissue revealed that this apparent hypertrophy was not due to increased muscle fiber size, but rather by increased deposition of fibro-fatty tissue that replaced atrophying or necrotic muscle cells(4, 5). Furthermore, Meryon reported that the nervous tissue of patients was generally unaffected(4), suggesting a primary defect originating within the muscle cells themselves.

In describing the disease that would come to be named Duchenne muscular dystrophy, these early case studies touched on hallmark manifestations that are typical of this disorder and are shared by several forms of childhood muscular dystrophy. In DMD, the most common and prototypic form of these diseases, muscle weakness first becomes apparent in early childhood, with affected boys falling more than others, having difficulty standing up from a sitting position, and showing difficulty in everyday physical activity like climbing stairs(2, 3). Over time, the ability for patients to withstand exercise gradually declines, and patients report exaggerated fatigue following even moderate bouts of physical activity(5, 6). Evidence of these impairments can be seen as diminished performance in timed function tests or measurements of 6-minute walk distance(7, 8). Progressive muscle atrophy further exacerbates patient weakness, ultimately leading to patients losing the ability to walk and being confined to a wheelchair by their early teenage years(2-5). In some forms of muscular dystrophy, such as the mild version of DMD known as Becker muscular dystrophy (BMD), certain types of limb-girdle muscular dystrophy (LGMD), and facioscapulohumeral muscular dystrophy (FSHD), such signs of muscle dysfunction do not appear until adulthood(9), implying a wide clinical spectrum of disease progression in this group of disorders.

Historically, patients with DMD have died by their early twenties due to complications from respiratory failure associated with damage and degeneration of the diaphragm and other respiratory muscles(2, 10). However, over the past 20-30 years advances in respiratory

interventions, including the use of surgery to correct scoliosis and the use of nocturnal ventilators, has allowed patients to survive in to their thirties and in some cases their forties(11). Unfortunately, this increase in lifespan has revealed that in addition to the limb and respiratory muscles, the cardiac muscle is also affected to varying degrees in as many as 90% of DMD patients(12). Cardiac muscle disease in DMD and related dystrophies ranges from subclinical electrocardiographic abnormalities, diastolic dysfunction, and cardiac hypertrophy, to overt dilated cardiomyopathy and heart failure(10, 12-15). Female carriers of DMD mutations do not typically exhibit the characteristic skeletal muscle disease seen in male DMD patients. However, DMD carriers are recognized has having a substantially increased rate incidence of cardiomyopathy compared to the population at large, indicating that they share some of the same cardiac muscle defects as male DMD patients(16-20).

Common pathological signs of the muscular dystrophies support a primary defect in muscle cell integrity as a unifying feature of these diseases. Clinically, muscular dystrophy patients exhibit elevated serum concentrations of proteins that are enriched in muscle, such as creatine kinase, lactate dehydrogenase, and cardiac- and skeletal-muscle specific troponin isoforms(21, 22). This observation indicates that there is excessive damage to or permeability of the plasma membrane of muscle cells in these diseases, allowing for leak of intracellular contents out of the muscle cells. Indeed, Meryon noted a breakdown of the sarcolemma upon microscopic evaluation of muscle biopsies taken from muscular dystrophy patients as early as 1852(4). This finding was later corroborated by electron microscopy studies in the 1970s, which showed discontinuities in the plasma membrane of DMD muscle that preceded muscle cell necrosis(23, 24). Interestingly, a report by Carpenter and Karpati noted the restoration of the plasma membrane in regions of the muscle fibers that appeared to have undergone recent sarcolemmal injury, providing evidence for endogenous repair mechanisms that exist to heal such breaches in the sarcolemma(24).

Histologically, evidence for muscle damage can be seen by the presence of necrotic muscle fibers and infiltration of the tissue with inflammatory cells(25). Dystrophic skeletal muscle also displays an increased proportion of small-diameter, centrally-nucleated muscle fibers, indicative of cells that have recently undergone regeneration from satellite cells in order

to replace dying fibers. As the regenerative capacity of the satellite cells is depleted, damaged skeletal muscle cells are instead replaced by fibrotic or fatty tissue (25). Similar signs of cellular injury can be seen in the dystrophic heart, with focal replacement of cardiomyocytes with fibrosis and fatty infiltrates(26, 27). Such loss of functional muscle cells is the primary driver of the severe skeletal muscle wasting, gradual thinning of the wall of the heart, and eventual dilated cardiomyopathy observed in DMD. The observation of plasma membrane breakdown and resulting cellular damage in DMD, as well as analogous histological findings in the skeletal muscle and/or heart in related diseases, led to the formulation of a "sarcolemmal hypothesis" for muscular dystrophy(2). This idea proposes that the underlying genetic defects of muscular dystrophies result in a common increase in the susceptibility of the muscle cell plasma membrane to lesions, especially when under mechanical stress, or otherwise a diminished capacity to repair normal amounts of damage incurred on otherwise healthy membranes.

Mutations in the X-linked *DMD* gene that encodes the 427-kDa, cytoskeletal protein dystrophin were identified as the genetic basis for Duchenne and Becker muscular dystrophy in 1986-1988(28-32). This breakthrough opened the door to a molecular understanding of the mechanisms of the muscular dystrophies. Since then, identification of the genetic causes of the various limb-girdle and congenital muscular dystrophies revealed a preponderance of genes encoding proteins that are functionally clustered at or near the muscle cell plasma membrane, in addition to proteins of the extracellular matrix(9). Subsequent biochemical studies revealed that dystrophin localizes to the interior of the muscle cell membrane and is concentrated at structures called costameres that overlie the z-line(33). There, dystrophin binds to the sub-sarcolemmal actin cytoskeleton and to an assembly of transmembrane glycoproteins including dystroglycan and the sarcoglycans in a complex that came to be known as the dystrophin-glycoprotein complex, or DGC(34-41) (Fig. 1.1). In muscle, α-dystroglycan binds in a glycosylation-dependent manner to extracellular matrix proteins such as laminin(36), agrin(42-44), and perlecan(45, 46) that contain multiple LamG domains. Thus, the core components of the dystrophin-glycoprotein complex form a structural linkage between the interior and exterior of the muscle cell. Finally, several additional accessory proteins complete the DGC: α- and β-syntrophin and α-dystrobrevin bind to dystrophin on the intracellular side of the DGC(47,

48), and the membrane-spanning protein sarcospan associates with the sarcoglycans(49) (Fig. 1.1).

Structural function of the dystrophin-glycoprotein complex in plasma membrane stabilization.

Resolution of the primary structure of the components of the dystrophin-glycoprotein complex and their protein interactions suggested a putative role for this complex as a molecular shock absorber that stabilizes the plasma membrane against the mechanical stresses experienced during muscle contraction or stretch(36, 50). Indeed, one recent study demonstrated a critical role for dystrophin and the DGC in transmitting the force of contraction from the interior of the muscle cell to the extracellular matrix and neighboring fibers(51). The authors of this work proposed that this function might help to maintain sarcomeres in register and to distribute the forces of muscle contraction evenly along the sarcolemma, preventing the development of excessive, injurious forces at any given point of the membrane. Such a force-dissipating, protective function of the DGC would explain why muscular dystrophies in which DGC components are mutated present with compromised integrity of the plasma membrane that ultimately leads to death of the muscle fibers. In recent years, the development of genetic animal models for these "DGC-opathies" has enabled the direct investigation of this stabilizing function of the complex. Given that the primary loss of dystrophin in DMD can result in secondary reductions in sarcolemmal expression of the remaining DGC proteins in both skeletal and cardiac muscle(52-55), the following paragraphs will focus on animal models of DMD as a prototypic example of DGC disruption and its consequences for membrane stability.

Like DMD patients, dystrophin-deficient *mdx* mice exhibit elevated plasma concentrations of creatine kinase (CK), necrotic muscle fiber degeneration, increased muscle fiber size variability and central nucleation, and increased muscle fibrosis(56, 57), features that are consistent with a defect in sarcolemmal integrity. Early experiments in *mdx* mice noted a particular susceptibility for muscle fiber damage in the diaphragm, and the investigators proposed that this increased susceptibility to injury compared to other skeletal muscles resulted from the continuous contractile activity of the diaphragm throughout life(58). These

same authors later used membrane-impermeant dyes to demonstrate that dystrophin specifically protects the sarcolemma from lesions during the increased mechanical stress imposed on it during muscle contraction(59). Consistent with this model, numerous *in vitro* and *in situ* investigations have reported exceptional susceptibility of dystrophin-deficient muscle to damage during "eccentric" or "lengthening" contractions, in which the load against which the muscle contracts is greater than the force generated by contraction and results in elongation of the muscle. Such eccentric contraction- or lengthening contraction-induced injury in *mdx* muscle is indicated by increased uptake of membrane impermeant dyes as well as a rapid drop in force production following repeated contractions(60-62). Delayed signs of eccentric contraction-induced injury include an increased incidence of fiber necrosis and IgG infiltration into damaged fibers(63). Experiments using intact *mdx* mice have shown that the stresses of eccentric muscle contraction during downhill treadmill running result in excessive CK release and increased dye uptake into the skeletal muscles, suggesting that such eccentric contraction-induced muscle injury is indeed a legitimate component of the muscle damage observed *in vivo* in DMD(64, 65). The phenotype of excessive membrane fragility can also be observed directly in isolated dystrophin-deficient muscle fibers. Studies using passive mechanical stretching report an exaggerated drop in force production and excessive permeability to extracellular cations, notably calcium, following eccentric contractions(66-68), recapitulating the findings from intact muscle. Furthermore, the application of hypo-osmotic stress to swell cells reveals an exaggerated sensitivity of dystrophin-deficient muscle fibers to membrane blebbing, hypercontracture, and leak of pyruvate kinase, suggesting increased sarcolemmal permeability when mechanical stress is placed on the membrane(69). However, the relative contribution of abnormal membrane lesions versus excessive activation of stretch-activated ion channels to calcium overload and cell death in these models remains a topic of debate(67, 68).

Dystrophin-deficient cardiac muscle also shows an exaggerated sensitivity to membrane injury when under mechanical stress. Aged *mdx* mice exhibit increased focal cardiomyocyte damage compared to wild-type mice(70), a phenotype that is exacerbated when animals are subjected to aortic banding and pressure overload(71). Over time, damaged cardiomyocytes are replaced by fibrosis and *mdx* animals develop dilation of the left ventricle, consistent with

features of the cardiomyopathy seen in DMD patients(10, 12, 26, 27). When subjected to acute β-adrenergic stress, as would be experienced during exercise, *mdx* hearts rapidly decompensate, showing a dramatic reduction in systolic pressure generation within as little as 30 minutes of administration of a β-agonist(55, 72, 73). This decline in cardiac function is associated with increased release of lactate dehydrogenase(74, 75) and increased staining of cardiomyocytes with membrane-impermeant Evan's blue dye(73, 75). These observations demonstrate that like dystrophin-deficient skeletal muscle fibers, dystrophin-deficient cardiomyocytes are more susceptible than healthy cardiomyocytes to sarcolemmal disruption under conditions of increased mechanical stress. Direct investigation of isolated *mdx* cardiomyocytes that are passively stretched *in vitro* revealed a reduced tolerance for such mechanical deformations. Compared to wild-type cardiomyocytes, *mdx* cardiomyocytes were more susceptible to stretch-induced calcium overload that resulted in cell contracture and death(72). This study further demonstrated that these defects in *mdx* cells could be corrected by treatment with a poloxamer 188, a polymer that can insert into the lipid bilayer and is thought to seal breaches in the plasma membrane. Together with the observations noted in isolated skeletal muscle fibers above, these cellular findings support a cell-intrinsic defect in sarcolemmal stability as a causative mechanism for the functional impairments and muscle damage evident following periods of increased mechanical stress in dystrophin-deficient striated muscle. Analogous findings of primary sarcolemmal instability have been observed in models of α-dystroglycan glycosylation-deficient muscular dystrophies(76, 77) and sarcoglycanopathies(78), suggesting a role not only for dystrophin itself, but rather for the function of the DGC as a whole to mechanically couple the interior and exterior of the muscle cell, in protecting the sarcolemma.

The dystrophin-glycoprotein complex in cellular signaling.

Beyond the classically-accepted role for dystrophin and the DGC in supporting the sarcolemma, recent work suggests additional, signaling functions of the complex. Several groups noted early on that the soleus muscle of dystrophin-deficient mice is specifically protected from contraction-induced injury(60, 61, 79), despite the fact that it still exhibits

hallmark features of dystrophic muscle pathology such as an increased proportion of recently-regenerated fibers(80). The soleus muscle of α-dystroglycan glycosylation-deficient mice similarly demonstrates an increased resistance to contraction-induced injury compared to other skeletal muscles, yet still remain weaker than the wild-type soleus(81). Finally, muscle degeneration has been observed in the absence of mechanical muscle injury in mice lacking γ-sarcoglycan(82). Collectively, these observations indicate that additional mechanisms distinct from sarcolemmal instability may contribute to the pathophysiology of the various diseases caused by DGC disruption. The inability for the sarcolemmal hypothesis to explain such membrane integrity-independent aspects of the DGC-opathies has contributed to the development of a complementary, "signaling hypothesis" for muscular dystrophy.

Initial investigations of a putative signaling function for the DGC compared the response of dystrophin-deficient and wild-type muscle cells to applied mechanical stretch, a stimulus that has long been known to activate a variety of signaling pathways in striated muscle(83, 84). One early study reported excessive activation of MAP kinase signaling upon stretch of fibers from the dystrophin-deficient *mdx* diaphragm, an effect that the authors attributed to excessive entry of extracellular calcium(85). However, it was not fully clear from this work whether excessive calcium flux and its effects on signaling was a direct consequence of lesions in the plasma membrane as would be suggested by the sarcolemmal hypothesis, or rather a consequence of increased activity of stretch-activated ion channels consistent with the signaling hypothesis. Additional evidence in favor of a signaling function of the DGC in calcium homeostasis was provided by the later finding that blocking mechanosensitive ion channels could reduce the incidence of abnormal calcium release events, or "sparks," from the sarcoplasmic reticulum during osmotic challenge of dystrophin-deficient muscle fibers(86). Parallel investigations have shown additional pathways, notably PI3K/Akt signaling, to be hyper-activated by stretch of dystrophin-deficient muscle(87).

How might the dystrophin-glycoprotein complex exert its effects upon cell signaling? A leading hypothesis is that the DGC regulates cell signaling via direct physical interactions with signaling proteins. This notion is perhaps most clearly illustrated by the finding that the syntrophins mediate direct protein-protein interactions between the DGC and TRPC cation

channels(88, 89). Such a model could help to explain how disruption of the DGC leads to altered mechano-activation of these channels and excessive calcium entry upon muscle stretch. Apart from ion channels , components of the DGC also bind to a variety of known signaling molecules including MEK and ERK(90), archvillin(91), a Grb2-Sos1 complex(92, 93), and heterotrimeric G protein subunits(94, 95). These observations have led to the notion that in addition to direct regulation of the activity of signaling proteins, the DGC may play a critical role in scaffolding signaling proteins near important interacting partners or sites of action, thus serving a permissive function in the transduction of the pathways in which these proteins participate. To date, the effects of DGC disruption on many of these proteins, and the contribution of the signaling pathways in which they act to the pathophysiology of muscular dystrophy, remain uncertain. However, in the last twenty years it has become apparent that the DGC has a clear and critical role in the regulation of neuronal nitric oxide synthase (nNOS), an enzyme that is essential for normal striated muscle function.

Nitric oxide as a critical regulator of the cardiovascular system and striated muscle.

Neuronal nitric oxide synthase is one of a family of three mammalian enzymes that catalyze the production of nitric oxide from the reduction of molecular oxygen and oxidation of the amino acid, L- arginine (Fig. 1.2). Both nNOS and the closely-related isoform, endothelial nitric oxide synthase (eNOS), are constitutively expressed in select tissues, whereas the expression of inducible nitric oxide synthase (iNOS) is stimulated by cytokines in disease states(96). The enzymatic activity of nNOS and eNOS requires the binding of calcium to calmodulin, which reversibly associates with homodimers of these isoforms(96). nNOS and eNOS activity is further subject to post-translational regulation by phosphorylation of a number of stimulatory or inhibitory residues, notably nNOS serine 1412/eNOS serine 1177 (stimulatory) and nNOS serine 841/eNOS threonine 495 (inhibitory)(96-98). Finally, all three NOS isoforms are subject to endogenous competitive inhibition by methylated arginine species generated by the proteolysis of proteins that have been modified by a class of enzymes known as protein arginine methyltransferases (PRMTs)(99). The presence of endogenous inhibitors such as methylated arginines and active arginase that depletes arginine in cells is thought to explain the

"arginine paradox," where supplemental arginine can increase NOS activity despite circulating arginine levels being 25- to 30-fold higher than the Michaelis-Menten constant of isolated purified NOS *in vitro*(100).

In skeletal muscle, nNOS is found near the sarcolemma, where it binds to the dystrophin-glycoprotein complex via direct interactions with α1-syntrophin as well as dystrophin itself(101-104) (Fig. 1.3A). This anchoring of nNOS at the sarcolemma and near the periphery of the muscle fiber facilitates the diffusion of nitric oxide out of the muscle fiber and to local arterioles within the vascular bed of the muscle tissue. Within the arteriolar smooth muscle, skeletal muscle-derived NO stimulates soluble guanylyl cyclase to produce cyclic guanosine monophosphate (cGMP), which stimulates the activity of protein kinase G (PKG). PKG in turn promotes smooth muscle relaxation via the activating phosphorylation of myosin light chain phosphatase, the phosphorylation of phospholamban to increase calcium uptake into the sarcoplasmic reticulum, and the activation of potassium channels to oppose depolarization and contraction of the smooth muscle cell(105-108). Investigations measuring blood flow in the rodent hindlimb demonstrated that such skeletal muscle-derived NO is critical for the local regulation of muscle blood flow in the face of increased sympathetic tone, as occurs during exercise. In this process, known as sympatholysis, contraction of the skeletal muscle triggers nNOS to produce NO, which attenuates α-adrenergic vasoconstriction of the local arterioles and thus helps to maintain blood flow and the delivery of oxygen and nutrients to the contracting skeletal muscle(109, 110). Mechanical stimulation such as contraction or stretch also triggers the activation of nNOS and eNOS within cardiomyocytes(111, 112). While endothelial cell-derived NO is thought to be a major determinant of coronary artery blood flow, it is possible that an analogous mechanism of mechanically-activated cardiomyocyte-derived NO production may also contribute to local blood flow regulation within the heart(113-115).

In addition to paracrine effects on blood flow, there is an increasing body of evidence to suggest that nNOS has intrinsic effects on the contractile function of both skeletal and cardiac muscle cells. In healthy skeletal muscle, nNOS has been reported to attenuate contractile force, thought to occur through activation of cGMP production as in smooth muscle(116). Paradoxically, studies using nNOS knockout mice have demonstrated reduced maximal force

generating capacity in nNOS-deficient skeletal muscle, although this feature could presumably be explained by a decrease in cross-sectional area of the muscles tested, as overall muscle mass was reduced in nNOS knockout animals and specific force was not altered(117). This same work reported that nNOS-deficient muscles were more susceptible than controls to contraction-induced fatigue, even though they did not exhibit any increase in susceptibility to eccentric contraction-induced injury. *In vivo* experiments demonstrating that nNOS-null mice exhibit a dramatic reduction in voluntary physical activity following an acute bout of treadmill running(118) suggest that the muscle fatigue observed *in situ* in nNOS knockout animals is recapitulated as a behavioral phenotype at the level of the intact animal. Together, these data support the contention that nNOS exerts important physiological roles within the muscle, and may do so via effects that are independent of plasma membrane stability. Percival et al. also later noted localization of an nNOS splice variant in the Golgi apparatus of skeletal muscle, and showed it to be critical for the regulation of muscle fatigue resistance and post-exercise force generation, distinct from effects on blood flow(119). This observation indicated that skeletal muscle NO production may be compartmentalized in discrete functional domains, and that additional mechanisms besides vasoregulation may contribute to the physiological control of muscle function by nNOS.

nNOS also regulates the contractility of cardiomyocytes, tending to favor relaxation and enhance the diastolic function of the myocardium. These effects are mediated in part by PKG- or protein kinase A-dependent phosphorylation of phospholamban serine 16(120-122), leading to enhanced SERCA function and calcium uptake into the sarcoplasmic reticulum. Studies of nNOS knockout mice suggest that myocardial nNOS also reduces calcium current density and calcium transient amplitude, thus attenuating contractile force development(111, 123, 124). These effects are likely mediated by direct actions of NO to nitrosylate and inhibit L-type calcium channels(125), although evidence also exists supporting inhibitory phosphorylation of the L-type calcium channel by PKG(126, 127). Furthermore, as PKG can target the myofilament proteins titin(128) and troponin I(129), nNOS- or eNOS-derived cardiomyocyte NO may also facilitate diastolic function by increasing the passive distensibility of the myocardium and decreasing myofilament sensitivity to calcium(130, 131).

Disruption of nitric oxide signaling and phenotypic consequences in muscular dystrophy.

In the mid-1990s, immunohistochemical studies of tissue from DMD patients and *mdx* mice noted a striking loss of nNOS from the sarcolemma of dystrophin-deficient skeletal muscle cells (Fig. 1.3B). In contrast to wild-type skeletal muscle, nNOS tended to become localized to the cytosol of dystrophin-deficient fibers, and total cellular nNOS activity was reduced compared to healthy controls(101, 102). This reduction in total nNOS activity was partially explained by an overall reduction in nNOS mRNA and protein content, indicating some degree of transcriptional silencing of nNOS and possibly increased degradation of the mislocalized nNOS protein(101, 102). Similar observations of altered nNOS localization and function were later reported in other instances of DGC disruption, including Becker muscular dystrophy(132), dystroglycan deficiency(133), sarcoglycan deficiency(134), syntrophin deficiency(135, 136), and dystrobrevin deficiency(137). These findings suggest that nNOS dysregulation may be a common feature underlying the pathophysiology of multiple forms of muscular dystrophy.

The functional consequences of disrupted nNOS function in dystrophin-deficient skeletal muscle were made clear by studies of vascular function in *mdx* mice. Thomas et al. reported that sympatholysis is attenuated in dystrophin-deficient animals, recapitulating a characteristic phenotype of nNOS-null animals(1). These observations suggested that disruption of nNOS signaling in dystrophin-deficient muscle specifically contributed to an impaired regulation of muscle blood flow. Indeed, whereas wild-type mice respond to exercise with an increase in limb muscle blood flow, this phenomenon is abrogated in *mdx* mice(118). The additional findings of abnormal vascular function and reduced contraction-induced skeletal muscle cGMP production that paralleled the loss of sarcolemmal nNOS in δ- or β-sarcoglycan(138, 139) or α-dystrobrevin deficient mice(137) provided further support for the notion that sarcolemmal NO production is critical for the ability of the skeletal muscle to exert regulatory control over the local vasculature. Given a demonstrated cell-intrinsic role for nNOS in muscle fatigue resistance(117), defective nNOS signaling may also help to explain the increased susceptibility of dystrophin-deficient muscle fibers to fatigue(140).

Recent *in vivo* investigations indicate that disrupted nNOS signaling contributes to impaired exercise tolerance in dystrophin-deficient *mdx* mice. Compared to wild-type, these animals exhibit exaggerated fatigue following treadmill running that closely resembles the phenotype of nNOS-null animals(118), as well as reduced endurance during exercise(141). Studies by the Campbell lab showed that post-exercise muscle blood flow could be increased and exercise-induced fatigue could be prevented in *mdx* mice treated with a phosphodiesterase 5 (PDE5) inhibitor to boost cGMP signaling. This intervention failed to correct exercise-induced fatigue in nNOS-null mice(118), implying that NO/cGMP-dependent regulation of muscle blood flow is critical to the prevention of such fatigue. Impaired nNOS-dependent vasoregulation has also been demonstrated in Duchenne and Becker muscular dystrophy patients(142, 143), prompting several investigations into whether PDE5 inhibition might also improve blood flow regulation and exercise tolerance in these populations(144-146).

Current model of DGC-dependent regulation of nNOS.

The work described above supports the notion that misregulation of muscle nNOS activity is an important component of the disease phenotype in DMD and suggests that enhancement of muscle nNOS signaling may improve exercise tolerance in this disease. However, the precise regulatory mechanisms that normally activate nNOS during muscle contraction, and the exact pathways by which DGC disruption alters nNOS function, remain poorly understood.

Collectively, the skeletal muscle investigations described in the preceding paragraphs led to the development of a model in which dystrophin or the DGC as a whole is critical for scaffolding nNOS near the plasma membrane of the muscle cell, where the NO that it produces during muscle contraction can signal in a paracrine fashion to promote the dilation of nearby arterioles (Fig. 1.3A). This model also proposes that the binding of nNOS to the DGC is required for basal and contraction-induced nNOS enzymatic activity. When the DGC is disrupted, as occurs with genetic loss of dystrophin in DMD, nNOS is mislocalized from the sarcolemma to the cytosol, where its enzymatic activity is reduced (Fig. 1.3B). Although this model is consistent with a requirement for a physical interaction between nNOS and the DGC in the stimulation of

muscle nNOS activity, direct evidence for this hypothesis is lacking. Indeed, the observation that transgenic over-expression of nNOS in dystrophin-deficient muscle can increase total muscle NO production, even without restoration of nNOS-DGC scaffolding interactions(147, 148), argues against an absolute necessity for protein-protein interactions with the DGC for nNOS activity.

This current model also fails to explain recent data regarding nNOS activity in dystrophin-deficient cardiac muscle. nNOS activity was first demonstrated to be decreased in total homogenates of *mdx* mouse hearts in 1999(149), and this reduction in cardiac nNOS activity was later localized specifically to the dystrophin-deficient cardiomyocytes (150). nNOS impairment could explain some of the characteristic functional defects of dystrophin-deficient hearts, such as decreased cardiomyocyte compliance(72), impaired relaxation and diastolic distensibility(14, 55, 73), and aberrant calcium handling(151). These observations suggest that like in skeletal muscle, impaired nNOS signaling is a relevant component of the pathophysiology of the dystrophin-deficient heart. They also raise the possibility that cardiac effects of disrupted nNOS, in addition to local effects within the skeletal muscle and skeletal muscle vasculature, contribute to the impaired exercise phenotype common to dystrophin-deficient and nNOS-deficient animals(118).

Notably, the study by Ramachandran et al. reported that the total mRNA and protein content of nNOS was not altered in *mdx* versus wild-type cardiomyocytes, indicating that the overall decrease in nNOS activity in *mdx* cells was due to impaired activation of nNOS, rather than attributable to a net reduction in total nNOS expression. Immunohistochemical staining of nNOS in mouse cardiac tissue revealed that in contrast to skeletal muscle, nNOS was not specifically enriched at the sarcolemma of either wild-type or dystrophin-deficient cardiomyocytes(150). This finding corroborated results from another group that demonstrated that nNOS co-immunoprecipitates with dystrophin and other DGC components in skeletal muscle but not cardiac muscle(152). Taken together, these results indicate that in cardiac muscle, dystrophin expression does affect the activity of nNOS, but does so via some mechanism that is independent of a direct physical scaffolding interaction with nNOS (Fig. 1.3 C and D). As nNOS protein levels and localization are not affected by loss of dystrophin, I

hypothesized that dystrophin- or DGC-dependent regulation of this enzyme likely occurs at the level of post-translational modification of nNOS. Importantly, this alternative model also calls into question whether skeletal muscle nNOS activity does in fact rely on direct protein-protein interactions between nNOS and the DGC, or rather may be regulated by intermediate dystrophin-dependent signaling pathways.

Rationale and Experimental Approach.

Studies of skeletal and cardiac muscle have suggested an important role for neuronal nitric oxide synthase in striated muscle. Furthermore, evidence from animal models of Duchenne muscular dystrophy indicates that impaired muscle nNOS activity contributes to impaired skeletal muscle and heart function, and, consequently, a diminished capacity for exercise in this disease. These observations point to a critical role for dystrophin in striated muscle NO signaling. However, the specific mechanisms by which muscle NO production is regulated, and the precise role of dystrophin and the dystrophin-glycoprotein complex in this process, are unclear. Therefore, the overall goal of the work in this thesis was to determine the mechanistic role of dystrophin in the regulation of striated muscle nitric oxide production. **The overall hypothesis is that dystrophin regulates biochemical signaling pathways that allow the transduction of mechanical cues into the stimulation of nitric oxide synthase activity.** This hypothesis was tested with the following specific aims:

Specific Aim 1: Determine the direct, cell-intrinsic mechanisms by which dystrophin affects striated muscle NO signaling.

The purpose of this set of experiments, described in Chapter 2, was to investigate the involvement of dystrophin in mechanosensitive signaling pathways that participate in the post-translational regulation of nitric oxide synthase activity. A novel live-cell imaging assay was developed that used *in vitro* stretch of adult mouse cardiomyocytes as a model to investigate mechanosensitive nitric oxide signaling in fully-differentiated striated muscle. The mechanical activation of the upstream nNOS kinase, AMP-activated protein kinase (AMPK), and the

subsequent stimulatory phosphorylation of nNOS at serine 1412 and activation of NO production was impaired in dystrophin-deficient cardiomyocytes as compared to wild-type. These data support a model in which dystrophin-dependent mechanoactivation of AMPK is critical for the mechanical stimulation of nNOS activity in striated muscle. This model is consistent with a role for dystrophin and the dystrophin-glycoprotein complex as an important mechanotransducer within muscle. Finally, this study shows that acute pharmacologic activation of AMPK, either with the AMP-analog AICAR or with salicylate, is sufficient to bypass defective mechanosignaling and increase nNOS activity in dystrophin-deficient cardiomyocytes *in vitro,* providing proof-of-concept evidence for this approach to increase striated muscle NO production in Duchenne muscular dystrophy.

Specific Aim 2: Test pharmacologic activation of AMPK-NO signaling as a strategy to improve exercise tolerance in the *mdx* mouse model of Duchenne muscular dystrophy.

The experiments performed in this Aim expanded on the findings from Aim 1 and sought to determine whether direct pharmacologic AMPK activation could stimulate increased NO production in dystrophin-deficient skeletal muscle *in vitro*, and whether acute pharmacologic AMPK activation *in vivo* could improve exercise tolerance in dystrophin-deficient mice. The results, discussed in Chapter 3, demonstrate that acute treatment with AICAR or salicylate increases the exercise capacity of dystrophin-deficient mice. This study also shows that like in dystrophin-deficient cardiac muscle, acute treatment with the AMPK-activating drug, salicylate, is sufficient to stimulate nitric oxide production in isolated dystrophin-deficient skeletal muscle fibers. In contrast, treatment with AICAR does not stimulate NO production in dystrophin-deficient skeletal muscle fibers, suggesting there may be important differences between cardiac and skeletal muscle in the subunit composition of AMPK heterotrimers that target nitric oxide synthase. Together with the findings from Chapter 2, the results of this investigation suggest that acute AMPK activation may a viable therapeutic strategy to improve exercise tolerance in DMD, and might do so via restoration of skeletal and/or cardiac muscle NO production.

Specific Aim 3: Understand the impact of secondary effects of dystrophin-deficiency on striated muscle NO signaling and exercise tolerance in Duchenne muscular dystrophy.

The increased degree of muscle damage, cell death, and inflammation within dystrophin-deficient striated muscle raises the possibility that the pathologic milieu of this tissue may perturb cell signaling, thus affecting cellular nitric oxide production not as a direct consequence of the loss of dystrophin, but rather as a secondary consequence of changes in the muscle cell microenvironment. Indeed, patients with Duchenne muscular dystrophy and dystrophin-deficient *mdx* mice exhibit elevated circulating concentrations of methylated arginines, modified amino acids that can act as endogenous inhibitors of the nitric oxide synthases. Chapter 4 describes experiments designed to test the hypothesis that excessive asymmetric dimethylarginine inhibits muscle NO production and contributes to exercise intolerance in DMD. Injury of otherwise healthy skeletal muscle with cardiotoxin resulted in increased circulating methylated arginine levels, supporting the notion that these compounds are elevated in DMD as a result of increased damage to the dystrophin-deficient muscle. Impairment of exercise performance in wild-type mice infused with exogenous ADMA indicated that this compound was sufficient to affect exercise, even in the absence of dystrophy. Dystrophin-deficient and dystrophin-heterozygous mice expressing a transgene encoding dimethylarginine dimethylaminohydrolase 1 (DDAH), an enzyme that degrades ADMA, were generated and assessed on treadmill running assays in order to examine the contribution of ADMA to exercise tolerance in DMD. Surprisingly, DDAH transgene expression attenuated exercise-induced fatigue in female dystrophin-heterozygous animals, but not in male dystrophin-deficient animals. This improvement in exercise tolerance was associated with reduced heart weight, improved cardiac contractile function, and improved chronotropic responsiveness to β-adrenergic stimulation in transgenic female dystrophin-heterozygous mice. Collectively, these observations support a model in which methylated arginines diminish resistance to exercise-induced fatigue in DMD carriers, potentially by promoting the pathological remodeling and impairing the function of the dystrophin-heterozygous heart.

Chapter 5 will summarize the principle findings of this thesis and describe how they fit into a new integrated model for the DGC's structural and signaling roles in the regulation of striated muscle nitric oxide signaling. The chapter concludes by discussing some unanswered questions regarding the mechanisms of AMPK regulation by the dystrophin-glycoprotein complex, the potential contribution of ADMA to cardiac remodeling in muscular dystrophy, and the potential applications of this thesis work given the current state of therapies targeting blood flow regulation and exercise tolerance in muscular dystrophy.

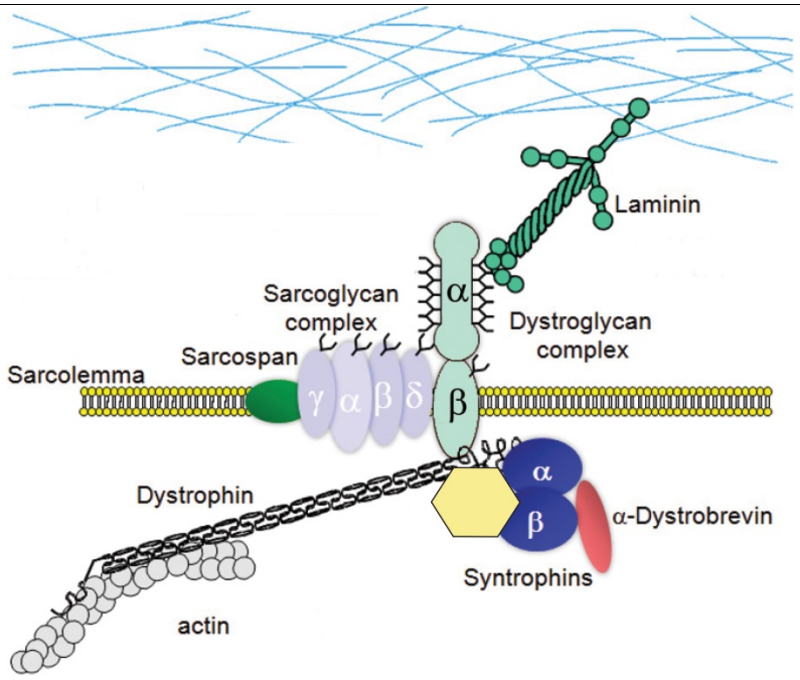

Figure 1.1. The structure of the dystrophin-glycoprotein complex. The dystrophin-glycoprotein-complex (DGC) is found at the plasma membrane of muscle cells, where it forms a functional linkage between the cytoskeleton and the extracellular matrix. This linkage is formed by binding of dystrophin to sub-sarcolemmal actin and to the transmembrane protein, β-dystroglycan. β-dystroglycan in turn associates with the α-dystroglycan, which is heavily glycosylated and mediates binding of the complex to matrix proteins such as laminin. β-dystroglycan also binds to a sub-complex comprised of the sarcoglycans and sarcospan. Additional accessory proteins including α- and β-syntrophin and α-dystrobrevin bind near the C-terminus of dystrophin to complete the DGC. (Adapted from Michele and Campbell, 2003 (153)).

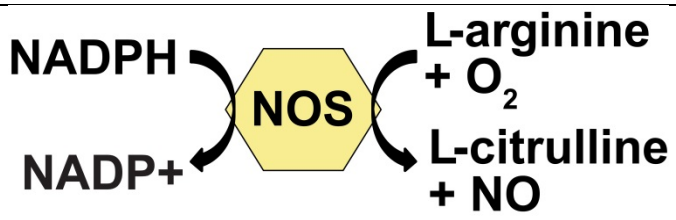

Figure 1.2. The production of nitric oxide by nitric oxide synthases. The three mammalian forms of nitric oxide synthase (NOS) (neuronal nitric oxide synthase, endothelial nitric oxide synthase, and inducible nitric oxide synthase) catalyze the production of nitric oxide (NO). In this reaction, NADPH provides electrons for the reduction of molecular oxygen, which is coupled to the oxidation of the amino acid L-arginine, to form NO. L-citrulline is formed as a byproduct.

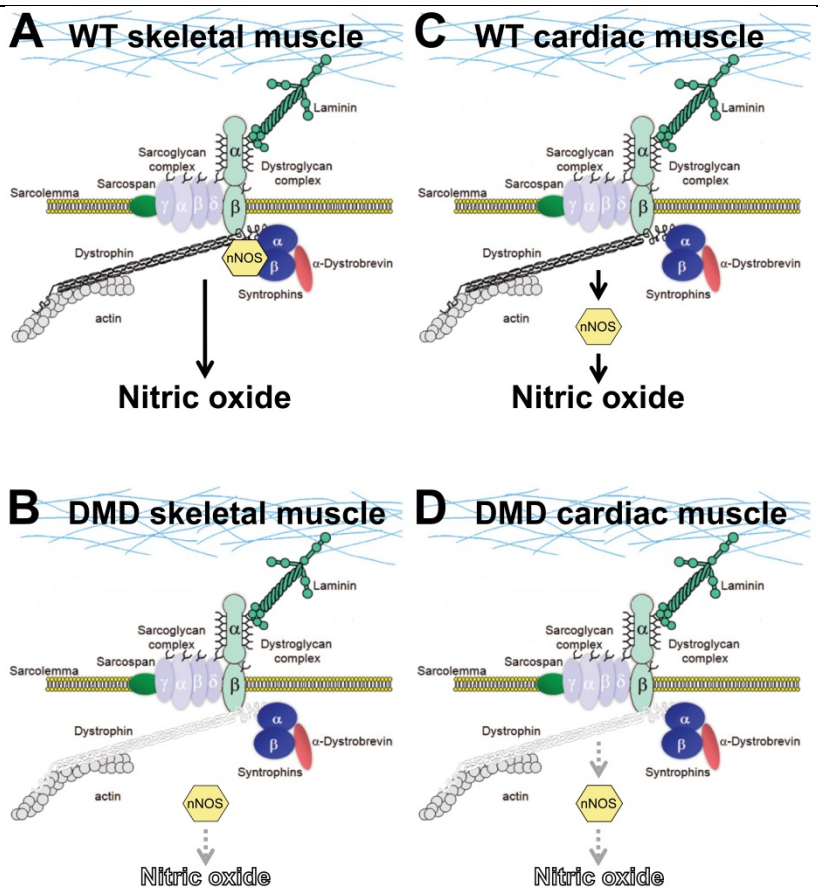

Figure 1.3. Functional relationships between nNOS and the dystrophin-glycoprotein complex in striated muscle. (**A**) The current model for dystrophin-dependent regulation of nNOS activity relies on a direct physical interaction between nNOS and the dystrophin-glycoprotein complex (DGC), as is observed in healthy wild-type (WT) skeletal muscle. (**B**) When dystrophin is lost, as occurs in Duchenne muscular dystrophy (DMD), nNOS-DGC binding is disrupted and nNOS is mislocalized to the cytosol. Mislocalization of nNOS is correlated with diminished nNOS enzymatic activity. (**C**) In healthy cardiac muscle, nNOS is expressed but does not bind to the DGC. (**D**) Diminished cardiac muscle nNOS activity in DMD supports an alternative model in which dystrophin is required for the appropriate regulation of nNOS activity, but does so via a mechanism that does not depend on direct binding between nNOS and the DGC.

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CHAPTER 2

Dystrophin-glycoprotein complex regulates muscle nitric oxide production through mechanoregulation of AMPK signaling.

ABSTRACT

Patients deficient in dystrophin, a protein that links the cytoskeleton to the extracellular matrix via the dystrophin-glycoprotein complex (DGC), exhibit muscular dystrophy, cardiomyopathy, and impaired muscle nitric oxide (NO) production. We used live-cell nitric oxide imaging and *in vitro* cyclic stretch of isolated adult mouse cardiomyocytes as a model system to investigate if and how the DGC directly regulates the mechanical activation of muscle NO signaling. Acute activation of NO synthesis by mechanical stretch was impaired in dystrophin-deficient *mdx* cardiomyocytes, accompanied by loss of stretch-induced neuronal nitric oxide synthase (nNOS) S1412 phosphorylation. Intriguingly, stretch induced the acute activation of AMP-activated protein kinase (AMPK) in normal cardiomyocytes but not in *mdx* cardiomyocytes, and specific inhibition of AMPK was sufficient to attenuate mechanoactivation of NO production. Therefore, we tested whether direct pharmacologic activation of AMPK could bypass defective mechanical signaling in order to restore nNOS activity in dystrophin-deficient cardiomyocytes. Indeed, activation of AMPK with 5-aminoimidazole-4-carboxamide riboside or salicylate increased nNOS S1412 phosphorylation and was sufficient to enhance NO production in *mdx* cardiomyocytes. We conclude that the DGC promotes the mechanical activation of cardiac nNOS by acting as a mechanosensor to regulate AMPK activity, and that pharmacologic AMPK activation may be a suitable therapeutic strategy to restore nNOS activity in dystrophin-deficient hearts and muscle.

INTRODUCTION

The muscular dystrophies are a group of muscle wasting disorders characterized by progressive weakening and degeneration of striated muscle. The most common form is Duchenne muscular dystrophy (DMD), an X-linked disorder caused by genetic disruption of dystrophin(1) that affects 1 in 3500-5000 males(2, 3). DMD and several other types of muscular dystrophy result from disruption of the dystrophin-glycoprotein complex (DGC), a structure that spans the sarcolemma and forms a mechanical linkage between the cytoskeleton and the extracellular matrix via the association of dystrophin with subsarcolemmal γ-actin and the binding of α-dystroglycan to laminin(4). The generally accepted role for this complex is to act as a molecular shock absorber and stabilize the plasma membrane during muscle contraction. Disruption of the DGC's linkage between the cytoskeleton and extracellular matrix, such as occurs in DMD(1, 5-7) or with the disruption of α-dystroglycan-laminin binding in glycosylation-deficient muscular dystrophies(8, 9), leads to destabilization of the plasma membrane, rendering skeletal muscle fibers and cardiomyocytes susceptible to stretch- or contraction-induced injury and cell death(10-14).

In addition to this structural role, a signaling function for the DGC has been proposed based on its association with several signaling proteins including Grb2-Sos1(15), MEK and ERK(16), heterotrimeric G-protein subunits(17, 18), archvillin(19), and neuronal nitric oxide synthase (nNOS)(20-22). The current dogma is that the DGC serves as a passive scaffold for these molecules, anchoring them near sites of action or important partners, with genetic disruption of the DGC leading to mislocalization, destabilization, or ineffective recruitment of these molecules to the complex(23). In healthy skeletal muscle, nNOS binds to the DGC via direct physical interactions with dystrophin and α-1 syntrophin(20, 22, 24) and is thus anchored near the sarcolemma. During muscle contraction, nitric oxide (NO) produced from nNOS is thought to signal in a paracrine fashion to the vascular smooth muscle of arterioles that supply the skeletal muscle with blood, thereby locally counteracting vasoconstriction induced by increased sympathetic tone during exercise(25, 26). In DMD, genetic loss of dystrophin results

in secondary mislocalization of nNOS from the skeletal muscle sarcolemma(20, 21) and impaired muscle NO production is observed. Disrupted muscle NO signaling impairs muscle blood flow regulation in dystrophin-deficient mice(27) and results in markedly exaggerated fatigue following exercise(28). Severe muscle fatigue is also a prominent feature of human DMD, and its improvement is considered an important clinical outcome for therapies, such that the six minute walk test is an important endpoint for clinical trials in DMD(29). Despite growing appreciation of the physiological implications of disrupted muscle NO production, it remains unclear whether impaired nNOS activation in dystrophin-deficient skeletal muscle is a consequence of disrupted scaffolding of nNOS to the DGC, or whether the DGC acts as a mechanosensor in a mechanosignaling pathway that regulates nNOS activity. Furthermore, mechanical stimulation enhances nNOS and endothelial nitric oxide synthase (eNOS) activity in cardiac muscle(30, 31), but whether such mechanosensitive cardiac NOS signaling occurs via one of these two possible mechanisms or depends on dystrophin is unknown.

Two reports have suggested that dystrophin-deficient hearts(32) and cardiomyocytes(33) exhibit impaired nNOS activity. Despite this apparent similarity of the effect of dystrophin-deficiency on cardiac and skeletal muscle NOS signaling, another recent study demonstrated that in contrast to skeletal muscle, nNOS is not physically associated with the DGC in cardiac muscle(34). These interesting observations suggest that dystrophin and the DGC's impact on NO signaling may not result from a purely scaffolding function of the DGC for nNOS in striated muscle. Therefore, we hypothesized that dystrophin and the DGC act as a mechanosensor to couple the muscle cell's mechanical activity to a biochemical signaling pathway leading to NO production, independent of a direct physical interaction between NOS and the DGC.

We used cardiomyocytes as a model for striated muscle, and performed *in vitro* mechanical stretching to investigate the direct involvement of dystrophin in mechanically-activated muscle NO signaling and the potential role of the DGC as a mechanosensor. Here we report that acute mechanical activation of NO production is impaired in dystrophin-deficient mouse cardiomyocytes, and identify disrupted mechano-AMPK-nNOS signaling as a key component of altered NO production in these cells. These findings suggest that dystrophin and

the DGC play more than a passive structural role in striated muscle, and rather may be active participants in mechanotransduction. Finally, we demonstrate that acute pharmacologic activation of AMPK is sufficient to restore nNOS activity and NO production in dystrophin-deficient cardiomyocytes, and propose that this may be a suitable strategy to bypass disrupted mechanical signaling and restore NO production in dystrophin-deficient striated muscle.

MATERIALS AND METHODS

Mice: Control wild-type (WT) C57 and dystrophin-deficient *mdx* mice were used at 8-25 weeks, before the development of dystrophic cardiomyopathy(35). All animals were housed at the University of Michigan's Unit for Laboratory Medicine and all procedures were approved by the University of Michigan Committee for the Use and Care of Animals.

Cardiomyocyte isolation and general plating conditions: Cardiomyocytes were isolated from adult mouse hearts using methods we have described previously(36). After isolation, cardiomyocytes were resuspended in plating medium (Table 2.1) and plated for 2 hours as described below.

***In vitro* cardiomyocyte nitric oxide imaging:** Cardiomyocytes were plated on individual 35mm-diameter FlexCell membranes (FlexCell International) coated with 50ug/mL mouse laminin (Invitrogen; 23017-015) at 30,000 cells/membrane. Following plating, cardiomyocytes were switched to culture medium (plating medium without serum), and then loaded with the nitric oxide-sensitive dye, 4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate (DAF-FM-DA) (Molecular Probes; D-23842). Cardiomyocytes on FlexCell membranes were incubated in imaging buffer (Table 2.2) containing 20μM DAF-FM-DA for 40 minutes in the dark at 37^{O}C. Cardiomyocytes were then washed in fresh imaging buffer without DAF-FM-DA for 20 minutes in the dark at 37^{O}C, then washed again for 5 minutes at room temperature. In experiments using enzyme inhibitors, inhibitors or appropriate vehicle controls were first added to buffer during this 5-minute wash. Membranes were then loaded into a StageFlexer Jr. stretch chamber connected to a FX-4000T Flexercell Tension Plus system (FlexCell International) and covered with fresh imaging buffer. The stretch chamber was secured on the stage of an Olympus BX51

upright epifluorescence microscope and a 10x field of view was visualized under the FITC channel using a 6% neutral density filter. Images of the same field of view were recorded at 0, 10, 30, and 60 minutes of 12% 1Hz stretch or drug treatment. After imaging, the fluorescence of all rod-shaped, non-fibrillatory cardiomyocytes was quantified using ImageJ, with background fluorescence subtracted. Changes in cellular DAF-FM fluorescence intensity over the 1-hour imaging period were expressed as the change in fluorescence over initial (0-minute) fluorescence ($\Delta F/F_0$). Inhibitors and other reagents used in these studies were as follows: N^G-Nitro-L-arginine-methyl ester hydrochloride (L-NAME) (100µM) (Enzo; ALX-105-003), S-Nitroso-N-acetylpenicillamine (SNAP) (100µM) (Calbiochem; 487910), vinyl-L-NIO hydrochloride (VLNIO) (100µM) (Cayman Chemical; 80330), Compound C (10µM) (Cayman Chemical; 11967), 5-aminoimidazole-4-carboxamide riboside (AICAR) (2mM) (Cayman Chemical; 10010241), and sodium salicylate (10mM) (Sigma; S2679).

***In vitro* stretch protocol:** Cardiomyocytes were plated on 6-well BioFlex culture plates (FlexCell International) coated with laminin at 30,000 cells/well in plating medium supplemented with 25µM blebbistatin (Toronto Research Chemicals; B592500).

Stretch for western blotting: Following plating, cardiomyocytes were changed to culture medium containing 25µM blebbistatin and serum-starved for 4 hours. BioFlex plates were loaded into a baseplate housed within a tissue culture incubator and connected to a FX-4000T Flexercell Tension Plus system (FlexCell International). Cardiomyocytes were subjected to 15% cyclic stretch at 1Hz for 0-60 minutes based on methods we have described previously(14).

Stretch for cGMP analysis: Cardiomyocytes were stretched for 60 minutes as described above. Stretched cardiomyocytes and non-stretched controls were then washed in DPBS and lysed in 0.1M HCl for 20 minutes. Lysates were centrifuged for 10 minutes at 1000 x g, and the supernatants were stored at -80^{o}C until use. cGMP concentration in cardiomyocyte lysates was analyzed using a cyclic GMP EIA kit (Cayman Chemical; 581021) according to the manufacturer's instructions, and was normalized to protein concentration based on the *DC* Protein Assay (Bio-Rad; 500-0116).

Drug treatments for western blot analysis: Cardiomyocytes were plated on 60mm tissue cultures dishes coated with laminin in plating medium supplemented with 25 µM

blebbistatin. After plating, cardiomyocytes were switched to culture medium containing 25μM blebbistatin and serum-starved for 4 hours. Cardiomyocytes were treated with AICAR or sodium salicylate for 0-60 minutes.

Cardiomyocyte lysis for western blot analysis: After stretch or drug treatment, cardiomyocytes were washed in DPBS and then lysed in ice-cold Triton-X lysis buffer supplemented with protease and phosphatase inhibitors (Table 2.3). Lysates were sonicated for 10 seconds and then centrifuged for 2 minutes at 14000 x g at 4^{o}C. Lysate supernatants were collected and stored at -80^{o}C until use.

Heart collection and tissue preparation for western blot analysis: Hearts were collected from anesthetized mice. The atria and large vessels were removed, then the ventricles were drained of blood, snap frozen in liquid nitrogen, and stored at -80^{o}C. Approximately 50mg of tissue per heart was added to ice-cold Triton-X lysis buffer containing protease and phosphatase inhibitors, minced with scissors, homogenized, and then sonicated twice for 10 seconds. Heart lysates were centrifuged at 14000 x g for 10 minutes at 4^{o}C. Lysate supernatants were collected and stored at -80^{o}C until use.

Western blot analysis: Protein concentration in lysates was quantified using a *DC* Protein Assay. 100 μg protein of each sample was separated on 3-15% (wt/vol) gradient polyacrylamide gels and then transferred to polyvinylidene fluoride membranes (Millipore; IPVH00010). Membranes were then blocked in 5% (wt/vol) nonfat dry milk (NFDM) or 5% (wt/vol) BSA (Fisher; BP1600) in Tris-buffered saline + 0.05% Tween 20 (TBST) for 1 hour. Following blocking, membranes were incubated overnight at room temperature with the appropriate primary antibody. Primary antibodies included the following rabbit polyclonal antibodies: dystrophin (Abcam; ab15277), phospho-nNOS-serine 1412 (Abcam; ab5583), nNOS (Cell Signaling; 4234), and AMPKα (Cell Signaling; 2532); and the following rabbit monoclonal antibodies: phospho-AMPKα-threonine 172 (Cell Signaling; 2535), phospho-acetyl-CoA carboxylase-serine 79 (Cell Signaling; 11818), and acetyl-CoA carboxylase (Cell Signaling; 3676). After incubation with primary antibodies, blots were washed 3 times for 10 minutes each in TBST and then incubated for 1 hour at room temperature with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories; 111-

035-144). Membranes were washed 3 times for 10 minutes each in TBST, then exposed using SuperSignal West Pico or Dura chemiluminescent substrate (ThermoFisher; 34080 and 34075). Images of blots were recorded with an Alpha Innotech western blot documentation system, and band intensities quantified with AlphaEaseFC software.

Cardiomyocyte Immunofluorescence: WT and *mdx* cardiomyocytes were plated on 6-well BioFlex culture plates and stretched for 0-10 minutes as described for Western blot analysis. After stretching, the cells were fixed for 15 minutes in 3% paraformaldehyde, washed three times for 5 minutes each in PBS, and then incubated overnight at 4^{o}C in block solution consisting of 1x PBS, 5% BSA, 0.5% Triton-X, and 50mM ammonium chloride. After blocking, the FlexCell membranes with attached cardiomyocytes were excised from wells of the BioFlex culture plates with a scalpel and placed on glass slides. The excised membranes were then incubated for 1.5 hours at room temperature with the appropriate primary antibodies diluted in block solution without ammonium chloride. Primary antibodies included the following rabbit polyclonal antibodies: nNOS (Cell Signaling; 4234) and AMPKα (Cell Signaling; 2532); and mouse monoclonal MANDRA1 dystrophin (Sigma-Aldrich; D8043). After incubation with primary antibodies, membranes were washed three times for 5 minutes each in PBS and then incubated for 1 hour at room temperature with secondary antibodies diluted in block solution without ammonium chloride containing DAPI (Sigma-Aldrich; D9564). Secondary antibodies were as follows: Alexa Fluor 488-conjugated goat anti-rabbit (Invitrogen; A-11034), Cy3-conjugated rat anti-mouse (Jackson ImmunoResearch Laboratories; 415-165-166), Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories; 111-165-144), and Alexa Fluor 488-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories; 115-546-062). Membranes were then washed three times for 5 minutes each in PBS, and a glass coverslip was mounted on top of each membrane using PermaFluor (Thermo Scientific; TA-030-FM). Images were captured using a 60x objective on a Nikon A-1 Spectral confocal microscope.

Statistics: All data are presented as mean ± SE. Significance was determined by the Student *t* test and was set at $P<0.05$.

RESULTS

Mechanical stretch activates NO production in adult mouse cardiomyocytes.

To investigate mechanically-activated muscle NO signaling, we established an assay using the NO-sensitive dye, DAF-FM-DA, to monitor cellular NO production during *in vitro* cyclic stretch of isolated adult mouse cardiomyocytes. Upon entering cells, DAF-FM-DA is cleaved to form 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM), which increases in fluorescence upon irreversible binding with nitric oxide(37) and thus can be used to assess cumulative cellular NO production over time. 1Hz cyclic stretch of wild-type cardiomyocytes elicited a significant increase in DAF-FM dye fluorescence over the course of an hour compared to non-stretched cells (Fig. 2.1 A and B). This stretch-induced increase in DAF-FM fluorescence was abolished by incubation of the cardiomyocytes with the general nitric oxide synthase inhibitor, L-NAME, verifying that the stretch-induced increase in dye fluorescence was due to increased NO production (Fig. 2.1B). DAF-FM fluorescence was also increased by treating cells with the exogenous NO donor, SNAP (Fig. 2.1B).

Stretch-dependent NO signaling is disrupted in dystrophin-deficient cardiomyocytes.

We then used this assay to test whether dystrophin is required for mechanically-activated NO signaling in cardiac muscle. Under resting conditions, we detected no difference in changes in DAF-FM dye fluorescence between WT and dystrophin-deficient *mdx* cardiomyocytes (Fig. 2.1C). However, the increase in DAF-FM dye fluorescence during stretch was significantly attenuated in *mdx* cardiomyocytes compared to WT cardiomyocytes (Fig. 2.1C), suggesting that dystrophin is required for appropriate mechanical activation of NO production in cardiac muscle. Treatment with SNAP after the stretch period produced a significant increase in DAF-FM fluorescence in both WT and *mdx* cardiomyocytes (Fig. 2.1D). This finding verifies that *mdx* cardiomyocytes did effectively retain the dye, and that the diminished increase in dye fluorescence during stretch of these cells was indeed attributable to impaired NO production, rather than an artifact of the dye leaking out of the excessively permeable or damaged plasma membranes that are characteristic of dystrophic cardiomyocytes(14, 38). Measurement of cellular cGMP content downstream of NO production

revealed no differences between WT and *mdx* cardiomyocytes at rest (Fig. 2.1E). One hour of 1Hz stretch increased cGMP content in WT cardiomyocytes, but this effect was abrogated in *mdx* cardiomyocytes (Fig. 2.1F). These data further support the idea that the loss of dystrophin disrupts mechanically-activated cardiac NO production and may have physiologically-relevant consequences on downstream signaling.

Stretch-induced nNOS phosphorylation is impaired in dystrophin-deficient cardiomyocytes.

This defect in mechanically-activated NO signaling prompted us to investigate the mechanism coupling dystrophin to cardiac NOS activity. Given that mRNA and protein content of the constitutively-expressed nitric oxide synthases, nNOS and eNOS, are not reduced in *mdx* hearts(33), we hypothesized that impaired mechano-NO production in dystrophin-deficient cardiomyocytes was related to misregulation of post-translational modification required for NOS activity. The enzymatic activity of both nNOS and eNOS is under multiple levels of regulatory control that include phosphorylation of several stimulatory or inhibitory residues(39, 40). Biochemical analysis revealed a significantly reduced ratio of serine 1412-phosphorylated to total nNOS in *mdx* versus WT heart lysates (Fig. 2.2). Interestingly, phosphorylation at this site increases nNOS activity(41).

To investigate whether decreased nNOS phosphorylation in dystrophin-deficient hearts *in vivo* was due to disrupted mechanical signaling, we compared the impact of stretch on nNOS-serine 1412 phosphorylation state in WT and *mdx* cardiomyocytes *in vitro*. Phosphorylation of this residue increased significantly over basal levels within 10 minutes of 1Hz stretch in WT cardiomyocytes (Fig. 2.3A). Incubation of WT cardiomyocytes with the nNOS-specific inhibitor vinyl-L-NIO attenuated the stretch-induced increase in NO production (Fig. 2.3B), verifying that increased nNOS-serine 1412 phosphorylation during stretch was indeed associated with increased nNOS activity. Intriguingly, stretch failed to elicit an increase in nNOS-serine 1412 phosphorylation in *mdx* cardiomyocytes (Fig. 2.3A). Immunofluorescence labeling revealed that nNOS was localized in the cytoplasm, in a striated pattern, and in the nucleus and was not specifically enriched at the sarcolemma of WT or *mdx* cardiomyocytes at rest (Fig. 2.4), in agreement with previous reports (33, 34). Moreover, stretch did not lead to membrane

recruitment of nNOS or otherwise discernibly alter nNOS localization in either WT or *mdx* cells (Fig. 2.4). Collectively, these results suggest that the loss of dystrophin impairs the mechanical activation of cardiac nNOS not by disrupting a physical interaction between nNOS and the DGC, but rather by disrupting mechanical signaling that regulates nNOS phosphorylation state and activity.

Stretch-induced AMPK phosphorylation is impaired in dystrophin-deficient cardiomyocytes.

We next sought to determine the mechanosensitive signaling pathway that couples dystrophin to stretch-induced activation of nNOS. AMP-activated protein kinase (AMPK) phosphorylates nNOS-serine 1412 in multiple cell types including skeletal muscle(42-45), and can be activated by mechanical stimulation of cardiac muscle(46, 47). Therefore, we investigated the involvement of AMPK in dystrophin-dependent mechano-nNOS signaling in cardiomyocytes. Western blot analysis demonstrated that stretch of WT cardiomyocytes increased phosphorylation of the catalytic alpha subunit of AMPK at threonine 172 (Fig. 2.3A), a modification that reflects increased AMPK activity(48). Stretch of WT cardiomyocytes also increased phosphorylation of the classical AMPK substrate acetyl-CoA carboxylase (ACC) at serine 79 (Fig. 2.3A), reinforcing the idea that stretch activates AMPK. Incubation with the AMPK inhibitor Compound C attenuated the stretch-induced increase in DAF-FM fluorescence in WT cardiomyocytes (Fig. 2.3C), confirming that AMPK activation contributes to mechanically-induced NO production in these cells. Notably, 1Hz stretch of *mdx* cardiomyocytes failed to increase phosphorylation of AMPKα-threonine 172 and ACC-serine 79 (Fig. 2.3A), indicating that dystrophin is required for appropriate mechanical activation of AMPK. These findings further imply that impaired mechanoactivation of AMPK contributes to impaired mechanoactivation of nNOS in dystrophin-deficient muscle. Similar to nNOS, AMPK displayed striated cytoplasmic and nuclear staining and was not associated with the sarcolemma in WT or *mdx* cardiomyocytes either at rest or after stretch (Fig. 2.5), raising the possibility that additional intermediate signaling factors couple the DGC to the regulation of AMPK.

Pharmacologic AMPK activation restores nNOS activity in dystrophin-deficient cardiomyocytes.

There is increasing interest in using AMPK activation as a therapeutic strategy to improve muscle pathology in Duchenne muscular dystrophy. Chronic activation of AMPK with administration of the AMP analog AICAR(49) in *mdx* mice has been shown to promote a slow, oxidative muscle phenotype, increase utrophin expression, and enhance sarcolemmal stability in skeletal muscle(50), and to reduce ongoing muscle damage and increase skeletal muscle strength(51, 52). Whether any of these effects depend on increased NO production, and whether AMPK activation can enhance NO production in the dystrophic heart, are unclear. Therefore, we tested whether acute pharmacologic activation of AMPK is sufficient to restore nNOS activity in dystrophin-deficient cardiomyocytes. Indeed, treatment with AICAR or with the AMPK-activating drug salicylate(53) significantly increased DAF-FM dye fluorescence in *mdx* cardiomyocytes compared with vehicle controls and at levels similar to treated WT cardiomyocytes (Fig. 2.6 A and C). Inhibition of nNOS with vinyl-L-NIO attenuated AICAR- and salicylate-induced increases in DAF-FM fluorescence in *mdx* cardiomyocytes (Fig. 2.6 B and D). Consistent with these data, acute 60-minute treatment with AICAR or salicylate increased the activating phosphorylation of AMPKα-threonine 172, and increased stimulatory phosphorylation of nNOS-serine 1412, in both WT and *mdx* cardiomyocytes (Fig. 2.7 A and B). Together, these results indicate that AMPK stimulates NO production in cardiomyocytes, and that acute pharmacologic AMPK activation is sufficient to restore nNOS activity in dystrophin-deficient cardiomyocytes.

DISCUSSION

Thirty years of research into the molecular mechanisms of the muscular dystrophies have supported an important structural role for the dystrophin-glycoprotein complex in stabilizing the plasma membrane of striated muscle cells. Increasing evidence suggests that the integrity of the DGC also has important consequences for cell signaling(15-22); however, the passive effects of the DGC to scaffold signaling proteins(23) have not yet been distinguished from the

potential for the DGC to participate actively as a mechanosensor to regulate cellular signaling. This question is particularly pertinent to elucidating the mechanisms of muscle NO signaling, considering that whether impaired nNOS function in DMD is a direct structural consequence of disrupted localization of nNOS to the sarcolemma by the DGC, or whether disruption of the DGC leads to altered post-translational regulation of nNOS activity, has remained unclear.

Here, we used adult cardiomyocytes as a model of differentiated striated muscle to address the hypothesis that the DGC functions directly as a mechanosensor to regulate nitric oxide signaling in muscle. Our observation of impaired mechanically-stimulated nNOS-serine 1412 phosphorylation in dystrophin-deficient cardiomyocytes provides direct mechanistic evidence of why nNOS activity is impaired in dystrophin-deficient cardiac muscle. It is also consistent with an earlier report of reduced nNOS activity in total lysates from *mdx* mouse hearts(32), given that the heart is stimulated mechanically by contraction *in vivo.* Moreover, the fact that such dystrophin-dependent mechanical nNOS signaling is independent of a physical interaction between nNOS and the cardiac DGC suggests that the DGC is not merely a passive scaffold for nNOS, but rather is an active regulator of a signaling pathway that controls nNOS activity in cardiac muscle, and possibly in skeletal muscle as well. A model in which dystrophin loss results in loss of AMPK-dependent regulation of nNOS would also help to explain why deletion of nNOS does not dramatically worsen muscle pathology in *mdx* mice(54, 55), because the activation of any nNOS expressed in dystrophin-deficient muscle would already be impaired.

One potential candidate kinase for regulating nNOS phosphorylation was AMPK. Our finding that mechanical activation of AMPK contributed to NO production in WT cardiomyocytes and was impaired in *mdx* cardiomyocytes suggests that impaired stretch-induced activation of nNOS with loss of dystrophin is due to impaired mechanical activation of AMPK. The identification of a dystrophin-AMPK-nNOS mechanical signaling axis in adult striated muscle is particularly interesting given the finding that the activation of AMPK by stretch of alveolar epithelial cells depends on dystroglycan(56), a core component of the muscle DGC. Together, these observations indicate that DGC-AMPK signaling may be an important component of mechano-sensing across multiple cell and tissue types. AMPK expressed in

cardiac muscle did not appear to colocalize with dystrophin at the sarcolemma. Thus, in future studies it will be important to determine the mechanism by which the DGC regulates AMPK activity, such as through regulation of the putative AMPK kinases liver kinase B1 (LKB1) or calcium-calmodulin-activated protein kinase kinase-β (CaMKKβ), or perhaps by preventing the excessive production of reactive oxygen species during stretch(57).

AMPK has been identified as an important therapeutic target in a variety of disorders, including diabetes and aging, and plays an important role in long-term adaptation of muscle to exercise. We demonstrate that acute, direct pharmacologic AMPK activation by either AICAR or salicylate mechanistically targets altered nNOS regulation and is sufficient to restore nNOS activity in dystrophin-deficient cardiac muscle. Several other strategies to boost NO and downstream cGMP signaling in the dystrophin-deficient heart have been tested, including transgenic myocardial expression of an nNOS transgene(58) and chronic administration of phosphodiesterase 5 inhibitors(35). Such studies have yielded mixed results in animal models, with modest improvements in cardiac function and pathology. Furthermore, human trials have offered little evidence of a beneficial cardiac effect of sildenafil treatment in adults with Duchenne or Becker muscular dystrophy(59, 60). In light of our findings, it is possible that the shortcomings of these previous studies reflect the existence of other functional targets of muscle-derived NO, or additional targets of altered AMPK regulation, that contribute to dysfunction in dystrophic muscle(45, 61, 62). This possibility warrants further investigation into whether acute AMPK activation can yield therapeutic benefit to dystrophic patients through the restoration of heart or skeletal muscle NO signaling.

Together, our data support a model in which dystrophin or an intact DGC is required as a mechanosensor for the appropriate activation of AMPK by mechanical stimulation in cardiac muscle. Dystrophin-dependent mechanical activation of AMPK then triggers stimulatory phosphorylation of nNOS-serine 1412, leading to increased cellular NO production. Loss of dystrophin expression in DMD uncouples mechanical stimulation from the activation of AMPK, ultimately leading to a failure to appropriately activate nNOS and resulting in impaired mechano-NO production. Further study is needed to clarify the contributions of disrupted mechano-AMPK-NO signaling to cardiac dysfunction in DMD and to determine whether

disrupted mechano-AMPK signaling similarly contributes to the disease phenotype, including nNOS impairment, in dystrophin-deficient skeletal muscle. Importantly, AMPK signaling may provide a new pharmacological target for restoring defective NO regulation in striated muscle and for improving cardiac and skeletal muscle blood flow in muscular dystrophy and other diseases where muscle blood flow is compromised.

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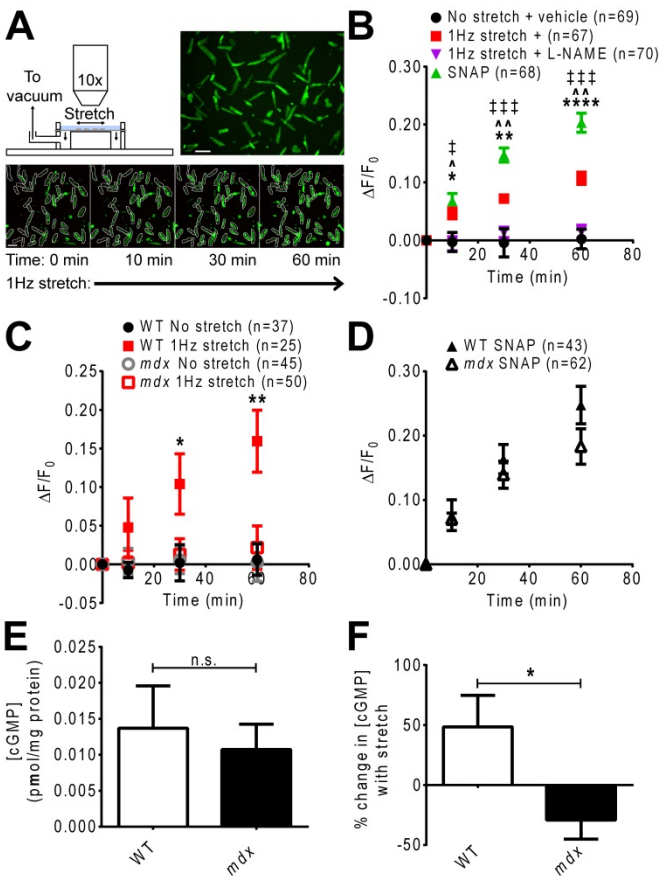

Figure 2.1. Stretch-dependent NO signaling is impaired in dystrophin-deficient cardiomyocytes. (**A**) Experimental setup. Adult mouse cardiomyocytes loaded with DAF-FM are visualized under 10x magnification using an epifluorescence microscope throughout 1 hour of 1Hz stretch. (Scale bars = 100 µm). DAF-FM fluorescence in each rod-shaped, non-fibrillatory cardiomyocyte is quantified throughout the protocol. (**B**) Validation of NO imaging assay. Stretch of WT cardiomyocytes induces a significant increase in DAF-FM fluorescence (*P<0.05, **P<0.01, ****P<0.0001 stretch vs. no stretch + vehicle). Incubation with the NOS inhibitor L-NAME abolishes the stretch-induced increase in DAF-FM fluorescence (^P<0.001, ^^P<0.0001 stretch + vehicle vs. L-NAME). Treatment with the NO donor SNAP yields a significant increase in DAF-FM fluorescence (‡P<0.01, ‡‡‡P<0.0001 SNAP vs. no stretch + vehicle). (**C**) The increase in DAF-FM fluorescence during stretch is significantly lower in *mdx* cardiomyocytes compared to WT cardiomyocytes (*P<0.05, **P<0.01 WT vs. *mdx* stretch). (**D**) Treatment with SNAP after stretch yields similar increases in DAF-FM fluorescence in WT and *mdx* cardiomyocytes. (**E**) There is no significant difference in cGMP concentration between WT and *mdx* cardiomyocytes before stretch (n=8 mice). (**F**) The change in cGMP concentration downstream of NO in response to stretch is significantly altered in *mdx* versus WT cardiomyocytes (n=8 mice) (*P<0.05).

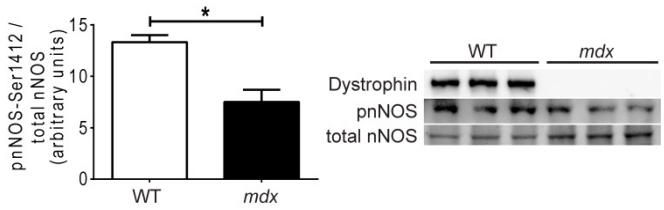

Figure 2.2. Stimulatory nNOS phosphorylation is impaired in dystrophin-deficient hearts *in vivo.* The ratio of phospho-Ser1412-nNOS to total nNOS is significantly reduced in *mdx* hearts versus WT hearts (n=3 mice) (*P<0.05).

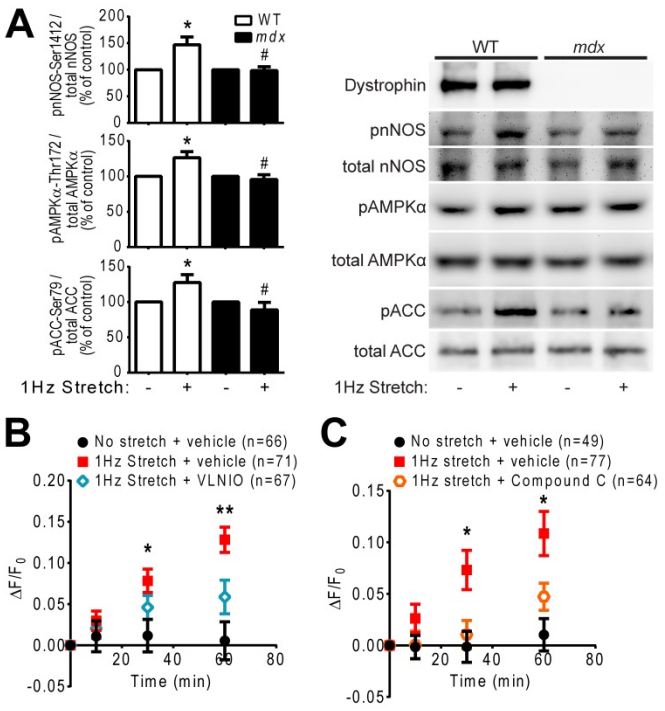

Figure 2.3. Stretch-induced nNOS and AMPK activation are impaired in dystrophin-deficient cardiomyocytes. (**A**) Acute stretch of WT cardiomyocytes elicits increased phosphorylation of nNOS at the stimulatory residue serine 1412, increased activating phosphorylation of AMPKα at threonine 172, and increased phosphorylation of ACC at serine 79 (*P<0.05 vs. WT control). These effects are attenuated in *mdx* cardiomyocytes ($^{\#}$P<0.05 vs. WT stretch) (n=6 mice). (**B**) The increase in DAF-FM fluorescence during stretch of WT cardiomyocytes is significantly reduced in cells incubated with the nNOS-selective inhibitor vinyl-L-NIO (*P<0.05, **P<0.01 stretch + vehicle vs. vinyl-L-NIO). (**C**) The increase in DAF-FM fluorescence during stretch of WT cardiomyocytes is significantly reduced in cells incubated with the AMPK inhibitor Compound C (*P<0.05 stretch + vehicle vs. Compound C).

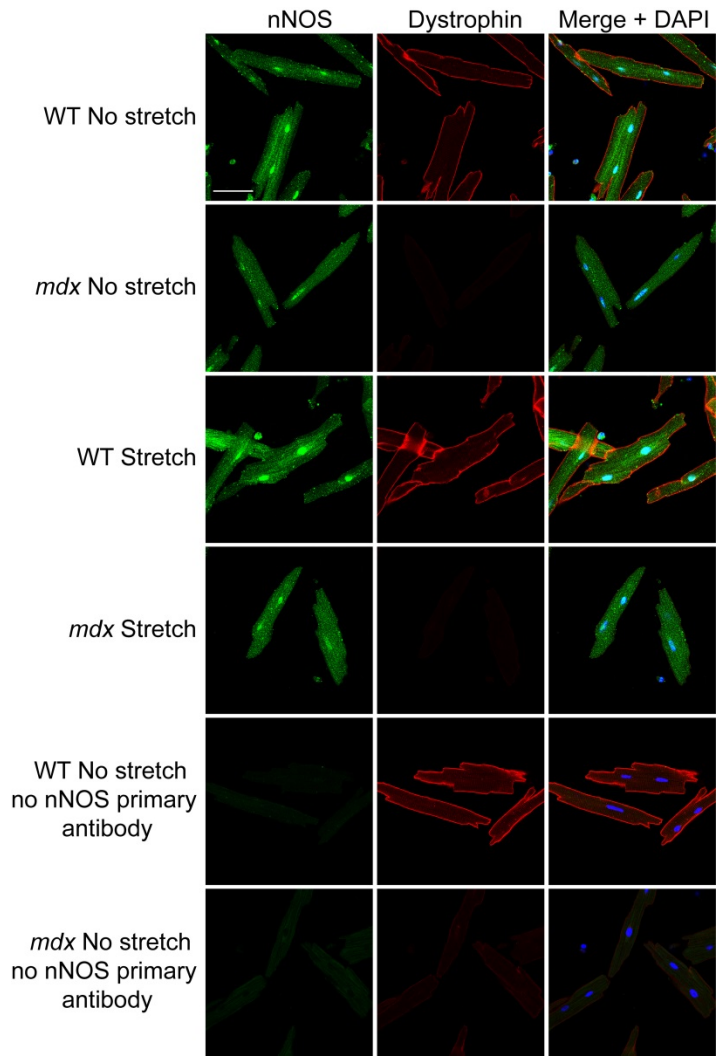

Figure 2.4. nNOS localization in adult mouse cardiomyocytes is not altered by acute stretch or dystrophin deficiency. WT and *mdx* cardiomyocytes exhibit cytoplasmic and nuclear localization of nNOS (green) at rest. nNOS is not specifically enriched at the sarcolemma and does not co-localize with dystrophin (red). 1Hz cyclic stretch does not lead to a discernible change in nNOS localization in either WT or *mdx* cardiomyocytes. (Scale bar = 50µm.)

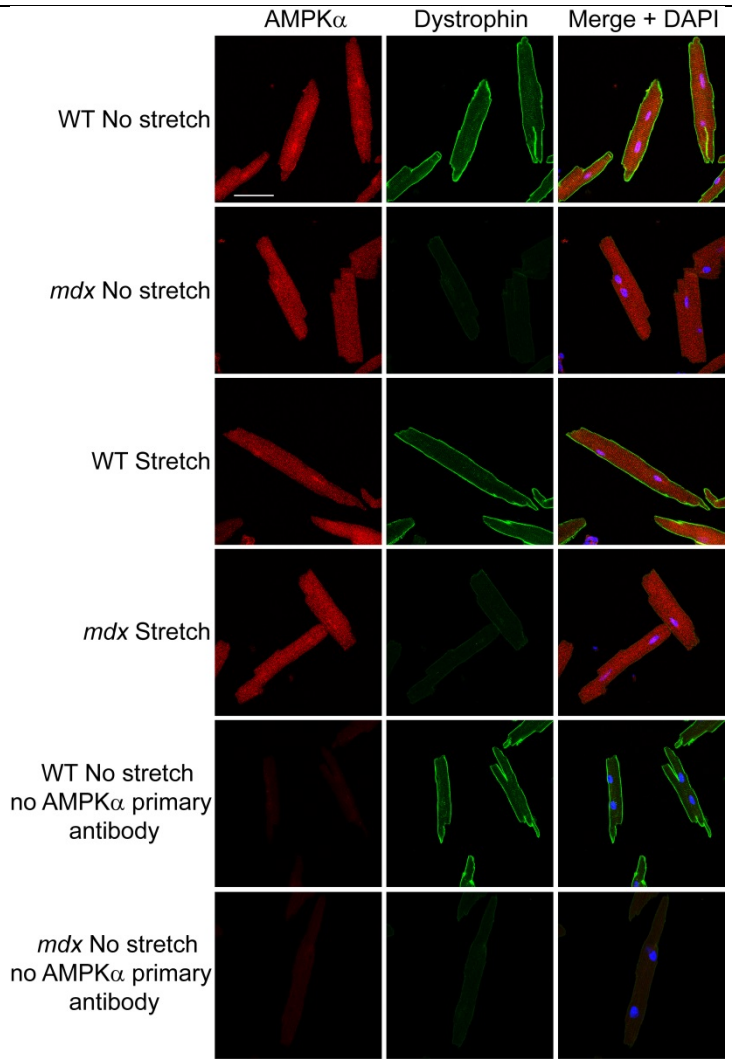

Figure 2.5. AMPKα localization in adult mouse cardiomyocytes is not altered by acute stretch or dystrophin deficiency. WT and *mdx* cardiomyocytes exhibit cytoplasmic and nuclear localization of AMPKα (red) at rest. AMPKα is not specifically enriched at the sarcolemma and does not co-localize with dystrophin (green). 1Hz cyclic stretch does not lead to a discernible change in AMPKα localization in either WT or *mdx* cardiomyocytes. (Scale bar = 50µm.)

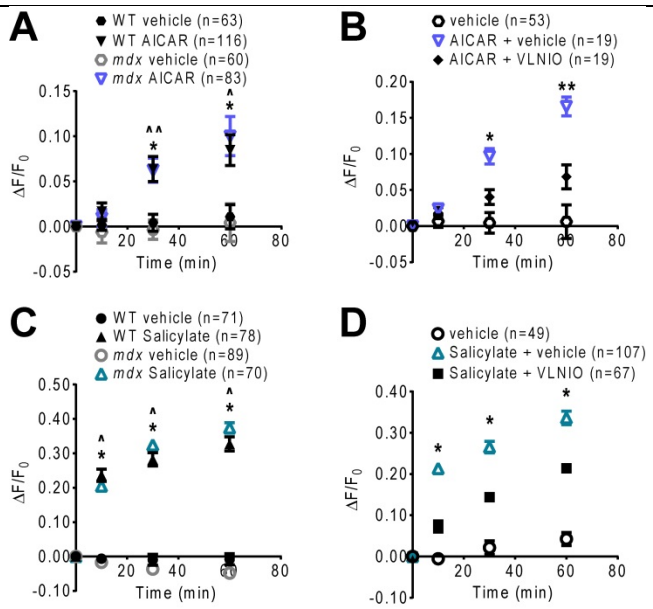

Figure 2.6. Pharmacologic AMPK activation increases nNOS activity in dystrophin-deficient cardiomyocytes. (**A**) Treatment with AICAR elicits a significant increase in DAF-FM fluorescence in both WT and *mdx* cardiomyocytes (*P<0.01 WT AICAR vs. vehicle; ^P<0.01, ^^P<0.001 *mdx* AICAR vs. vehicle). (**B**) nNOS inhibition with vinyl-L-NIO attenuates the increase in DAF-FM fluorescence induced by AICAR in *mdx* cardiomyocytes (*P<0.001, **P<0.0001 AICAR + vehicle vs. AICAR + vinyl-L-NIO). (**C**) Treatment with salicylate elicits a significant increase in DAF-FM fluorescence in both WT and *mdx* cardiomyocytes (*P<0.0001 WT salicylate vs. vehicle; ^P<0.0001 *mdx* salicylate vs. vehicle). (**D**) nNOS inhibition with vinyl-L-NIO attenuates the increase in DAF-FM fluorescence induced by salicylate in *mdx* cardiomyocytes (*P<0.0001 salicylate + vehicle vs. salicylate + vinyl-L-NIO).

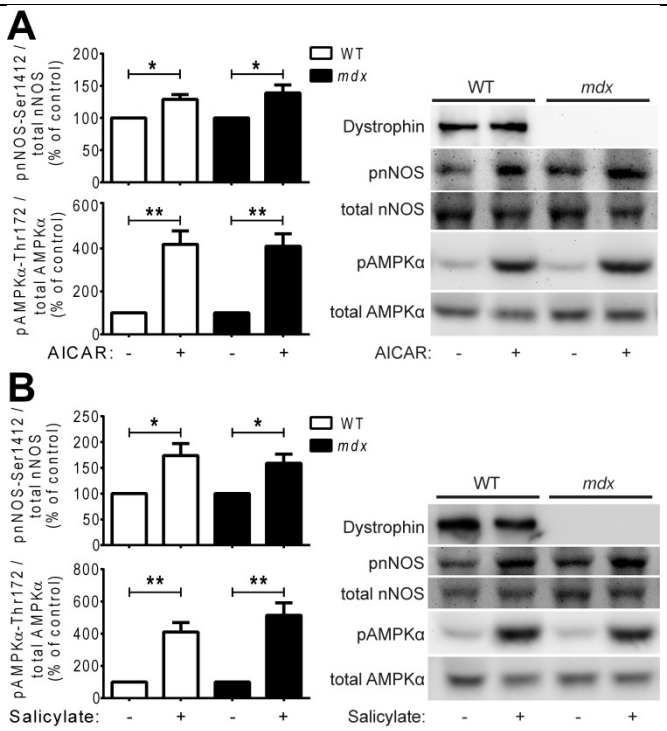

Figure 2.7. Pharmacologic AMPK activation increases stimulatory nNOS phosphorylation in dystrophin-deficient cardiomyocytes. (**A**) Acute treatment of WT and *mdx* cardiomyocytes with the AMPK activator AICAR increases activating phosphorylation of AMPKα at threonine 172, and increases stimulatory phosphorylation of nNOS at serine 1412 (*P<0.05, **P<0.01 vs. control) (n=6 mice). (**B**) Acute treatment of WT and *mdx* cardiomyocytes with the AMPK activator salicylate increases activating phosphorylation of AMPKα at threonine 172, and increases stimulatory phosphorylation of nNOS at serine 1412 (*P<0.05, **P<0.01 vs. control) (n=7 mice).

Table 2.1. **Plating Medium Composition.**	
Reagent	Final Concentration
MEM (Gibco 1157-032)	
Fetal bovine serum	5%
Penicillin G sodium salt	100U/mL
Glutamine	2mM
Bovine serum albumin	0.2%
$NaHCO_3$	4mM
HEPES	10mM

Table 2.2. Imaging Buffer Composition.	
Reagent	Final Concentration (mM)
NaCl	113
KCl	4.7
$MgSO_4$	1.2
KH_2PO_4	0.6
NaH_2PO_4	0.6
HEPES	10
$NaHCO_3$	1.6
Taurine	30
Glucose	20
$CaCl_2$	1.2
L-arginine HCl	0.01
pH = 7.4	

Table 2.3. Triton-X Lysis Buffer Composition.	
Reagent	Final Concentration
NaCl	150mM
Tris pH 7.5	50mM
Triton X-100	1%
EDTA	1mM
Benzamidine	0.6mM
PMSF	0.4mM
Pepstatin	0.5ug/mL
Aprotinin	2 KIU/mL
Leupeptin	1ug/mL
NaF	50mM
Sodium orthovanadate	1mM
Sodium pyrophosphate	10mM

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CHAPTER 3

Acute pharmacologic activation of AMPK improves exercise tolerance in a mouse model of Duchenne muscular dystrophy.

ABSTRACT

Duchenne muscular dystrophy (DMD) results from mutations in dystrophin and causes progressive muscle wasting and excessive fatigue upon exercising. Exercise intolerance in DMD has been attributed to impaired neuronal nitric oxide synthase (nNOS)-dependent nitric oxide (NO) production in the striated muscle, which leads to misregulation of local blood flow and inadequate perfusion of the muscles during exercise. We previously demonstrated that in dystrophin-deficient cardiomyocytes, impaired activation of AMP-activated protein kinase (AMPK) contributes to diminished activation of nNOS in response to mechanical stress, and that acute activation of AMPK with 5-aminoimidazole-4-carboxamideriboside (AICAR) or salicylate restores nNOS-dependent NO production. Therefore, we hypothesized that by increasing striated muscle nNOS activity, acute pharmacologic activation of AMPK would improve muscle blood flow and the exercise phenotype of dystrophin-deficient mice. Here, we used live-cell NO imaging and treadmill running assays to investigate whether acute pharmacologic AMPK activation stimulates NO production in dystrophin-deficient skeletal muscle *in vitro*, and whether acute pharmacologic AMPK activation improves exercise tolerance *in vivo* in the *mdx* mouse model of DMD. Incubation with an AMPK-activating drug, salicylate, caused a rapid increase in cellular fluorescence in isolated *mdx* flexor digitorum brevis fibers loaded with the NO-sensitive dye, DAF-FM-DA. In treadmill running assays, acute treatment with salicylate or AICAR increased the time and distance that *mdx* mice were able to run before reaching

exhaustion. We conclude that acute pharmacologic AMPK activation stimulates NO production in dystrophin-deficient skeletal muscle like it does in cardiac muscle, and that AMPK activation *in vivo* is sufficient to improve exercise tolerance in dystrophin-deficient animals. This work extends upon our previous findings in cardiomyocytes and provides proof-of-concept evidence for acute pharmacologic AMPK activation as a therapy to restore striated muscle NO signaling and ameliorate related defects in exercise performance in Duchenne muscular dystrophy.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a fatal, X-linked muscle wasting disease caused by mutations in the gene encoding the cytoskeletal protein, dystrophin(1, 2). Loss of dystrophin and disruption of the associated transmembrane dystrophin-glycoprotein complex (DGC) is thought to destabilize the sarcolemma of striated muscle cells, rendering these cells susceptible to contraction- or stretch-induced injury and cell death(3-8). Beyond playing an important structural function to protect striated muscle cells against mechanical injury, growing evidence suggests that the DGC also regulates biochemical signaling pathways, often by scaffolding signaling proteins at critical sites of action or near important interacting proteins, as has been recently reviewed by Constanin(9). The most widely-studied role of dystrophin and the DGC in cell signaling is their impact on nitric oxide (NO) production induced by the mechanical activity of striated muscle. In healthy skeletal muscle, this phenomenon of contraction-induced NO production is critical for sympatholysis, the process by which skeletal muscle contraction attenuates sympathetic vasoconstriction of the arterioles supplying the skeletal muscle with blood(10). In the heart, local NO production is similarly stimulated by stretch or contraction, and is thought to contribute to the regulation of coronary blood flow as well as the intrinsic systolic and diastolic properties of the myocardium(11-19).

When dystrophin expression is disrupted in DMD, NO signaling becomes disrupted in both skeletal muscle fibers and cardiomyocytes(20-23). In dystrophin-deficient skeletal muscle, such impaired NO signaling is associated with the loss of scaffolding interactions between nNOS and the DGC and consequent mislocalization of nNOS away from the sarcolemma and into the

cytoplasm(20, 24). Disruption of sympatholysis secondary to impaired nNOS activation during exercise has been demonstrated to contribute to excessive exercise-induced fatigue in the dystrophin-deficient *mdx* mouse model of DMD(25, 26), and may help to explain impaired sympatholysis in boys with this disease(27). It has also been proposed that transient ischemia resulting from impaired muscle NO signaling contributes to muscle cell injury and necrosis in DMD(28), and that disruption of local NO production contributes to impaired function and excessive fatigability of dystrophin-deficient hearts and skeletal muscle in a manner distinct from the vascular effects of NO(21, 29, 30). Thus, the restoration of striated muscle NO production has gained considerable attention as a therapeutic goal to help maintain skeletal muscle and cardiac function and to alleviate exercise intolerance in DMD patients.

Early reports demonstrating the ability for transgenic approaches that boost nNOS expression in the dystrophin-deficient striated muscle of *mdx* mice to improve neuromuscular junction architecture, reduce membrane damage, reduce cardiac fibrosis, and correct electrocardiographic abnormalities(31-33) provided foundational evidence for the potential for augmentation of nNOS signaling to ameliorate dystrophic muscle pathology in DMD. Translational studies over the past ten years have focused on the use of phosphodiesterase 5 (PDE5) inhibitors to attenuate the breakdown of cyclic guanosine monophosphate (cGMP), which is normally produced by guanylyl cyclase in response to NO production, and thereby enhance downstream effects of NO signaling in order to improve blood flow to dystrophin-deficient muscle during exercise. In dystrophin-deficient *mdx* mice, this strategy has been shown to alleviate exercise-induced fatigue and ischemic muscle fiber damage(26, 34, 35), improve cardiac function and the ability of the myocardium to withstand mechanical stress, and slow the progression of dystrophic cardiomyopathy(36-38). Unfortunately, human clinical trials of the PDE5 inhibitors sildenafil and tadalafil have produced mixed results at best, with different groups reporting alleviated skeletal muscle ischemia during exercise(39, 40), no effect on muscle blood flow during exercise or on heart function(41), and no to worsening effects on the progression of cardiomyopathy(42) in DMD or in Becker muscular dystrophy (BMD), the milder form of this disease. Thus, alternative approaches to correct defects in striated muscle NO signaling in dystrophinopathies remain a subject of substantial interest.

We recently demonstrated that in cardiomyocytes, the activation of nNOS is mechanically regulated via phosphorylation by the upstream kinase, AMP-activated protein kinase (AMPK), and that the mechanical activation of AMPK by muscle stretch depends in turn on the expression of dystrophin ((23), described above in Chapter 2). These findings suggested that defective NO production in dystrophin-deficient cardiac muscle is due in part to the impaired mechanical activation of AMPK. We further showed that direct pharmacologic activation of AMPK in dystrophin-deficient cardiomyocytes *in vitro* could be used to bypass defective mechanosignaling and restore nNOS-dependent NO production within these cells(23). While it remains to be determined whether nNOS activity is similarly regulated by dystrophin-dependent mechano-AMPK signaling in skeletal muscle, our work in cardiomyocytes prompted us to investigate whether the same pharmacologic approach could be used to augment NO production in dystrophin-deficient skeletal muscle fibers, and whether a strategy of acute AMPK activation could improve exercise tolerance at the integrated, *in vivo* level in a mouse model of DMD.

Here, we used fluorescence imaging of nitric oxide production and treadmill running assays in order to test the hypothesis that acute pharmacologic activation of AMPK restores skeletal muscle NO production and improves exercise tolerance in dystrophin-deficient animals. We report that acute pharmacologic stimulation of AMPK increases NO production in dystrophin-deficient skeletal muscle fibers *in vitro*, and increases exercise capacity *in vivo* in dystrophin-deficient mice. Together with our previous work, these findings indicate that acute pharmacologic activation of AMPK *in vivo* is sufficient to modulate exercise performance in dystrophin-deficient subjects, and may do so via NO-dependent effects at the heart and/or skeletal muscle. We therefore propose acute pharmacologic targeting of AMPK-NO signaling as a novel approach to improve exercise tolerance in DMD.

MATERIALS AND METHODS

Mice: Male control wild-type (WT) C57BL/10SnJ mice (The Jackson Laboratory, Bar Harbor, ME, USA, stock number: 000666) and dystrophin-deficient *mdx* mice (The Jackson Laboratory, Bar

Harbor, ME, USA, stock number: 001801) were used at 8-12 weeks of age. Mice were housed at the University of Michigan's Unit for Laboratory Animal Medicine, and all procedures were approved by the University of Michigan's Institutional Animal Care and Use Committee.

Skeletal muscle fiber isolation and general plating conditions: Fibers were isolated from the mouse flexor digitorum brevis (FDB) muscle as described previously(43, 44). FDB fibers were plated in MEM (Gibco, Grand Island, NY, USA, #1157-032) + 10% fetal bovine serum (Gibco, Grand Island, NY, USA, #16000-044) on 35-mm tissue culture dishes coated with 10% phenol red-free, growth factor-reduced matrigel (BD Biosciences, Franklin Lakes, NJ, USA, #356231). Plated fibers were allowed to adhere to tissue culture dishes for 1 hour before use in imaging experiments.

***In vitro* FDB fiber nitric oxide imaging:** Nitric oxide production was assessed in isolated adult mouse FDB fibers following a protocol modified from that which we have used to measure cardiomyocyte NO production(23). Briefly, fibers were incubated in imaging buffer (Chapter 2, Table 2.2) containing 10 μM of the nitric oxide-sensitive dye, 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM-DA) (Cayman Chemical, Ann Arbor, MI, USA, #18767), for 40 minutes in the dark at 37^{o}C. Fibers were then washed in fresh imaging buffer without DAF-FM-DA for 20 minutes in the dark at 37^{o}C to allow complete de-esterification of the dye. Next, fibers were acclimated to room temperature for 5 minutes before being covered in fresh room temperature imaging buffer containing AMPK-activating drugs or appropriate vehicle controls. Tissue culture plates were then immediately placed on the stage of an Olympus BX51 upright epifluorescence microscope and FDB fibers were imaged under the FITC channel at 4x magnification using a 25% neutral density filter. Images of the same field of view were recorded at 0, 10, 30, and 60 minutes of drug or vehicle treatment. Following imaging, fluorescence of all FDB fibers that retained their elongated, rod-shaped morphology throughout the imaging period was quantified using ImageJ as described previously(23). Drugs and other reagents used in these studies were as follows: 5-aminoimidazole-4-carboxamide riboside (AICAR) (2mM; Cayman Chemical, Ann Arbor, MI, USA, #10010241), and sodium salicylate (30mM; Sigma-Aldrich, St. Louis, MO, USA, #S2679).

Treadmill exercise: Assessment of exercise-induced fatigue and exercise capacity was performed in the University of Michigan Physiology Phenotyping Core. Mice were subjected to running exercise on variable speed belt treadmills (Columbus Instruments, Columbus, OH, USA, #0257-901M) with the shock grid at the back of the treadmill lanes set to deliver shocks at an intensity setting of 0.50, about 0.4 milliamps, in 200ms pulses at maximum rate of 11 shocks per 10 seconds. In preliminary experiments, naïve *mdx* mice that were run on treadmills for the first time had difficulty avoiding the shock grid even at moderate running speeds. Therefore, in order to minimize behavioral effects due to the stress of being shocked on our functional analyses, mice were first acclimated to the treadmills by exercising them on a low intensity running paradigm on each of the two days immediately preceding the days of functional assessment. These training sessions consisted of exercise for 2 minutes at 4 m/min, followed by 5 minutes at 8 m/min, and then 5 minutes at 12 m/min, with the treadmill set at 0^{o} inclination. For all experiments, the treadmill operator was blinded to mouse genotype and treatment condition.

Fatigue assay: The pre-exercise voluntary ambulatory activity of mice was assessed by using an ANY-maze automated video tracking system (Stoelting, Wood Dale, IL, USA, version 5.11) to measure the total distance that each animal walked within individual 44.6cm x 44.6cm Plexiglass open field chambers during a 6-minute monitoring session. Mice were then exercised on treadmills set at 0^{o} inclination for a total of 15 minutes, starting at a speed of 8 m/min for the first 5 minutes, followed by 10 minutes at a speed of 12 m/min. This intensity of exercise was moderate enough that *mdx* mice, as well as wild-type mice, were generally able to complete the running protocol without being shocked. Immediately following the completion of treadmill running, mice were returned to their respective open field chambers for measurement of their post-exercise voluntary ambulatory activity. For mock exercise groups, mice were placed in individual empty cages for 15 minutes instead of being run on the treadmills in between the first and second open field monitoring sessions. To compare changes in the voluntary ambulatory activity between mice of different genotypes or treatment groups, the raw open field activity for each animal was normalized by expressing the distance that it

walked during each open field session as a percentage of the distance that it walked during the first open field session.

Exhaustion assay: The day following assessment of fatigue, mice were run on treadmills at a 0^{o} inclination starting at a speed of 3 m/min, and the treadmill belt speed was increased by 3 m/min every 2 minutes up to a maximum speed of 21 m/min. Mice were exercised in this manner until reaching exhaustion, defined as the point at which they could no longer avoid the shock grid at the back of the treadmill lanes despite 3 successive manual boosts from the treadmill operator.

Power analyses: The statistical power of the exercise assays described above to detect changes in the exercise phenotype of dystrophin-deficient mice was assessed using DSS Research Statistical Power Calculator software (Decision Support Systems, Fort Worth, TX, USA, https://www.dssresearch.com/knowledgecenter/toolkitcalculators/statisticalpowercalculators.aspx). Two-sample power calculations were performed assuming a two-tailed test with the significance level set to $\alpha = 0.05$.

***In vivo* drug treatments:** Pharmaceutical grade sildenafil citrate (Greenstone, Peapack, NJ, USA, #F000154334) was administered to mice in the drinking water at a concentration of 400mg/L(35, 38) starting 2 days prior to exercise testing and continuing through completion of the exhaustion assay. In acute assays, mice were given intraperitoneal (IP) injections of 0-500mg/kg AICAR (Toronto Research Chemicals, Toronto, Ontario, ON, Canada, #A611700) or 0-50mg/kg veterinary-grade sodium salicylate (HorsePreRace.com, Tyler, TX, USA) prepared in sterile pharmaceutical grade saline. AICAR or saline as a vehicle control was administered 8 minutes prior to the start of treadmill running (i.e., 2 minutes before the start of the first open field session in fatigue assays), and salicylate or control saline was administered 1 hour prior to the start of treadmill running (54 minutes before the start of the first open field session in fatigue assays).

Statistics: All data are presented as mean ± SE, and significance for all analyses was set at $P<0.05$. Direct comparisons between two groups were assessed by the Student *t* test. For exercise-induced fatigue assays, data were analyzed by two-way repeated measures ANOVA followed by Sidak's multiple comparisons tests.

RESULTS

Acute pharmacologic AMPK activation increases nitric oxide production in dystrophin-deficient skeletal muscle fibers *in vitro*.

In order to investigate the effects of acute AMPK activation on nitric oxide production in skeletal muscle, we modified the assay we previously developed for measuring NO production in isolated mouse cardiomyocytes(23) for use in isolated mouse flexor digitorum brevis fibers (Fig. 3.1A). In contrast to our previous findings in cardiomyocytes, treatment with the AMP analog, AICAR, at a concentration of up to 2mM did not significantly increase DAF-FM dye fluorescence compared to vehicle controls in either wild-type or dystrophin-deficient *mdx* FDB fibers (Fig. 3.1 B and C). These results are inconsistent with previous observations of AICAR-induced NO production in L6 myotubes(45), although it is possible that the lack of AICAR responsiveness in our study reflects developmental differences between myotubes and adult muscle cells. Additionally, we did observe a decrease in the baseline cellular fluorescence of both WT and *mdx* FDB fibers over the course of the 1 hour imaging period (Fig. 3.1 B and C), possibly due to photobleaching of fiber autofluorescence. This overall decrease in basal cell fluorescence may have obscured detection of any small effects of AICAR to stimulate NO production in adult fibers. Unlike AICAR, salicylate, a drug that activates AMPK via the β1 subunit(46), did yield a significant increase in DAF-FM fluorescence in both WT and *mdx* fibers (Fig. 3.1 D and E). This finding indicates that similar to cardiomyocytes, direct pharmacologic activation of AMPK by salicylate is sufficient to restore nitric oxide production in skeletal muscle fibers regardless of their dystrophin expression.

Exaggerated exercise-induced fatigue and diminished exercise capacity in *mdx* mice can be detected with treadmill running assays.

Our finding that acute pharmacologic AMPK activation can stimulate NO production in dystrophin-deficient striated muscle cells suggests that this treatment has the potential to increase blood flow to dystrophic muscle during exercise, and prompted us to investigate

whether this strategy could affect exercise tolerance in DMD. Various testing paradigms involving measurement of voluntary exercise on running wheels, exhaustive forced running on treadmills, or analysis of voluntary activity immediately following acute exercise have been put forward as strategies to evaluate integrated skeletal and cardiac muscle function in mouse models of muscular dystrophy(26, 47-51). However, the translatability of pre-clinical findings from these assays to human muscular dystrophy patients is debated, and even the power of analogous clinical assessments such as the six-minute walk test is limited by potential subjective influences on the part of both participants and investigators(52-56). Furthermore, few of the studies that rely on behavioral parameters such as the animals' voluntary activity in order to assess effects of dystrophy on exercise, and the ability of therapeutic interventions to modulate exercise phenotypes, have published appropriate control data to demonstrate that any observed effects are due to changes specifically in exercise performance or tolerance, rather than simple behavioral adaptations throughout the course of the experiments. Therefore, before testing effects of pharmacologic AMPK activation *in vivo*, we first set out to validate the use of two common treadmill running assays to measure exercise intolerance in dystrophin-deficient *mdx* mice.

To compare exercise-induced fatigue in healthy versus dystrophin-deficient mice, we measured the animals' voluntary open field ambulatory activity immediately before and again immediately following 15 minutes of moderate intensity treadmill running, similar to the approach used by Kobayashi et al.(26, 51). Both wild-type BL10 and *mdx* mice that were exercised on treadmills walked a shorter distance during the second open field monitoring session than they did during the first open field session (Fig. 3.2A). However, mice of both genotypes that were mock exercised did not exhibit a significant decrease in voluntary ambulatory activity between the first and second open field monitoring sessions (Fig 3.2A). These data indicate that the decrease in activity of exercised mice was indeed an effect of treadmill running, rather than an effect of behavioral acclimation to a second exposure to the open field chambers. As WT mice had approximately twice the basal level of open field activity than *mdx* mice, we normalized each animal's post-exercise voluntary activity to its pre-exercise voluntary activity in order to more directly compare the effects of exercise between these two

strains of mice. This analysis revealed that whereas the open field activity of WT mice drops about 35% following exercise, the open field activity of *mdx* mice decreases by up to 80% following exercise (Fig. 3.2B). The slight decrease in activity of mock exercised mice in the second versus first open field session did not differ between genotypes (Fig. 3.2B). These findings substantiate previous reports that dystrophin-deficient animals exhibit excessive post-exercise fatigue(26, 51, 57), and demonstrate that the minimal behavioral effects of acclimation due to repeat exposure to the open field chambers are not altered by the dystrophic phenotype of *mdx* mice.

In the second assay we performed, mice were exercised on treadmills at progressively increasing speeds until they reached exhaustion and could no longer remain on the treadmill belt. As expected, *mdx* mice were able to run on treadmills for a shorter time and therefore distance before reaching exhaustion compared to WT mice (Fig. 3.2 C and D). Moreover, unlike WT, most *mdx* mice failed to achieve the maximum running velocity in this assay (21m/min starting at minute 12 of the protocol), suggesting that the maximal exercise capacity or work rate is impaired in dystrophin-deficient animals.

To evaluate the relative sensitivity and potential statistical power of each of these exercise assays to detect changes in the exercise tolerance of *mdx* mice, post hoc power analyses were conducted using the observed mean and standard deviation for *mdx* normalized post-voluntary activity (Fig. 3.2B, mean ± S.D. = 17.85 ± 26.36 % of pre-exercise) and *mdx* time to exhaustion (Fig. 3.2C, mean ± S.D. = 11.51 ± 1.899 minutes). These calculations suggest that the fatigue assay has sufficient 60-80% power to detect only relatively large changes in post-exercise fatigue (approximately 85-100% change compared to control group) at a sample size of 20-30 animals per group (Fig. 3.2E). In contrast, the power analysis indicates that exhaustion assay is more sensitive to changes in exercise performance, as with the same sample size of 20-30 animals per group it can detect as small as a 10-15% change in the time to exhaustion at a power of 60-80% (Fig. 3.2F).

Phosphodiesterase 5 inhibition improves exercise-induced fatigue, but not exercise capacity, in dystrophin-deficient mice.

We next set out to determine whether these two exercise assays were sensitive enough to detect an improvement in *mdx* exercise tolerance upon pharmacologic intervention by using them to assess the exercise phenotype of *mdx* mice treated with the phosphodiesterase 5 inhibitor, sildenafil citrate. This drug has been reported to alleviate exercise-induced fatigue in dystrophin-deficient mice and is presumed to do so by inhibiting the degradation of cGMP that is produced by vascular smooth muscle cells in response to a local increase in NO levels, thus improving vasodilatory function and helping to improve sympatholysis in dystrophic skeletal muscle(26). Chronic sildenafil treatment has also been shown to improve the function of dystrophin-deficient hearts(36, 38), suggesting that some of its effects on exercise tolerance may be related to changes in cardiac performance. In our hands, administration of sildenafil for two days was sufficient to increase both the pre- and post-exercise voluntary ambulatory open field activity of *mdx* mice (Fig 3.3A). Sildenafil also attenuated the animals' decrease in ambulatory activity following exercise (Fig. 3.3B), demonstrating that it was effective at alleviating exercise-induced fatigue. These results also confirm that this exercise-induced fatigue assay is indeed sensitive enough to detect effects of pharmacologic intervention on exercise tolerance of *mdx* mice, despite having less statistical power than the exhaustion assay. We did not observe any significant effect of sildenafil treatment on the time or distance that *mdx* mice were able to run prior to reaching exhaustion (Fig. 3.3 C and D).

Acute pharmacologic AMPK activation improves exercise capacity in dystrophin-deficient mice.

Having validated our exercise assays, we tested whether acute pharmacologic AMPK activation could improve the exercise phenotype of dystrophin-deficient *mdx* mice. Earlier studies reported that chronic administration of AICAR at 500mg/kg/day improved skeletal muscle function and pathology in *mdx* animals(58, 59). Therefore, in pilot experiments we used a dose of 500mg/kg for acute AICAR treatment. Unexpectedly, several WT mice that were given this dose of AICAR experienced seizures soon after completing treadmill exercise, raising important concerns about the safety of high doses of this compound. The seizure activity we observed may have been due to post-exercise hypoglycemia, as AICAR, like muscle contraction

itself, is known to stimulate glucose uptake(60, 61), and hypoglycemia has been reported as a consequence of overdose with AICAR or metformin, another AMPK-activating drug(62, 63). To avoid such deleterious effects of AICAR, we reduced the dose of AICAR given to animals in all subsequent experiments.

A single, acute IP injection of a low, 50mg/kg dose of AICAR did not significantly attenuate the exercise-induced drop in voluntary ambulatory activity in *mdx* mice (Fig. 3.4 A and B). However, acute AICAR administration did increase the time and distance *mdx* mice were able to run before reaching exhaustion (Fig. 3.4 C and D). Acute injection with salicylate revealed that this drug tended to attenuate the exercise-induced drop in voluntary ambulatory activity in *mdx* mice, though this effect fell short of statistical significance (Fig. 3.5 A and B). Similar to AICAR, acute salicylate administration increased the time and distance that *mdx* mice ran prior to exhaustion (Fig 3.5 C and D). Together, these results suggest that acute pharmacologic AMPK activation is sufficient to improve exercise capacity in dystrophin-deficient animals. The rapidity of the effect of the AMPK activators on treadmill running capacity, which occurred within the first half hour following AICAR administration or the first hour and 15 minutes following salicylate administration, suggests that it is mediated by acute changes in AMPK-dependent signaling pathways rather than by changes in the expression of metabolic genes, upregulation of utrophin, or increased mitochondrial biogenesis within the dystrophic striated muscle, as has been observed in studies of chronic pharmacologic AMPK activation in *mdx* mice(58, 59, 64, 65).

DISCUSSION

Research from multiple groups supports the idea that restoration of nNOS signaling is beneficial to the function and pathology of dystrophin-deficient striated muscle and can ameliorate exercise deficits in dystrophin-deficient mice. However, translation of these findings from animal models to human Duchenne muscular dystrophy patients has proved challenging. Phosphodiesterase 5 inhibition, the only pharmacologic approach currently available for modulating downstream NO signaling, has rapidly advanced to clinical trials, but has had only

limited success in improving muscle blood flow, striated muscle function, and overall exercise capacity in DMD and Becker muscular dystrophy patients(39-42). These shortcomings may reflect the inability of PDE5 inhibition to restore essential, direct effects of nitric oxide on striated muscle physiology that are independent of changes in cGMP. One recent clinical study demonstrated a down regulation of PDE5A in dystrophic muscle(41), suggesting that the very enzyme that the PDE5 inhibitor approach aims to target may also be disrupted in dystrophin-deficient subjects.

A growing understanding of the physiological mechanisms that regulate nNOS activity in striated muscle opens up the possibility of targeting the endogenous pathways that control nNOS function as an alternative approach to increase muscle NO production in dystrophinopathies. Here, we used a combination of *in vitro* nitric oxide imaging and *in vivo* exercise assays to address the hypothesis that acute pharmacologic stimulation of AMPK, an endogenous upstream kinase for nNOS, rescues skeletal muscle NO production and improves exercise tolerance in dystrophin-deficient mice. Our observation that acute treatment with salicylate increases NO production in in dystrophin-deficient skeletal muscle fibers, as it does in dystrophin-deficient cardiomyocytes(23), provides direct evidence that nitric oxide synthase activity is still targetable in these cells, despite the loss of physical binding between nNOS and the dystrophin-glycoprotein complex. This argues against the current model for nNOS regulation in skeletal muscle, which proposes that direct scaffolding interactions between nNOS and dystrophin or the DGC are required for stimulation of nNOS activity. Indeed, work demonstrating that increased muscle NO content and improvement of muscle pathology by expression of an nNOS transgene in dystrophin/utrophin double knockout mice do not require the localization of nNOS to the sarcolemma(66) provides further support for the idea that direct NOS-DGC interactions are not absolutely critical for nNOS activity within skeletal muscle.

Thus, rather than directly modulating the enzymatic activity of nNOS, the scaffolding interaction between nNOS and the DGC in healthy skeletal muscle may be important primarily for the appropriate targeting of a portion of the skeletal muscle's NO production to the periphery of the muscle cell. Such targeting of NO production to the sarcolemma would minimize the diffusion distance between skeletal muscle nNOS and the smooth muscle cells of

local resistance arterioles, the main target of regulation for skeletal muscle-derived NO in mediating sympatholysis(10, 25). Minimizing this diffusion distance is especially important for nitric oxide-mediated signaling, as NO is highly reactive(67) and has a half-life of only a few microseconds in muscle tissue due to rapid reaction with myoglobin(68-70). Mathematical modeling predicts that in muscle tissue, NO concentration declines sharply with increasing distance from its site of production, and that nanomolar concentrations sufficient to activate smooth muscle guanylyl cyclase may be reached only within a few micrometers of the source of NO production(71, 72). The limitation to the diffusion of NO may have minimal consequences for vasoregulation by cytosolic nNOS within cardiomyocytes, which have a diameter of about 20μm(73), because even nNOS located in the middle of a cardiomyocyte would be only about 10μm from the nearest neighboring cell. However, in larger skeletal muscle fibers that have a diameter of 40-60 μm(74, 75), scaffolding of nNOS at the DGC near the edge of the cell would be critical for allowing nNOS-derived NO to reach nearby arterioles in order to promote vasodilation. The idea that the specific targeting of nNOS near the skeletal muscle sarcolemma is critical for local vasoregulation by the skeletal muscle fibers is supported by experiments showing that only those transgenic dystrophin constructs that restore nNOS sarcolemmal localization are sufficient to restore functional sympatholysis in *mdx* mice(76), and that sympatholysis is disrupted in syntrophin-deficient mice that exhibit reduced sarcolemmal nNOS localization(77). The data in the present study showing that pharmacologic activation of AMPK was able to restore skeletal muscle NO production suggests that this may be a viable approach to increase skeletal muscle NO signaling in DMD patients. However, because acute AMPK activation presumably did not restore nNOS sarcolemmal localization, any beneficial effects of increased skeletal muscle NO production on exercise performance with this treatment may reflect targets of NO within the skeletal muscle fibers rather than a dramatic improvement in muscle blood flow. This notion also raises the possibility that a combined therapeutic approach restoring both sarcolemmal nNOS localization and acute pharmacologic nNOS activation through AMPK in DMD may be more effective at improving sympatholysis and improving exercise tolerance than either approach alone.

In contrast to the effects of salicylate and unlike our previous findings in cardiomyocytes(23), we did not observe a significant effect of AICAR to activate NO production in dystrophin-deficient skeletal muscle fibers. We also failed to observe any effect of AICAR on NO production in wild-type fibers, indicating that the lack of AICAR-induced NO production was not merely a consequence of dystrophin deficiency. The discrepancy between the effects of AICAR on NO production in cardiac versus skeletal muscle may reflect differences in the nucleotide-sensing, γ-subunit isoform composition of AMPK heterotrimers between these two tissues. Whereas cardiac muscle expresses γ2 AMPK subunits, skeletal muscle exhibits tissue-specific expression of γ3 AMPK(63). Fitting with this idea of tissue-specific differences in AICAR sensitivity, AMPK trimeric complexes containing γ2 subunits have been shown to be the most sensitive to activation by AMP, while complexes containing γ3 subunits are the least sensitive to activation by AMP(78). As there is correlative evidence for the activating phosphorylation of nNOS by AMPK in skeletal muscle(79), it is likely that in our experiments any subset of skeletal muscle AMPK heterotrimers that may have been effectively activated by AICAR, or that may have already been activated by any metabolic stress imposed by the fiber isolation, is simply distinct from the subset of AMPK heterotrimers that normally regulate nNOS. Our observation that salicylate, which specifically activates β1-subunit-containing AMPK heterotrimers(46), does activate NO production in FDB fibers provides further support for the notion that a specific fraction of the total population of AMPK trimeric complexes within skeletal muscle functionally targets nitric oxide synthases.

The effect of chronic pharmacologic activation of AMPK with AICAR on the pathology of dystrophin-deficient muscle has been the subject of several recent investigations(49, 58, 59, 64, 65). Although such studies have demonstrated a shift toward a more oxidative muscle phenotype and improved contractile function of the skeletal muscle after weeks of AICAR treatment, the ability for AMPK activation to affect integrated exercise phenotypes in animal models of DMD has so far received little attention. Our finding that acute treatment with the AMPK activators AICAR or salicylate increases the time and distance that dystrophin-deficient *mdx* mice are able to run on treadmills is the first demonstration that pharmacologic AMPK activation can indeed improve exercise performance in dystrophin-deficient subjects.

Moreover, that the improvement in exercise occurred on the timescale of minutes to hours following administration of these drugs suggests that this beneficial effect is mediated by rapidly-acting mechanisms. The one group that measured endurance running in *mdx* mice following 30 days of AICAR treatment did not observe any effect of the treatment on total running capacity despite upregulation of the dystrophin homolog, utrophin, and improvements in skeletal muscle integrity with this treatment paradigm(49, 58). This result provides further evidence that the exercise capacity of *mdx* mice is specifically affected by the immediate rather than long-term effects of increased AMPK activity. Both AICAR and salicylate can acutely regulate other proteins besides AMPK, such as the ryanodine receptor(80), cyclooxygenase(81), and IKKβ(82), and therefore may alter AMPK-independent signaling pathways that could have affected performance on the exhaustion assay we used in this investigation. However, the fact that we observed improved exercise capacity in *mdx* mice treated with either drug supports the idea that this improvement was due to AMPK itself, rather than some other unintended target of the drugs.

The disparate effects of AMPK activators compared to a PDE5 inhibitor that we observed in our two different exercise assays raises the intriguing possibility that these different classes of drugs affect distinct components of the exercise intolerance phenotype of dystrophin-deficient mice. Sildenafil treatment increases post-exercise skeletal muscle blood flow in *mdx* mice and attenuates the decrease in their voluntary activity following exercise(26), a phenomenon that we were able to reproduce. We found a similar trend toward attenuated post-exercise fatigue in *mdx* mice treated with salicylate, but not in animals treated with AICAR. In light of our *in vitro* data demonstrating that salicylate, but not AICAR, stimulates nitric oxide production in dystrophin-deficient skeletal muscle, it is likely that amelioration of post-exercise fatigue in *mdx* mice specifically depends on increasing NO/cGMP-dependent signaling, and possibly local blood flow, within the skeletal muscle tissue. On the other hand, the increase in total treadmill running capacity in *mdx* mice treated with AICAR or salicylate was not recapitulated in mice treated with sildenafil, suggesting that increased cGMP levels and any associated changes in sympatholysis are not sufficient to elicit this effect. Rather, the improvement in exercise capacity may reflect direct, cGMP-independent effects of NO to alter

calcium handling and increase the contractile function of the heart and, in salicylate-treated animals, the skeletal muscle(83, 84). Any such effects to improve cardiac function or increase muscle strength could increase the animals' maximum work output, enabling them to run at a greater maximal speed and therefore remain on the treadmills for a longer time over the course of our exhaustion assay. Because AICAR was able to improve the exercise capacity of *mdx* mice even without stimulating FDB fiber NO production, it is also possible that the benefits of AMPK activation on exercise capacity are attributable to increased NO signaling specifically within the cardiac rather than skeletal muscle, or else are due to NO-independent effects of AMPK, such as on metabolism, within either type of muscle or in even non-muscle tissue. It will be interesting for future studies to clarify the relative cardiac and skeletal muscle contributions to the improved exercise capacity in *mdx* mice treated with AMPK activators, and to determine the necessity of heart or skeletal muscle nNOS for this effect.

Together with our work in cardiomyocytes discussed in Chapter 2 of this thesis, our data support a model in which acute pharmacologic stimulation of AMPK is sufficient to enhance nitric oxide production in dystrophin-deficient striated muscle, regardless of any scaffolding interactions between nNOS and the dystrophin-glycoprotein complex. Further study will be required to identify the precise subunit composition of the AMPK heterotrimers that are targeted by this approach, as well as the specific nNOS splice variants that are regulated by AMPK. Our *in vivo* findings demonstrate the physiological relevance of modulating AMPK signaling in dystrophin-deficient animals and provide proof-of-concept evidence for acute pharmacologic AMPK activation as a novel approach to improve exercise tolerance in Duchenne muscular dystrophy.

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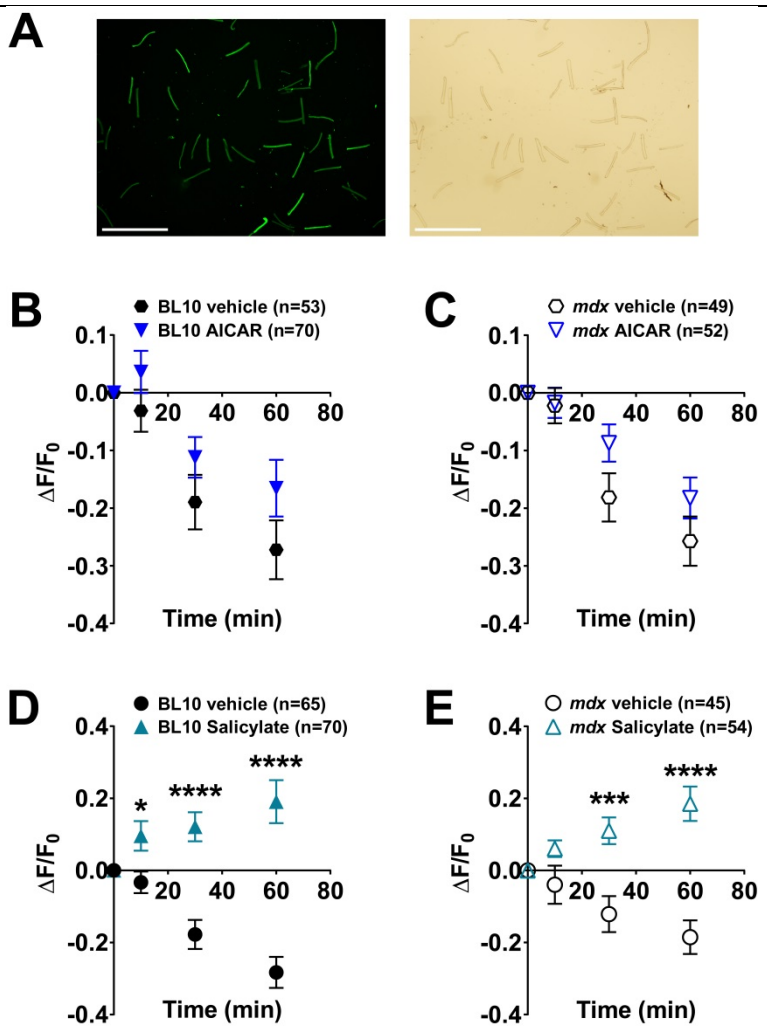

Figure 3.1. Acute pharmacologic AMPK activation with salicylate increases nitric oxide production in dystrophin-deficient skeletal muscle fibers *in vitro*. (**A**) Epifluorescence imaging of isolated *mdx* flexor digitorum brevis(FDB) fibers loaded with the nitric oxide sensitive dye, DAF-FM-DA, at 4x magnification under a FITC filter (left panel) or brightfield (right panel). (Scale bars = 1mm.) (**B**) Treatment of wild-type C57BL10 (BL10) FDB fibers with 2mM AICAR does not significantly increase cellular DAF-FM fluorescence compared to vehicle-treated cells. (**C**) Treatment of dystrophin-deficient *mdx* FDB fibers does not significantly increase cellular DAF-FM fluorescence compared to vehicle-treated cells. Cellular DAF-FM fluorescence is increased in BL10 (**D**) and *mdx* (**E**) FDB fibers treated with 30mM salicylate compared to vehicle control (*P<0.05,***P<0.001, ****P<0.0001 salicylate vs. vehicle).

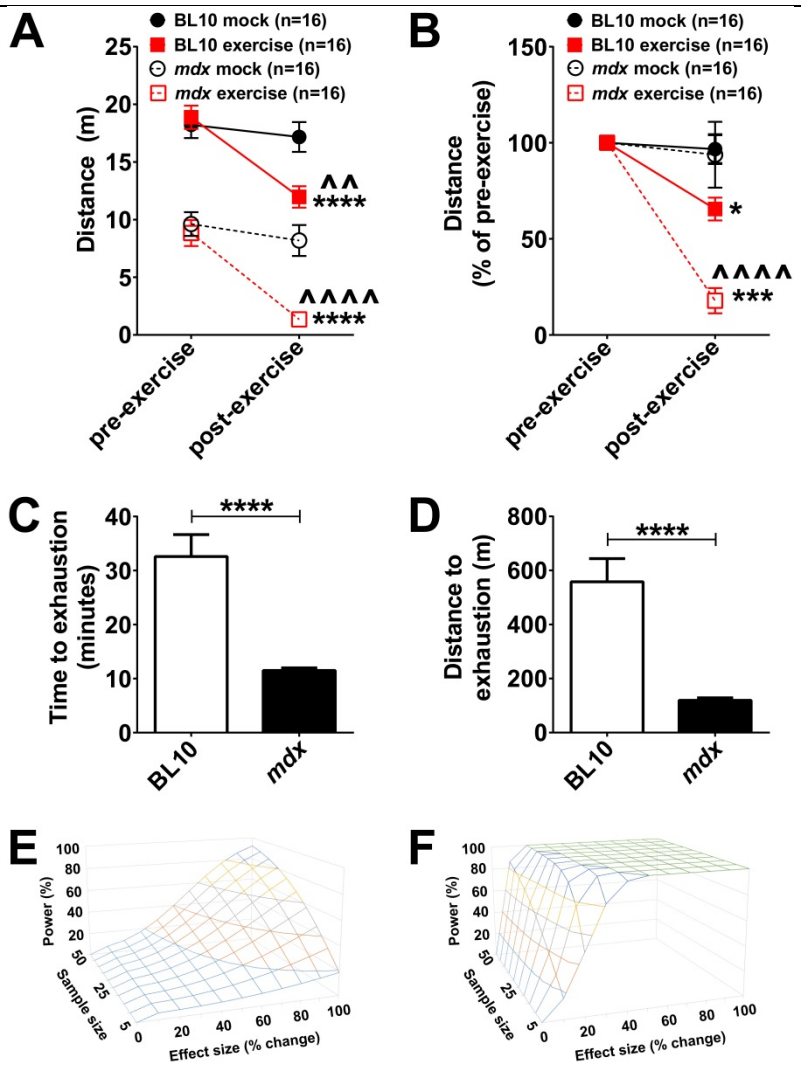

Figure 3.2. Exercise-induced fatigue is exaggerated and exercise capacity is diminished in dystrophin-deficient mice. (**A**) Wild-type BL10 and dystrophin-deficient *mdx* mice exhibit a decrease in voluntary ambulatory open field activity following a single bout of treadmill exercise, while voluntary open field activity is not affected in mice that are mock exercised (****P<0.0001 vs. same genotype, pre-exercise; ^^P<0.01, ^^^^P<0.0001 vs. same genotype, mock post-exercise). (**B**) The relative decrease in voluntary ambulatory open field activity following exercise is greater in *mdx* mice than in WT mice (*P<0.01, ***P<0.001 vs. mock post-exercise for same genotype; ^^^^P<0.0001 vs. BL10 post-exercise). *mdx* mice subjected to treadmill exercise run a shorter time (**C**) and distance (**D**) before reaching exhaustion than WT mice (****P<0.0001) (n=16). Surface plots representing the results of statistical power analyses for the measurement of the exercise phenotype of *mdx* mice in the fatigue assay (**E**) and exhaustion assay (**F**) reveal a greater sensitivity for the exhaustion assay in detecting small effect sizes (% change compared to control group).

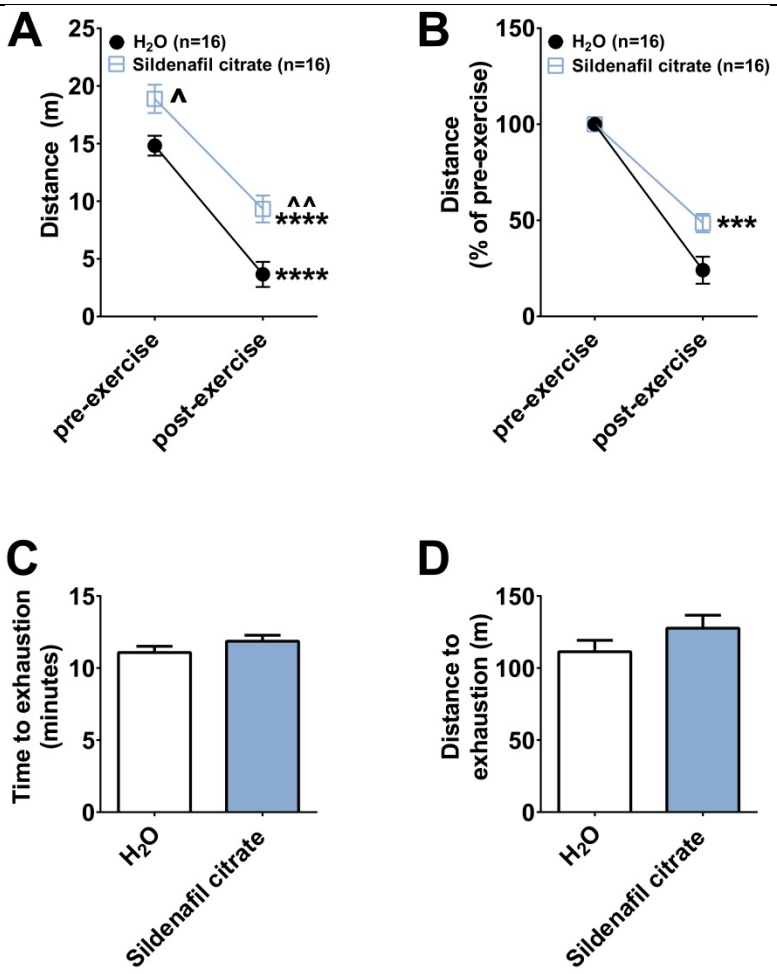

Figure 3.3. Phosphodiesterase 5 inhibition improves exercise-induced fatigue but not exercise capacity in dystrophin-deficient *mdx* mice. (**A**) Treatment with sildenafil increases the pre-and post-exercise voluntary ambulatory activity of dystrophin-deficient *mdx* mice (****P<0.0001 vs. pre-exercise for same treatment condition; ^P<0.05, ^^P<0.01 vs. H_2O within same open field session). (**B**) The drop in voluntary open field activity following exercise is attenuated in *mdx* mice treated with sildenafil (***P<0.001 vs. H_2O post-exercise). Sildenafil does not affect the time (**C**) or distance (**D**) that *mdx* mice are able to run before reaching exhaustion (n=16).

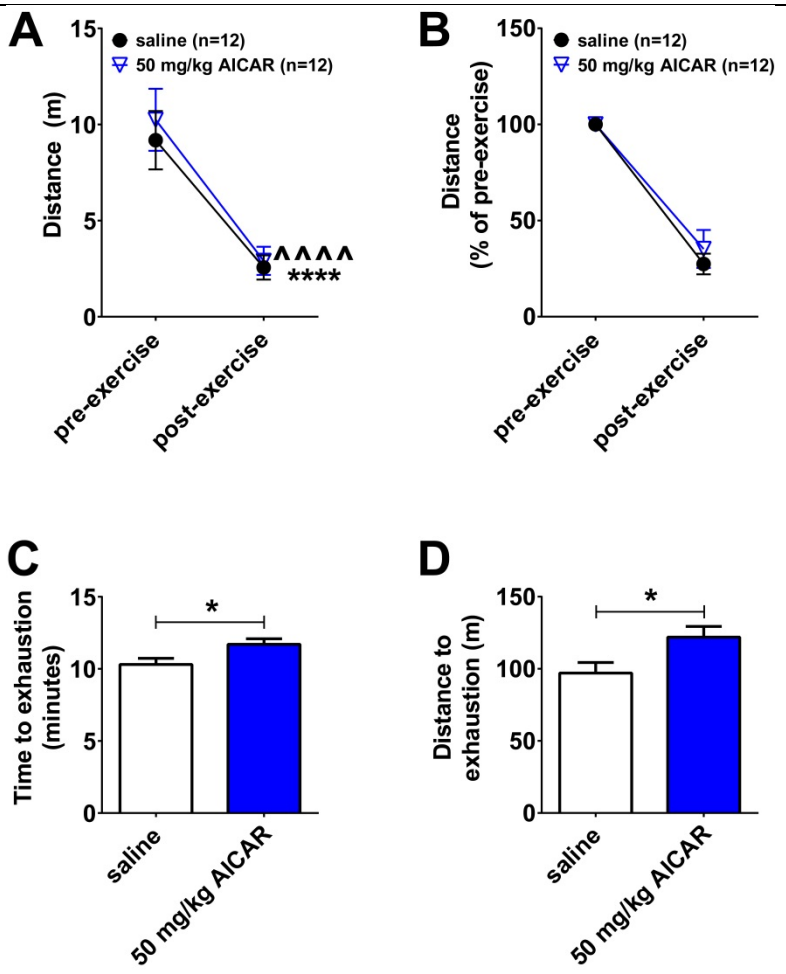

Figure 3.4. Acute treatment with AICAR improves exercise capacity in dystrophin-deficient mice. (**A**) Acute treatment with AICAR does not significantly alter pre- or post-exercise voluntary ambulatory activity in *mdx* mice (****P<0.0001, saline post- vs. pre-exercise; ^^^^P<0.0001, AICAR post- vs. pre-exercise). (**B**) The decrease in voluntary ambulatory activity following treadmill running is not significantly attenuated in *mdx* mice acutely treated with AICAR compared to saline control. Acute AICAR treatment increases the time (**C**) and distance (**D**) that *mdx* mice are able to run before reaching exhaustion (*P<0.05) (n=12).

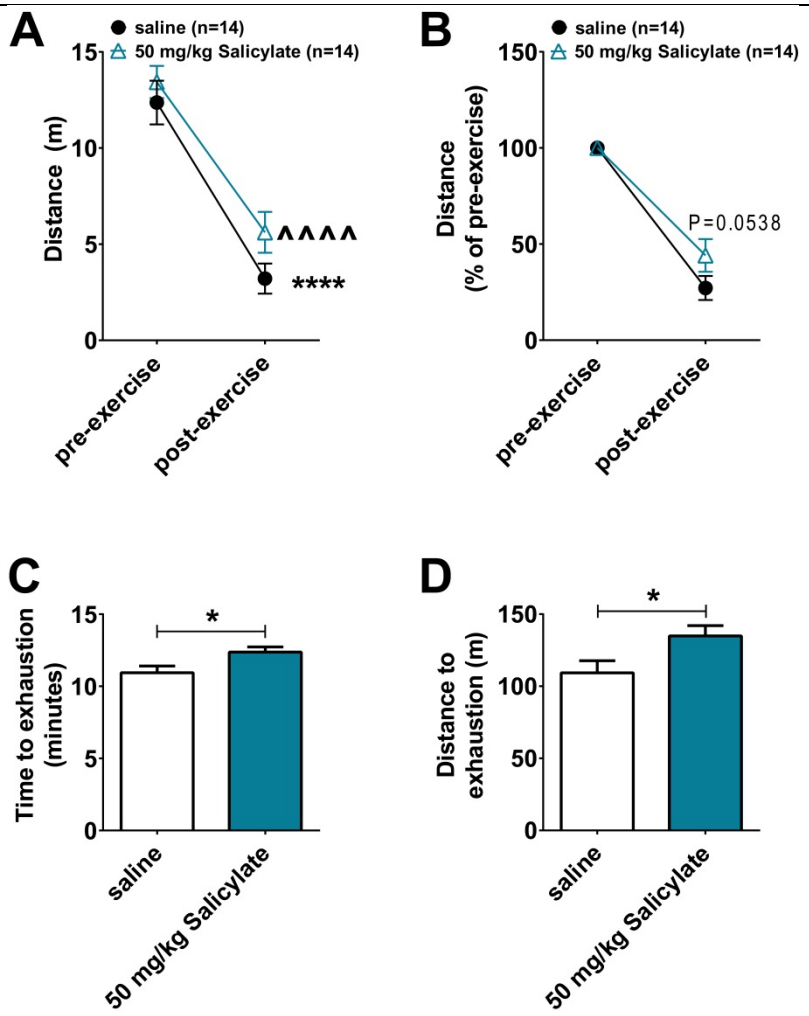

Figure 3.5. Acute treatment with salicylate improves exercise capacity in dystrophin-deficient mice. (**A**) Acute treatment with salicylate tends to increase the post-exercise voluntary ambulatory activity of *mdx* mice (****P<0.0001, saline post- vs. pre-exercise; ^^^^P<0.0001, salicylate post- vs. pre-exercise). (**B**) The decrease in voluntary ambulatory activity following treadmill running tends to be attenuated in *mdx* mice acutely treated with salicylate compared to saline control. Acute salicylate treatment increases the time (**C**) and distance (**D**) that *mdx* mice are able to run before reaching exhaustion (*P<0.05) (n=14).

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CHAPTER 4

Transgenic expression of dimethylarginine dimethylaminohydrolase attenuates exercise-induced fatigue in Duchenne muscular dystrophy carrier mice.

ABSTRACT

Duchenne muscular dystrophy (DMD) is an X-linked disease caused by mutations in dystrophin and characterized by muscle degeneration, cardiomyopathy, and impaired muscle nitric oxide (NO) production that disrupts muscle blood flow regulation and leads to excessive post-exercise fatigue. We show here that circulating levels of endogenous methylated arginines that act as NO synthase inhibitors, such as asymmetric dimethylarginine (ADMA), are elevated both by acute necrotic muscle damage and in chronically-necrotic dystrophin-deficient mice. Therefore, we hypothesized that excessive circulating ADMA impairs muscle NO production and thus negatively impacts exercise tolerance in DMD. We used transgenic expression of dimethylarginine dimethylaminohydrolase 1 (DDAH), the enzyme that degrades ADMA, to investigate the contribution of ADMA to exaggerated exercise-induced fatigue in DMD. Although infusion of exogenous ADMA was sufficient to impair exercise performance in healthy wild-type mice, transgenic DDAH expression aimed at reducing methylated arginine levels was not sufficient to affect exercise-induced fatigue in dystrophin-deficient male *mdx* mice. Surprisingly, DDAH transgene expression did attenuate exercise-induced fatigue in dystrophin-heterozygous female *mdx* carrier mice. This improvement in exercise tolerance was associated with reduced heart weight, improved cardiac contractile function, and improved chronotropic responsiveness to beta-adrenergic stimulation in DDAH-transgenic female *mdx* carriers. We conclude that DDAH transgene expression improves exercise tolerance in dystrophin-

heterozygous females, possibly by limiting pathological cardiac remodeling and preserving heart performance. These findings emphasize the importance of methylated arginines to cardiac function in dystrophinopathies and reveal a new therapeutic target to mitigate the risk of cardiomyopathy in DMD carriers.

INTRODUCTION

Mutations in the genes encoding the proteins of the sarcolemmal dystrophin-glycoprotein complex (DGC) lead to muscle wasting diseases known as muscular dystrophies. One of the most common forms, Duchenne muscular dystrophy (DMD), is caused by mutations in the X-linked DMD gene that encodes dystrophin(1, 2). This protein normally links the muscle cell's cytoskeleton and plasma membrane via its interactions with subsarcolemmal γ-actin and the transmembrane protein β-dystroglycan(3-5). Loss of dystrophin destabilizes the sarcolemma and contributes to excessive sarcolemmal permeability that can lead to calcium overload and death of the muscle cell, particularly during the mechanical stresses that occur with eccentric contractions or increased workloads(6-9). Evidence of striated muscle cell injury can be seen by leak of intracellular proteins such as creatine kinase and lactate dehydrogenase into the blood(10), and by an increased prevalence of regenerating skeletal muscle fibers and replacement of damaged cardiomyocytes by fibrosis. Over time, accumulated damage to the muscle cells results in debilitating muscle weakness and, in older DMD patients, compromised cardiac function and cardiomyopathy(11, 12). Cardiomyocyte damage likely also contributes to the elevated risk for cardiomyopathy observed in female carriers of DMD mutations, who display mosaic dystrophin expression in the heart due to random X-inactivation(13-15).

In addition to structural defects, dystrophin-deficient striated muscle cells exhibit impaired nitric oxide (NO) signaling(16-19). Production of NO by neuronal nitric oxide synthase (nNOS) in contracting skeletal muscle is critical for sympatholysis, the process by which muscle contraction locally attenuates sympathetic vasoconstriction and helps to maintain blood flow to exercising skeletal muscles(20). In the absence of dystrophin, the disruption of muscle NO production and resulting misregulation of muscle blood flow(21-24) is thought to contribute to

exaggerated exercise-induced fatigue observed in dystrophin-deficient mice and patients(25, 26). Given the importance of nitric oxide for regulating coronary blood flow(27) and the intrinsic contractile properties of the myocardium(28-33), it is also probable that disrupted cardiac muscle NO production contributes to cardiac dysfunction and, consequently, poor exercise tolerance in DMD.

Several mechanisms have been proposed to explain how the loss of dystrophin negatively affects striated muscle nitric oxide signaling. Various groups have suggested that a direct physical interaction between nNOS and the DGC or dystrophin itself is required for appropriate activation of nNOS(16, 25, 34), or alternatively that dystrophin is required for the normal regulation of biochemical signaling cascades that control the phosphorylation state and enzymatic activity of nNOS(19). However, understanding of the endogenous regulation of muscle NO production, and the mechanisms by which the loss of dystrophin impairs this phenomenon, remains incomplete. Reports from as early as the 1970s suggest that elevated concentrations of asymmetric dimethylarginine (ADMA) are present in the urine of DMD patients(35, 36), perhaps reflecting changes in circulating levels of ADMA. ADMA is generated via methylation of arginine residues within intact proteins, and following release during proteolysis can act as an endogenous competitive inhibitor of the nitric oxide synthases(37, 38). Therefore, we reasoned that excessive ADMA released from damaged, degenerating muscle cells may impair nNOS function and thus contribute to impaired muscle blood flow regulation and increased susceptibility to exercise-induced fatigue in dystrophin-deficient subjects.

We used transgenic expression of dimethylarginine dimethylaminohydrolase 1 (DDAH), an enzyme that degrades ADMA, to test the hypothesis that excessive circulating ADMA contributes to exaggerated exercise-induced fatigue in the *mdx* mouse model of Duchenne muscular dystrophy. Here, we report that administration of exogenous ADMA negatively affects exercise performance in otherwise healthy mice, and that transgenic DDAH expression improves resistance to exercise-induced fatigue in female dystrophin-heterozygous *mdx* carrier mice, but not in male dystrophin-deficient *mdx* mice. We further demonstrate that DDAH transgene expression improves the cardiac performance of female *mdx* carrier mice, suggesting that improved fatigue resistance in these animals is related to altered heart function. These

surprising results reveal a new role for methylated arginine metabolism in the regulation of cardiac function in DMD carriers and point to the manipulation of methylated arginine metabolism as new therapeutic strategy to target the development of cardiomyopathy in this population.

MATERIALS AND METHODS

Mice: Control wild-type (WT) C57BL/10SnJ (strain #000666) and dystrophin-deficient *mdx* mice (C57BL/10ScSn-*DMD*^{mdx}/J, strain #001801) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Dystrophin-deficient female *mdx* mice were crossed to wild-type males expressing a transgene encoding human dimethylarginine dimethylaminohydrolase 1 (DDAH) under the control of a β-actin promoter(39) (The Jackson Laboratory, Bar Harbor, ME, USA, strain #005863, C57BL/6J-Tg(ACTB-DDAH1)1Jpck/J) to generate DDAH-transgenic (TG) and non-transgenic (ntg) dystrophin-deficient male *mdx* mice, and TG and ntg dystrophin-heterozygous female mice ("*mdx* carriers" or "*mdx*hets"). DDAH-genotype was confirmed by polymerase chain reaction as has been described elsewhere(39). Primer sequences for the DDAH transgene were 5'-AGCACCAGCTCTACGTG-3' and 3'-GCCCTTTGTTGGGGATATT-5'. Internal control primers were 5'-CAAATGTTGCTTGTCTGGTG-3' and 5'-GTCAGTCGAGTGCACAGTTT-3'. For initial assessments of plasma methylated arginine concentrations, cardiotoxin injury experiments, and ADMA infusion experiments, WT and *mdx* mice were used at 3-4 months of age. All subsequent experiments involving DDAH-transgenic mice were performed in mice 11-12 months of age. All animals were housed in the University of Michigan's Unit for Laboratory Animal Medicine, and all procedures were approved by the University of Michigan's Institutional Animal Care and Use Committee.

Plasma collection: Blood was collected via the saphenous vein of restrained animals into ethylenediaminetetraacetic acid (EDTA)-coated tubes and then centrifuged at 1,000 x g for 5 minutes. Plasma was removed from cells and stored at -80^{o}C until analysis.

Serum collection: Mice were anesthetized with 250mg/kg tribromoethanol (Sigma-Aldrich, St. Louis, MO, USA, #T48402) dissolved in tert-amyl alcohol. Trunk blood was collected

following decapitation, allowed to clot for 15 minutes, and then centrifuged at 1,000 x g for 15 minutes at 4^{o}C. The serum was removed from the clot and stored at -80^{o}C until use.

Measurement of circulating methylated arginines: Concentrations of asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), and L-N-monomethylarginine (L-NMMA) in plasma and serum samples were determined by high-performance liquid chromatography (HPLC) using methods described previously(40-42).

Cardiotoxin injury: To induce acute muscle injury, 100uL of 10mM cardiotoxin (Sigma-Aldrich, St. Louis, MO, USA, #C3987) was injected into the gastrocnemius and quadriceps muscles of a single leg of wild-type C57BL/10 mice. Plasma was collected via the saphenous vein six hours following cardiotoxin injection and analyzed for methylated arginine concentration, as well as for creatine kinase (CK) concentration to confirm the efficacy of muscle injury.

Plasma creatine kinase analysis: Plasma was assessed for CK concentration using CK NADP Reagent (Cliniqa, San Marcos, CA, USA, #R85119) according to the manufacturer's instructions.

ADMA infusion: Osmotic minipumps (ALZET, Cupertino, CA, USA, model #1002) were surgically implanted in wild-type C57BL/10 mice and set to continuously deliver 60mg/kg/day of ADMA (Cayman Chemical, Ann Arbor, MI, USA, #80230) dissolved in saline, or saline alone as a vehicle control**.**

Voluntary running wheel activity: Three days following osmotic minipump implantation, mice were placed in cages with voluntary running wheels (Columbus Instruments, Columbus, OH, USA, #0297-0521) to measure exercise performance. Voluntary running wheel exercise was monitored for five consecutive nights using a DSI telemetry system (Data Sciences International, St. Paul, MN, USA) to record running wheel revolutions and total distance run per 24 hours. Bouts of running wheel exercise were defined as any length of time greater than 10 consecutive seconds that a mouse continued to run on the wheel without getting off.

Tissue collection and western blotting: Mice were anesthetized and the gastrocnemius muscle and heart were rapidly dissected and snap frozen in liquid nitrogen. Tissue samples were homogenized in Triton-X lysis buffer containing protease and phosphatase inhibitors(19).

Protein concentrations in tissue lysates were quantified using the *DC* protein assay (Bio-Rad, Hercules, CA, USA, #500-0116) and 100ug protein of each sample was separated on polyacrylamide gels, transferred to polyvinyldiene fluoride membranes (Millipore, Billerica, MA, USA, #IPVH00010), and subjected to quantitative Western blotting as we have described elsewhere (19). Primary antibodies included the following rabbit polyclonal antibodies: DDAH1 (Abcam, Cambridge, MA, USA, #ab82908) and dystrophin (Abcam, Cambridge, MA, USA, #ab14277), and rat monoclonal antibody against tubulin (Abcam, Cambridge, MA, USA, #ab61610). Secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA, USA, #111-035-144), and horseradish peroxidase-conjugated donkey anti-rat (Jackson ImmunoResearch Laboratories, West Grove, PA, USA, #712-035-153).

Treadmill exercise: Mice were exercised on an Exer-3/6 adjustable variable speed belt treadmill (Columbus Instruments, Columbus, OH, USA, #0257-901M) with the shock grid at the back of the treadmill set to deliver shocks at an intensity of approximately 0.4 milliamps in 200ms pulses separated at a maximum rate of 11 shocks per 10 seconds. For all experiments, the treadmill operator was blinded to mouse genotype. Before functional testing, mice were first acclimated to treadmill running by being exercised for a total of 15 minutes, starting at a speed of 8m/min for 5 minutes, followed by 5 minutes at 12m/min, and 5 minutes at 15m/min.

Fatigue assay: The day after treadmill acclimation, the animals' voluntary ambulatory open field activity was measured before and immediately following repeated bouts of treadmill exercise of increasing intensity in order to assess exercise-induced fatigue. For these open field measurements, individual mice were placed in the center of 44.6cm x 44.6cm Plexiglass cubes and the distance they walked during a 6 minute period was recorded using a ceiling-mounted camera with ANY-maze automated video tracking software (Stoelting, Wood Dale, IL, USA, version 5.11). In between open field measurement sessions, mice were exercised in 15-minute treadmill running sessions with the treadmill incline set at -15^{o}. The first treadmill running session consisted of 15 minutes of running at 8m/min. The second treadmill running session consisted of 5 minutes of running at 8m/min, followed by 10 minutes of running at 12m/min. Finally, the third treadmill running session consisted of 5 minutes of running at 8/min, followed

by 10 minutes of running at 15m/min. Mice in mock exercise groups were placed individually in empty cages for 15 minutes during these treadmill sessions instead of performing exercise. For direct comparisons of changes in voluntary ambulatory open field activity between mice of different genotypes or different exercise groups, the raw open field activity data for each mouse was normalized by expressing the distance each animal walked during each open field session as a percentage of the distance it walked during the first open field session.

Exhaustion assay: The day after assessment on the fatigue assay described above, mice were run on treadmills at a -15^{o} decline starting at a speed of 3m/min. Every two minutes, the treadmill belt speed was increased by 3m/min up to a maximum speed of 21m/min. Mice were exercised until they reached exhaustion, defined as the point at which they could no longer avoid the shock grid at the back of the treadmill despite 3 successive manual boosts from the treadmill operator.

Measurement of urine nitrite + nitrate: Mouse urine was collected on clean strips of parafilm and then stored at -80^{o}C until use. Total urine nitrite + nitrate (NO_x) concentration was measured using a nitrate/nitrite colorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA, #780001) according to the manufacturer's instructions.

Histology and immunofluorescence: The heart and the soleus and gastrocnemius muscles were collected from anesthetized mice, mounted in Tissue-Plus OCT (Fisher HealthCare, Houston, TX, USA, #23-730-571), frozen in isopentane-cooled liquid nitrogen, and cut into 8-micron cross sections as described previously(43). To quantify fibrosis, slides were stained with 0.1% sirius red in picric acid following the procedure described by Hadi et al.(44). Images of the heart or gastrocnemius and soleus muscles were captured using an Olympus BX-51 fluorescence microscope and the collagen area fraction (CAF), the percentage of the total area of each section that stained positively with picosirius red, was measured using ImageJ. To quantify the percentage of centrally-nucleated skeletal muscle fibers as well as fiber cross-sectional area (CSA), slides containing gastrocnemius and soleus sections were washed in phosphate-buffered saline (PBS), blocked for 1 hour in PBS containing 5% bovine serum albumin (Fisher Scientific, Fair Lawn, NJ, USA, #BP1600), and then incubated with a rabbit polyclonal antibody against laminin (Sigma-Aldrich, St. Louis, MO, USA, #L9393) for 1.5 hours.

Slides were washed 3 times for 5 minutes each in PBS, and then incubated with an AlexaFluor 488-conjugated goat-anti rabbit secondary antibody (Invitrogen, Carlsbad, CA, USA, #A-11034) and 4',6-diamino-2-phenyindole, dilactate (DAPI) (Sigma-Aldrich, S. Louis, MO, USA, #D9564) for 1.5 hours. Finally, slides were washed 3 times for 5 minutes each and mounted using PermaFluor (Thermo Fisher Scientific, Fremont, CA, USA, #TA-030-FM). Laminin staining outlining the muscle fiber boundaries was used to measure the dimensions of individual fibers in ImageJ. The variance coefficient of the fiber CSA was calculated as: (standard deviation of muscle fiber CSA / mean muscle fiber CSA) x 100(45). All assessments of fibrosis, central nucleation, and muscle fiber size were performed blinded to mouse genotype.

Echocardiography: Echocardiography was performed in mice under isoflurane anesthesia as described previously(46). Measurements of stroke volume and cardiac output were made in the 2-dimensional long axis view, and pulsed-wave Doppler imaging was used to assess the left-ventricular myocardial performance index (LVMPI), defined as: (isovolumic relaxation time + isovolumic contraction time)/aortic ejection time. To assess beta-adrenergic responsiveness, after baseline measurements of cardiac function, a subset of mice was injected intraperitoneally with 2mg/kg isoproterenol (Sigma-Aldrich, St. Louis, MO, USA, #I6504) and measurements were immediately repeated.

General statistical methods: All data are presented as mean ± SE. For simple comparisons between two groups, significance was determined by the Student *t* test. Direct comparisons between three or more groups were made by one-way or two-way ANOVA, as appropriate, followed by Tukey's multiple comparisons tests. For exercise-induced fatigue assays, data were analyzed by two-way repeated measures ANOVA followed by Fisher's least significant difference test for comparison of groups at each of multiple successive open field sessions. For all analyses, $P<0.05$ was considered significant.

RESULTS

Asymmetric dimethylarginine is elevated in dystrophin-deficiency or following acute necrotic muscle injury, and is sufficient to impair exercise performance in healthy mice.

We first sought to determine whether methylated arginine levels are elevated in dystrophin-deficient *mdx* mice as they are in human DMD patients. Compared to WT C57BL/10 controls, *mdx* mice exhibited elevated circulating concentrations of ADMA and the related endogenous nitric oxide synthase inhibitor, monomethylarginine (L-NMMA) (Fig. 4.1A). In contrast, circulating symmetric dimethylarginine (SDMA) concentrations did not differ between genotypes (Fig. 4.1A). Induction of degenerative muscle injury in WT mice using cardiotoxin injection triggered an increase in plasma creatine kinase (CK) concentration along with a concurrent increase in circulating L-NMMA and a tendency towards increased circulating ADMA (Fig. 4.1 B and C). These data demonstrate that methylated arginines are elevated following acute muscle injury, and support the idea that circulating methylated arginine concentrations are elevated in DMD because these compounds are released from dystrophic muscle cells as a direct result of muscle injury.

To investigate the potential for excessive circulating ADMA to impact exercise performance, we infused ADMA into healthy, WT mice and assessed their voluntary running wheel activity. Infusion of exogenous ADMA resulted in circulating ADMA levels approximately 3-fold higher than in control mice infused with saline (Fig. 4.1D). This elevation in circulating ADMA levels was associated with a reduction in the total distance that ADMA-infused WT mice ran on voluntary running wheels per 24 hours (Fig. 4.1E). Mice infused with ADMA also ran less total distance per individual bout on the running wheels compared to saline-infused mice (Fig. 4.1F), consistent with the notion that elevated circulating ADMA causes mice to become more easily fatigued following the onset of exercise.

DDAH transgene expression reduces circulating ADMA concentration in dystrophin-deficient male *mdx* mice.

To investigate whether elevated circulating ADMA contributes to exaggerated exercise-induced fatigue in Duchenne muscular dystrophy, we crossed dystrophin-deficient female *mdx* mice to wild-type males expressing a transgene encoding human dimethylarginine dimethylaminohydrolase 1 (DDAH), an enzyme that degrades ADMA. This cross produced transgenic (TG) and non-transgenic (ntg) dystrophin-deficient *mdx* male offspring, as well as TG

and ntg dystrophin-heterozygous *mdx* carrier female offspring (Fig. 4.2A). Western blot analysis showed robust DDAH transgene expression in the skeletal muscle (Fig. 4.2B) and hearts (Fig. 4.2C) of TG *mdx* males and *mdx* carrier females. Interestingly, transgenic DDAH expression was lower in the skeletal muscle of TG *mdx* males compared to TG WT males and TG *mdx* carrier females (Fig. 4.2B). In contrast, transgenic DDAH expression was reduced in the hearts of TG *mdx* carrier females compared to TG WT males and TG *md*x males (Fig. 4.2C). We also observed low levels of endogenous DDAH expression in the skeletal muscle and hearts of ntg animals (Fig. 4.2 B and C), although expression of endogenous DDAH did not differ between ntg WT males, ntg *mdx* males, and ntg *mdx* carrier females in either tissue. Dystrophin protein was not detected in the skeletal muscle or hearts of *mdx* male mice (Fig. 4.2 B and C). As expected based on the typical expression of dystrophin at relatively normal levels in multinucleated skeletal muscle cells, but mosaic expression pattern due to random-X inactivation in cardiomyocytes(47, 48), dystrophin expression was significantly reduced compared to WT in the hearts but not skeletal muscle of female *mdx* carriers (Fig. 4.2 B and C).

HPLC analysis revealed that steady-state circulating ADMA concentration was reduced in TG versus ntg male *mdx* mice, while serum concentrations of L-NMMA and SDMA were not significantly altered (Fig. 4.3 A-C). In contrast, initial results indicated that DDAH transgene expression did not significantly alter basal serum methylated arginine concentrations in female *mdx* carriers (Fig. 4.3 D-F).

DDAH transgene expression improves resistance to exercise-induced fatigue in dystrophin-heterozygous *mdx* carrier female, but not in fully dystrophin-deficient *mdx* male mice.

We next established a strategy to assess the effects of DDAH transgene expression on exercise-induced fatigue and overall exercise capacity in dystrophin-deficient mice. To assess exercise-induced fatigue, the animals' voluntary ambulatory activity was measured in 6-minute open field sessions before and immediately after successive bouts of treadmill running at progressively increased speeds (Fig. 4.4A). To assess total exercise capacity, mice were run on a single treadmill session, with velocity increased every 2 minutes up to a maximum velocity of 21 m/min until they could no longer stay on the treadmill belt (Fig. 4.4B). Successive bouts of

treadmill running in the fatigue assay led to an exaggerated drop in voluntary ambulatory open field activity in both WT C57 and *mdx* males compared to animals that were mock exercised (Fig. 4.4 C and D). These observations established that the drop in activity following successive bouts of treadmill running was not due merely to acclimation effects during repeat exposure to the open field measurement chambers, suggesting that both WT and *mdx* male mice exhibit exercise-induced fatigue. Normalization of each animal's post-exercise voluntary activity to its own pre-exercise voluntary activity revealed that the drop in voluntary activity following treadmill exercise was greater in *mdx* than WT males (Fig. 4.4E) , in agreement with previous reports that exercise-induced fatigue is exaggerated in dystrophin-deficient animals(25, 49). In contrast, the drop in activity over repeated open field monitoring sessions in mock-exercised animals did not differ between wild-type and *mdx* males, indicating that these genotypes did not differ in their acclimation to the testing chambers (Fig. 4.4F). This control experiment verified that any differences between genotypes in the open field activity of exercised mice was indeed attributable to differential effects of or responses to exercise, rather than underlying differences in behavioral responses to repeat exposures to the open field chambers. Finally, male *mdx* mice were able to run for a shorter total time and distance prior to reaching exhaustion than WT males (Fig. 4.4 G and H), confirming that their total exercise capacity was diminished compared to healthy animals.

These same assays revealed that like male mice, WT, dystrophin-heterozygous *mdx* carrier, and fully dystrophin-deficient *mdx* females that were run on treadmills exhibited a greater drop in voluntary ambulatory open field activity compared to females that were mock exercised (Fig. 4.5 A-C), suggesting that females of all three genotypes are also susceptible to exercise-induced fatigue. However, in contrast to fully dystrophin-deficient *mdx* females, this exercise-induced fatigue was not exaggerated beyond wild-type levels in *mdx* carrier females (Fig. 4.5D). The drop in voluntary activity over repeated open field sessions was similar among mock exercised wild-type, *mdx* carrier, and *mdx* female mice (Fig. 4.5E), indicating that all three genotypes had similar behavioral responses to acclimate to the open field chambers. Finally, the time and distance that *mdx* carriers were able to run before reaching exhaustion was reduced compared to WT females but was still significantly greater than *mdx* females (Fig. 4.5 F

and G). The observation that *mdx* carrier performance on both exercise assays was significantly better than that of fully dystrophin-deficient *mdx* females is in agreement with reports that human DMD carriers suffer only relatively mild impairments in muscle function compared to male DMD patients(13, 15, 50) .

Following validation of these two exercise assays, we used them to test the effect of DDAH transgene expression on exercise tolerance in dystrophic animals. Comparison of TG and ntg *mdx* male littermates revealed no effect of the DDAH transgene on the absolute (Fig. 4.6A) or relative (Fig. 4.6B) drop in voluntary ambulatory open field activity following exercise. Similarly, we observed no significant effect of the DDAH transgene on time or distance that *mdx* males were able to run before reaching exhaustion (Fig. 4.6 C and D). Together, these data indicate that expression of the DDAH transgene was not sufficient to impact exercise-induced fatigue or exercise capacity in fully dystrophin-deficient males.

Surprisingly, the drop in voluntary activity following exercise was significantly attenuated in DDAH-transgenic female *mdx* carriers compared to non-transgenic female *mdx* carrier littermates (Fig. 4.7 A and B). This observation demonstrated that unlike in fully dystrophin-deficient *mdx* males, DDAH transgene expression did have a beneficial effect to alleviate exercise-induced fatigue in dystrophin-heterozygous animals. This positive influence was limited to exercise-induced fatigue, as DDAH transgene expression did not affect the time or distance that *mdx* carrier females were able to run before reaching exhaustion (Fig. 4.7 C and D). This discrepancy would indicate that while DDAH transgene expression in *mdx* carriers can influence functional responses immediately following moderate-intensity exercise, it does not change the overall total exercise capacity or endurance of these mice.

DDAH transgene expression alters heart size, but not skeletal muscle pathology, in female dystrophin-heterozygous *mdx* carriers.

We next sought to understand the mechanisms underlying the beneficial effect of the DDAH transgene on exercise-induced fatigue in female dystrophin-heterozygous *mdx* carrier mice. Analysis of total nitrite + nitrate (NO_x) concentration in the urine as an estimate of basal whole-body nitric oxide production revealed a slight but non-significant effect of the DDAH

transgene to increase urine NO_x concentration in *mdx* males (Fig. 4.8A), and no significant effect of the DDAH transgene on urine NO_x concentration in *mdx* carrier females (Fig. 4.8B). These data argue against any effect of the DDAH transgene to significantly modulate systemic NO levels in either *mdx* males or *mdx* carrier females. However, it remains possible that any local effects of the DDAH transgene on methylated arginine levels within specific tissues may in turn alter local NO production, any changes in which could be too small or too locally constrained to dramatically alter whole-body NO_x levels. Unexpectedly, our data indicated that urine NO_x concentration already tended to be elevated in ntg *mdx* males compared to WT males (Fig. 4.8A), and was already significantly elevated in ntg female *mdx* carriers compared to WT females (Fig. 4.8B). These finding provides further support to the notion that any small changes in NO production in TG animals may have been masked by an already elevated level of basal NO production in dystrophin-deficient and dystrophin-heterozygous animals. The elevated NO_x concentration we observed likely represents NO produced by inducible NOS (iNOS), the expression of which is elevated in both dystrophic skeletal and cardiac muscle(17, 51), as well as in inflammatory cells typically recruited to dystrophic muscle tissue(52).

Similar to urine NO_x levels, steady-state plasma creatine kinase concentration was also elevated in non-transgenic male dystrophin-deficient *mdx* mice compared to WT controls, suggesting that dystrophin-deficient mice still exhibit muscle necrosis or sarcolemmal damage even at advanced ages (Fig. 4.8C). However, CK concentration was not significantly altered by DDAH transgene expression (Fig. 4.8C). Plasma CK concentration also tended to be elevated in ntg *mdx* carrier females compared to WT females (Fig. 4.8D). Although this difference did not reach statistical significance, it would support the idea that like fully dystrophin-deficient males, dystrophin-heterozygous animals exhibit a greater degree of muscle damage than healthy controls. As for *mdx* males, DDAH transgene expression did not significantly affect plasma CK concentration in female *mdx* carriers (Fig. 4.8D).

To distinguish any tissue-specific effects of DDAH transgene expression, we examined the overall pathology of the heart and skeletal muscle. Although we saw no effect of DDAH transgene expression on heart weight in male *mdx* mice (Fig. 4.9 A and B), DDAH transgene expression resulted in a significantly lower heart weight in female *mdx* carriers (Fig. 4.9 C and

D). The specificity of this effect to female *mdx* carriers raises the intriguing possibility that the attenuation of exercise-induced fatigue in female TG *mdx* carriers and not male TG *mdx* mice is due to effects of the transgene that are unique to the dystrophin-heterozygous heart. We further assessed the effects of DDAH transgene expression on cardiac pathology by using Sirius red staining to quantify cardiac fibrosis. However, we did not observe any significant effect of transgene expression on this parameter in either *mdx* males or *mdx* carrier females (Fig. 4.9 E and F).

Examination of skeletal muscle histology showed a reduction in the percentage of centrally-nucleated fibers in the gastrocnemius of TG versus ntg *mdx* males (Fig. 4.10A), but no other significant effects of DDAH transgene expression on fiber size, fiber size variability, or fibrosis in the gastrocnemius or soleus muscles of *mdx* males (Fig. 4.10 B-G). Similarly, DDAH transgene expression did not significantly affect central nucleation, fiber size, fiber size variability, or fibrosis in the gastrocnemius or soleus muscles of *mdx* carrier females (Fig. 4.10 H-N). These data support the idea that DDAH transgene expression improves resistance to exercise-induced fatigue in *mdx* carrier females by exerting specific beneficial effects on the heart rather than in the skeletal muscle of these animals.

DDAH transgene expression improves cardiac function in *mdx* carriers.

Finally, we investigated whether DDAH transgene expression had any direct impact on cardiac function in female dystrophin-heterozygous *mdx* carriers. Echocardiography revealed that stroke volume was greater in DDAH-transgenic than non-transgenic female *mdx* carriers (Fig. 4.11A). This increase in stroke volume was associated with a reduction in basal heart rate in TG *mdx* carriers (Fig. 4.11B), resulting in similar cardiac output between the two genotypes (Fig. 4.11C). The left ventricular myocardial performance index, an integrated measure of both systolic and diastolic function, was also reduced in TG *mdx* carriers (Fig. 4.11D), lending additional support to the notion that contractile function is improved by DDAH transgene expression in these mice. The observation that an elevated heart rate seemed to compensate for diminished contractile performance in ntg *mdx* carriers prompted us to test the effects of DDAH transgene expression functional cardiac reserve in *mdx* carriers. Acute injection of

isoproterenol increased heart rate in TG *mdx* carriers, but failed to increase heart rate in ntg *mdx* carriers (Fig. 4.11E). This finding, coupled with the observation that resting heart rate is already elevated in ntg versus TG *mdx* carriers, indicates that much of the cardiac functional reserve is already used up in ntg *mdx* carriers under basal conditions, and therefore cardiac function cannot increase further in response to increased physiological demand. Furthermore, these results suggest that by improving contractile performance and lowering resting heart rates, DDAH transgene expression restores the functional reserve available for *mdx* carrier mice to increase cardiac function in response to acute stresses such as exercise.

DISCUSSION

A growing body of evidence suggests that deficient striated muscle nitric oxide signaling and consequent disruption of sympatholysis represents a considerable component of the exercise-induced fatigue phenotype in Duchenne muscular dystrophy. Recent reports demonstrating that deletion of nNOS worsens the phenotype of dystrophin-deficient mice(49, 53) and that restoration of nNOS expression or signaling downstream of NO production can improve the function of dystrophin-deficient muscle(25, 54-57) further support the idea that the regulation of striated muscle NO production plays an important role in modulating disease progression in DMD. While a number of studies have investigated how changes in muscle cell-intrinsic processes affecting nNOS expression and its activity may alter NO signaling in DMD(18, 19, 58), the potential effects of cell-extrinsic factors such as circulating endogenous NOS inhibitors, and their ultimate functional consequences for dystrophin-deficient patients, have received little attention. This question is particularly relevant given increasing interest in increasing nNOS expression(54, 55), restoring nNOS sarcolemmal localization(34), or activating upstream regulators of nNOS activity(19) as therapeutic strategies to increase muscle nNOS activity in this disease, because endogenous NOS inhibitors may limit the efficacy of these interventions.

Here, we used an *in vivo* approach to address the hypothesis that excessive circulating levels of the endogenous NO synthase inhibitor, ADMA, contributes to exaggerated exercise-induced fatigue in the *mdx* mouse model of Duchenne muscular dystrophy. Our findings that

methylated arginines are released to the circulation following muscle injury and are sufficient to impair exercise performance even in healthy mice with an intact dystrophin-glycoprotein complex support a causal role for these compounds in the exercise intolerance observed in DMD. They also emphasize the notion that secondary consequences of dystrophic muscle pathology, subsequent to the primary disruption of the DGC and destabilization of the sarcolemma, can have a substantial impact on the dystrophic phenotype.

Unexpectedly, transgenic expression of dimethylarginine dimethylaminohydrolase 1 (DDAH) in an attempt to increase the degradation of ADMA improved resistance to exercise-induced fatigue in female *mdx* carrier mice, but not male *mdx* mice. The discrepancy in the transgene's effects between the dystrophin-deficient *mdx* background and the dystrophin-heterozygous *mdx* carrier background raises interesting questions as to the mechanism by which changes in ADMA metabolism may affect exercise tolerance. We did not observe any effect of DDAH transgene expression on steady-state circulating methylated arginine concentrations in female *mdx* carriers. If changes in methylated arginine metabolism are in fact responsible for the alleviation of exercise-induced fatigue in female *mdx* carriers, these data suggest that the effects of transgenic DDAH expression on circulating methylated arginine levels may be appreciable only with acute elevation of these compounds during or immediately following a bout of exercise, and therefore would have been missed by our approach. Alternatively, any substantial effects of transgenic DDAH expression on ADMA or possibly L-NMMA concentrations may occur locally within specific tissues. The idea of local rather than generalized effects of DDAH expression on methylated arginines and NO production is consistent with our observation that total urine NO_x levels were not altered by TG expression. Indeed, the finding that total urine NO_x concentration was elevated compared to WT in *mdx* carriers and also tended to be elevated in *mdx* males argues against a generalized, systemic inhibition of the nitric oxide synthases by excessive circulating methylarginines in dystrophic animals.

Our findings that DDAH transgene expression reduced the mass and improved the contractile performance of the hearts of female *mdx* carriers indicate that local manipulation of ADMA metabolism can have consequential beneficial effects within dystrophin-heterozygous

cardiac muscle. This specific improvement in heart function may explain the efficacy of DDAH transgene expression in attenuating exercise-induced fatigue in *mdx* carriers, as we did not observe any changes in the skeletal muscle pathology with DDAH transgene expression in these animals. A cardiac-specific benefit of the DDAH transgene to affect exercise tolerance also makes sense in light of the phenotype of human DMD carriers, who generally suffer from little skeletal muscle pathology but are at increased risk for developing cardiomyopathy as they age(12-14). Fitting with this notion, the improvement in cardiac function and fatigue resistance that we observed with DDAH transgene expression occurred in relatively old (11-12 month) female *mdx* carriers. Although overt dilated cardiomyopathy has been reported to be absent in female *mdx* carrier mice up to 21 months of age(59), our observations that the extent of cardiac fibrosis is nearly the same in 11- to 12-month-old female *mdx* carriers as in 11-to 12-month-old *mdx* males, and that chronotropic responses to isoproterenol are lost in female *mdx* carriers, indicate that dystrophin heterozygosity has already started to affect cardiac structure and function at this age. Furthermore, our finding that improved cardiac performance was associated with improved resistance to exercise-induced fatigue but not with increased maximal exercise capacity in TG female *mdx* carriers suggests that fatigue resistance is the phenotype that is more sensitive to changes in cardiac function in dystrophin-heterozygous animals.

It will be interesting for future studies to determine the precise molecular mechanisms by which DDAH transgene expression improves heart function in female *mdx* carriers. Although the impact of mosaic dystrophin expression on cardiac NOS activity has not been reported, our data are consistent with a model in which ADMA released during necrosis of damaged, dystrophin-deficient cardiomyocytes has local effects to inhibit NO production in neighboring dystrophin-positive and dystrophin-null cells. As both nNOS and endothelial NOS are critical regulators of contractile function(30-33, 60, 61), relief of their inhibition with DDAH transgene expression and local reductions in ADMA concentration may contribute to improved contractile performance in TG *mdx* carriers. Locally-elevated ADMA may also exert either NO-dependent or NO-independent effects to promote expression of hypertrophic gene programs, and these effects may be attenuated in DDAH-transgenic carriers(62, 63).

Why we did not see an impact of DDAH expression on the susceptibility to exercise-induced fatigue despite a significant reduction in circulating ADMA concentration in fully dystrophin-deficient *mdx* males remains unanswered. The failure of DDAH transgene expression to affect heart size in male *mdx* mice may reflect that local reductions in ADMA concentration or subsequent effects on NO signaling within the heart are simply too small to significantly alter the progression of cardiac disease or dysfunction in the setting of total dystrophin deficiency. The findings presented in Chapter 2 of this thesis support the alternative hypothesis that NO production in dystrophin-deficient striated muscle cells is impaired due to distinct defects in mechanosignaling that are independent of methylated arginine metabolism. Thus, transgenic DDAH expression alone may in fact do little to improve muscle NO production in fully dystrophin-deficient males. Nevertheless, our work raises the interesting possibility that manipulating ADMA metabolism may have therapeutic implications not only for heterozygous DMD carriers, but also for instances in which exon-skipping or viral gene-delivery approaches result in only partial restoration of dystrophin expression in the hearts of male DMD patients. Given that exercise-induced fatigue was similar between aged *mdx* carriers and WT females, and that transgenic DDAH expression improved cardiac performance and fatigue resistance of *mdx* carriers, an intriguing question for future investigations is whether altered ADMA metabolism may also have beneficial effects on cardiac function or exercise tolerance as otherwise healthy individuals age.

Together, our findings support a model in which ADMA that is released from damaged cells contributes to dysfunction of the aged dystrophin-heterozygous heart. Impaired contractile function results in a compensatory increase in heart rate and a diminished functional cardiac reserve available for dystrophin-heterozygous subjects to respond to adrenergic stress, negatively affecting their ability to tolerate exercise. We demonstrate that DDAH transgene expression is sufficient to reduce heart size, improve contractile function, and restore chronotropic reserve in female dystrophin-heterozygous mice, possibly improving their immediate physiological responses to exercise. While additional studies will be required to uncover the pathways by which DDAH transgene expression modifies cardiac function in *mdx* carriers , this work places a new emphasis on the relevance of the regulation of methylated

arginine signaling to cardiac function in dystrophinopathies, and its potential to improve exercise phenotypes in these diseases.

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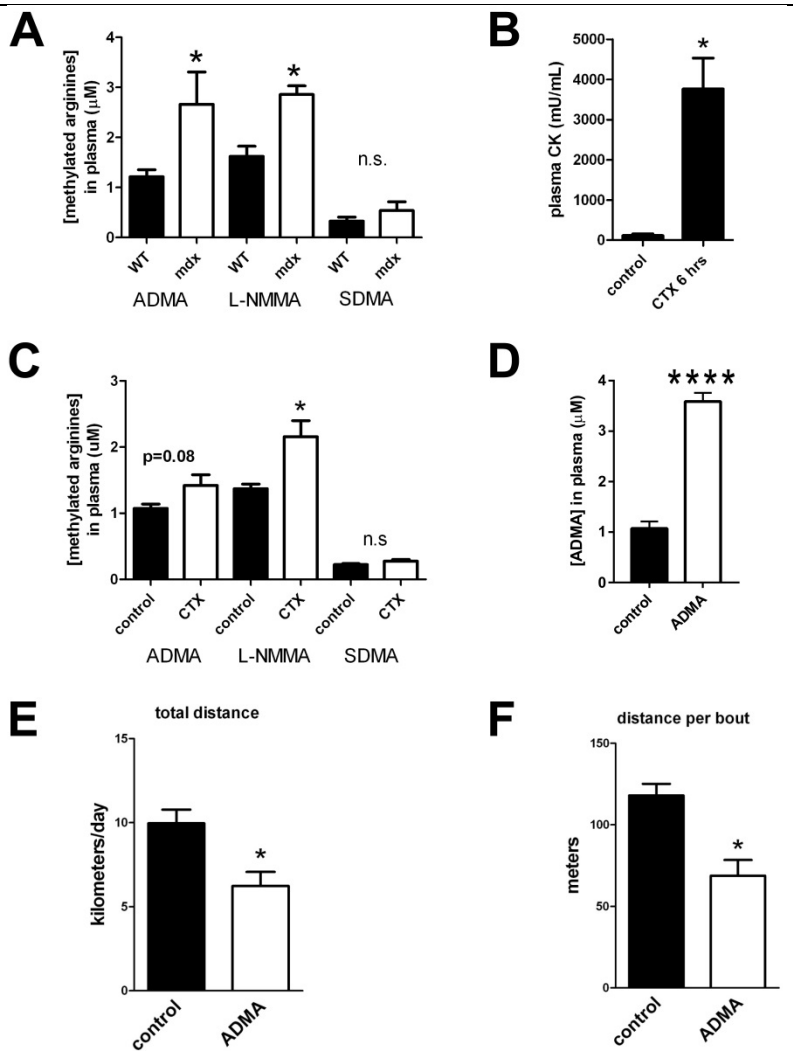

Figure 4.1. Circulating methylated arginines are elevated by dystrophin-deficiency or acute muscle injury, and are sufficient to impair exercise performance. (**A**) Plasma concentrations of asymmetric dimethylargine (ADMA) and monomethylarginine (L-NMMA) are increased in *mdx* versus wild-type (WT) C57BL/10 mice (n=4-7) (*P<0.05 vs. WT control). (**B**) Acute single leg muscle injury with cardiotoxin (CTX) leads to an elevation in plasma creatine kinase (CK) concentration in WT mice (n=3-7) (*P<0.05). (**C**) Acute cardiotoxin injury elevates circulating L-NMMA concentration and tends to elevate circulating ADMA concentration in WT mice (n=6-10) (*P<0.05 vs. non-injured control). (**D**) In WT mice, infusion of exogenous ADMA elevates steady-state circulating ADMA concentration compared to infusion of saline (n=5) (****P<0.0001). ADMA-infused WT mice run less total distance per day (**E**) and per bout (**F**) on voluntary running wheels compared to saline-infused controls (n=5) (*P<0.05).

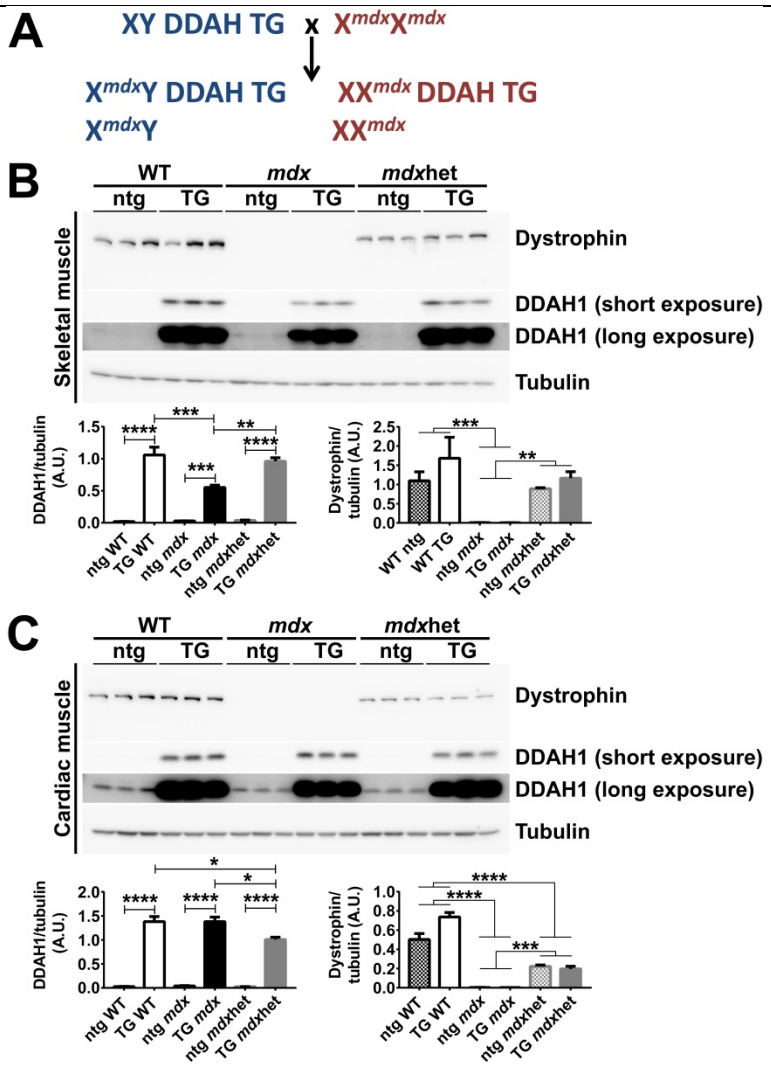

Figure 4.2. DDAH1 protein is robustly expressed in DDAH-transgenic mice. (**A**) Breeding scheme. Male DDAH-transgenic mice were crossed to female *mdx* mice, generating DDAH-transgenic (TG) and non-transgenic (ntg) dystrophin-deficient *mdx* male and dystrophin-heterozygous female offspring. (**B**) DDAH1 protein expression is increased in the gastrocnemius muscle of TG wild-type (WT) males, *mdx* males, and heterozygous *mdx* carrier females ("*mdx*het") compared to ntg controls (n=3) (**P<0.01, ***P<0.001, ****P<0.0001). Dystrophin protein is not detected in gastrocnemius muscle of male *mdx* mice (n=6) (**P<0.01, ***P<0.001). Low levels of endogenous DDAH1 are detected in the gastrocnemius muscle of ntg animals, but do not significantly vary between ntg WT males, ntg *mdx* males, and ntg *mdx* carrier females (n=3). (**C**) DDAH1 protein expression is increased in the heart of TG WT males, *mdx* males, and *mdx* carrier females compared to ntg controls (n=3) (*P<0.05. ****P<0.0001). Dystrophin protein is not detected in the hearts of male *mdx* mice, and is reduced compared to WT in the hearts of *mdx* carrier females (n=6) (**P<0.01, ****P<0.0001). Low levels of endogenous DDAH1 are detected in the hearts of ntg animals, but do not significantly vary between ntg WT males, ntg *mdx* males, and ntg *mdx* carrier females (n=3).

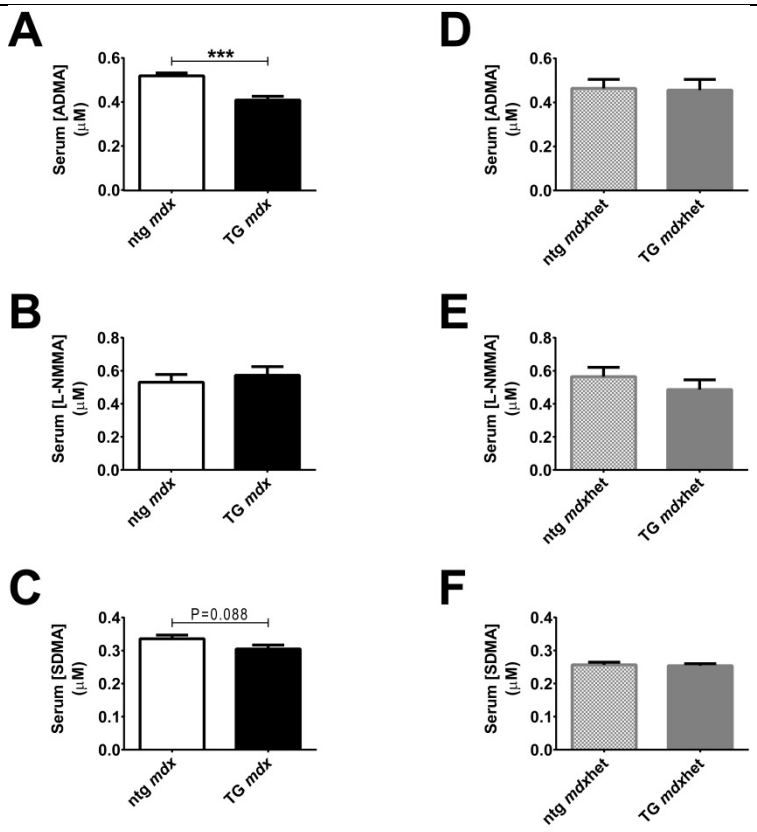

Figure 4.3. DDAH transgene expression reduces circulating ADMA concentration in male dystrophin-deficient *mdx* mice. (**A**) Serum concentration of asymmetric dimethylarginine (ADMA) is reduced in DDAH-transgenic (TG) versus non-transgenic (ntg) male dystrophin-deficient *mdx* mice (n=6) (***P<0.001). Serum concentrations of monomethylarginine (L-NMMA) (**B**) and symmetric dimethylarginine (SDMA) (**C**) do not differ between ntg and TG male *mdx* mice (n=6). Serum concentrations of ADMA (**D**), L-NMMA (**E**), and SDMA (**F**) do not differ between ntg and TG female dystrophin-heterozygous *mdx* carrier mice (n=4).

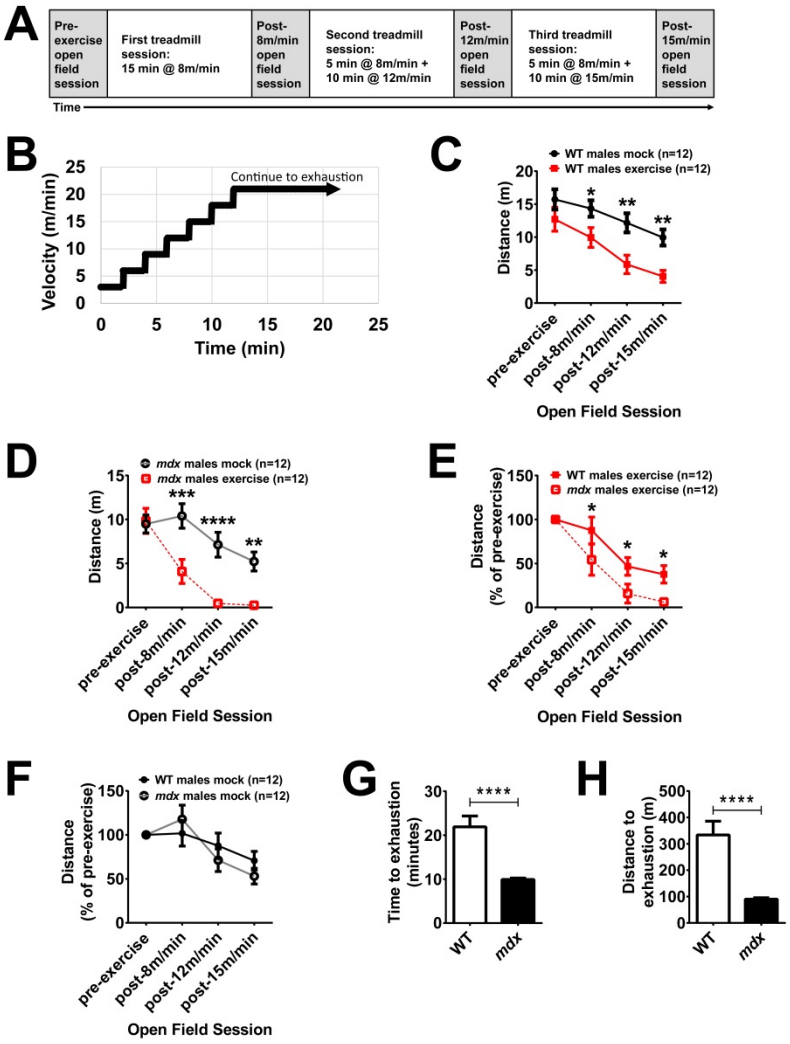

Figure 4.4. Validation of exercise assays and assessment of exercise tolerance in male *mdx* mice. (**A**) Sequence of open-field measurement sessions and treadmill exercise sessions used to assess exercise-induced fatigue. (**B**) Progressive increase of treadmill running speed in exhaustion assay. (**C**) Voluntary ambulatory activity decreases more over successive open field monitoring sessions in exercised than in mock-exercised male wild-type (WT) mice (*P<0.05, **P<0.01). (**D**) Voluntary ambulatory activity decreases more over successive open field monitoring sessions in exercised than in mock-exercised male dystrophin-deficient *mdx* mice (**P<0.01, ***P<0.001, ****P<0.0001). (**E**) Exercised male *mdx* mice exhibit a greater decrease in voluntary activity over successive open field monitoring sessions than exercised male WT mice (*P<0.05). (**F**) The decrease in voluntary ambulatory activity over successive open field monitoring sessions is similar in mock-exercised male WT and *mdx* mice. Male *mdx* mice run a shorter time (**G**) and distance (**H**) before reaching exhaustion than male WT mice (n=12) (****P<0.0001).

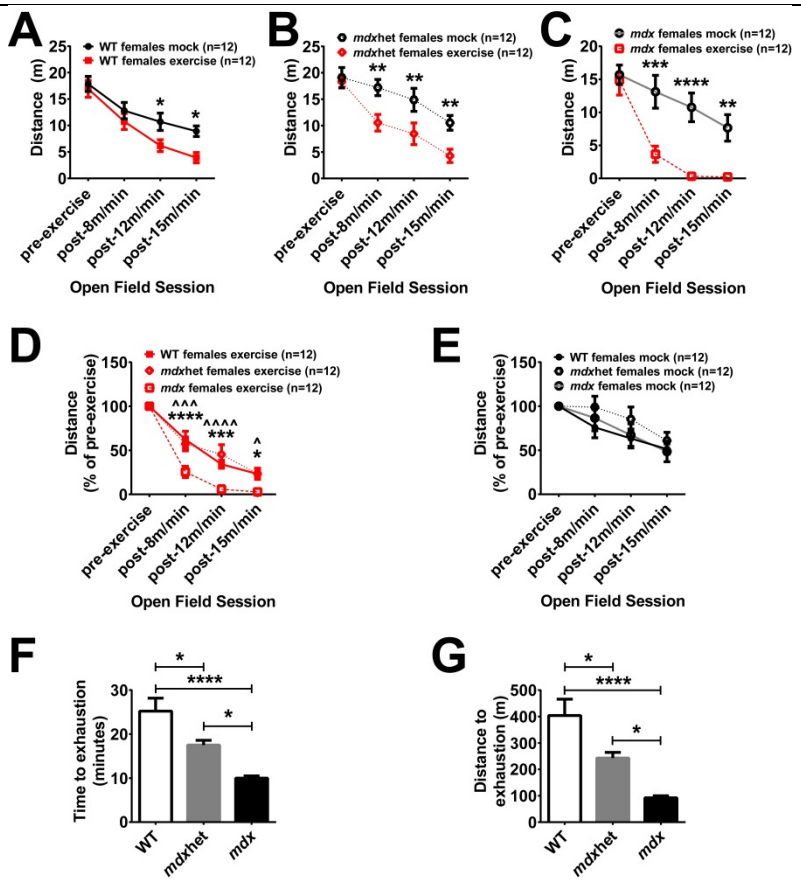

Figure 4.5. Validation of exercise assays and assessment of exercise tolerance in female *mdx* carrier mice. (**A**) Voluntary ambulatory activity decreases more over successive open field monitoring sessions in exercised than in mock-exercised female wild-type (WT) mice (*P<0.05). (**B**) Voluntary ambulatory activity decreases more over successive open field monitoring sessions in exercised than in mock-exercised female dystrophin-heterozygous *mdx* carrier mice (**P<0.01). (**C**) Voluntary ambulatory activity decreases more over successive open field monitoring sessions in exercised than in mock-exercised female dystrophin-deficient *mdx* mice (**P<0.01, ***P<0.001, ****P<0.0001). (**D**) Exercised female *mdx* mice exhibit a greater decrease in voluntary ambulatory activity over successive open field monitoring sessions than exercised female WT and *mdx* carrier mice (*P<0.05, ***P<0.001, ****<P<0.0001, WT vs. *mdx*; ^P<0.05, ^^^P<0.001, ^^^^P<0.0001 *mdx* carrier vs. *mdx*). (**E**) The decrease in voluntary ambulatory activity over successive open field monitoring sessions is similar in mock-exercised female WT, *mdx* carrier, and *mdx* mice. Female *mdx* carrier mice run a shorter time (**F**) and distance (**G**) before reaching exhaustion than female WT mice; this impairment is further exaggerated in female *mdx* mice (n=12) (*P<0.05, ****P<0.0001).

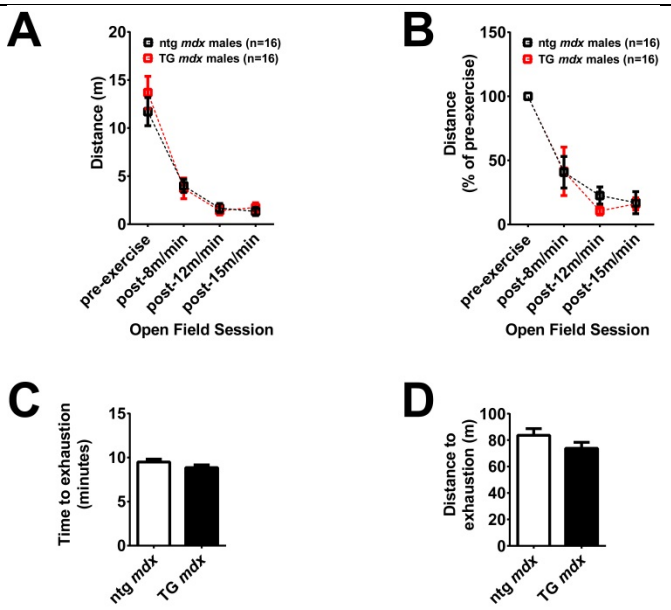

Figure 4.6. DDAH transgene expression does not affect exercise-induced fatigue or exercise capacity in male dystrophin-deficient *mdx* mice. (**A**) The absolute voluntary ambulatory activity over successive open field monitoring sessions does not differ between exercised DDAH-transgenic (TG) and non-transgenic (ntg) male *mdx* mice. (**B**) The relative decrease in voluntary ambulatory activity over successive open field monitoring sessions does not differ between exercised TG and ntg male *mdx* mice. Male TG and ntg *mdx* mice do not differ in the time (**C**) or distance (**D**) that they are able to run before reaching exhaustion (n=16).

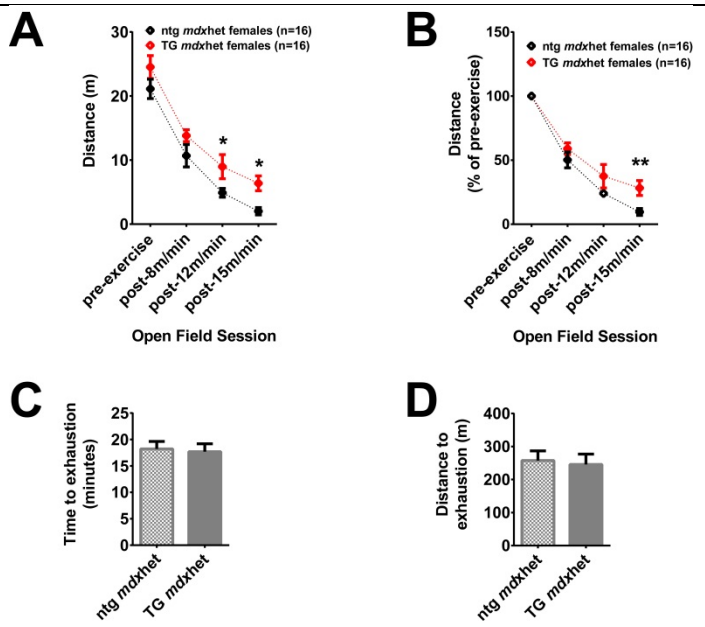

Figure 4.7. DDAH transgene expression attenuates exercise-induced fatigue in female dystrophin-heterozygous *mdx* carrier mice. (**A**) The absolute voluntary activity in open field sessions following successive bouts of treadmill exercise is greater in DDAH-transgenic (TG) than non-transgenic (ntg) female dystrophin-heterozygous *mdx* carrier mice (*P<0.05). (**B**) The relative decrease in voluntary ambulatory activity over successive open field monitoring sessions is attenuated in exercised TG versus ntg female *mdx* carrier mice (**P<0.05). Female TG and ntg *mdx* carrier mice do not differ in the time (**C**) or distance (**D**) that they are able to run before reaching exhaustion (n=16).

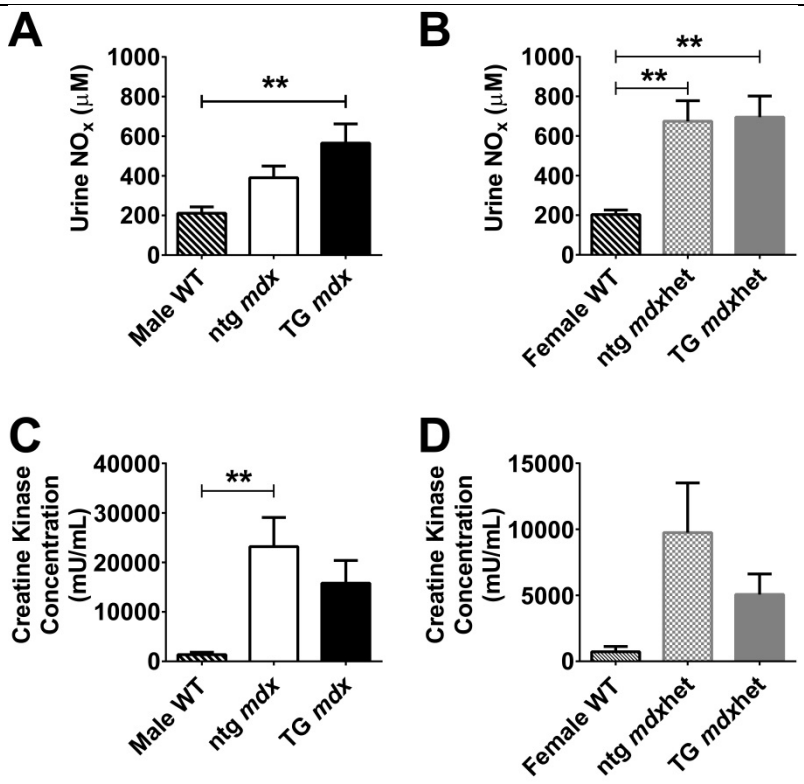

Figure 4.8. DDAH transgene expression does not significantly affect total body NO production or overall creatine kinase release in dystrophin-deficient *mdx* males or dystrophin-heterozygous *mdx* carrier females. (**A**) Urine nitrite + nitrate (NO_x) concentration in dystrophin-deficient *mdx* males is not significantly altered by DDAH transgene expression (n=12 for WT; n=16 for ntg and TG *mdx*) (**P<0.01). (**B**) Urine NO_x concentration is elevated in female dystrophin-heterozygous *mdx* carriers compared to wild-type (WT) but is not altered by DDAH transgene expression (n=12 for WT; n=16 for ntg and TG *mdx*hets) (**P<0.01). (**C**) Plasma creatine kinase concentration is elevated in ntg male *mdx* mice compared to WT, but is not significantly altered by DDAH transgene expression (n=12 for WT, n=16 for ntg and TG *mdx*) (**P<0.01). (**D**) Plasma creatine kinase concentration is slightly but not significantly elevated in female *mdx* carriers compared to WT, and is not significantly altered by DDAH transgene expression (n=12 for WT, n=16 for ntg and TG *mdx*hets).

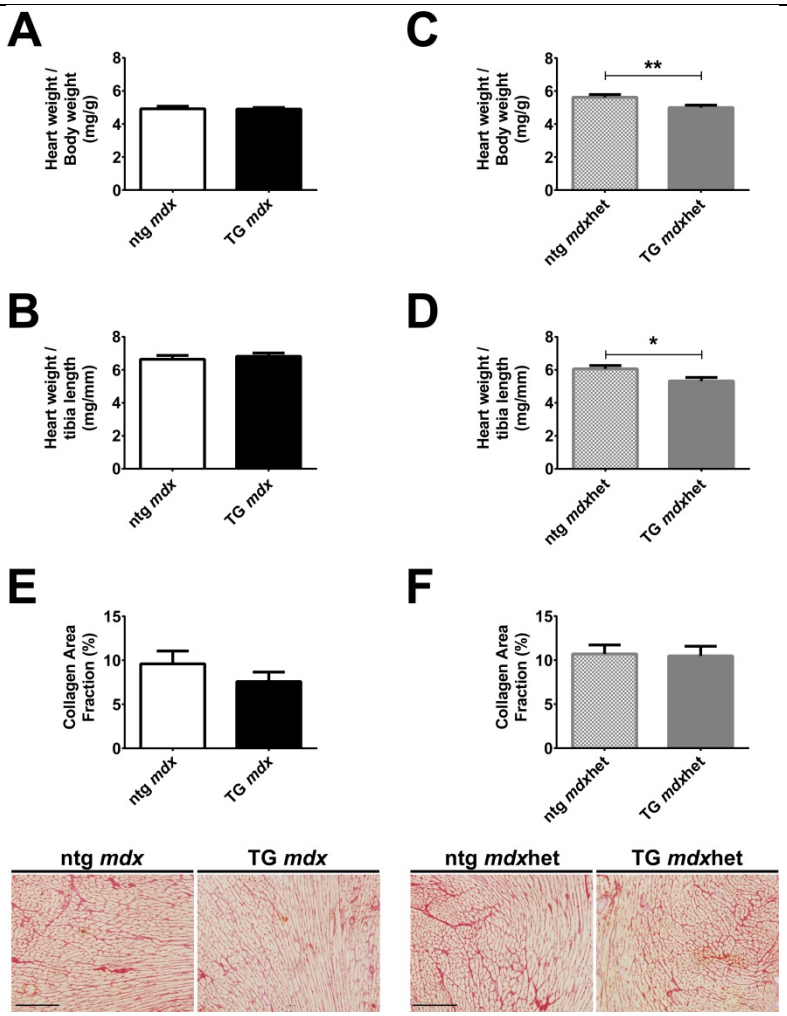

Figure 4.9. DDAH transgene expression reduces heart size in female dystrophin-heterozygous *mdx* carrier mice. The heart weight to body weight ratio (**A**) and heart weight to tibia length ratio (**B**) do not differ between non-transgenic (ntg) and DDAH-transgenic (TG) dystrophin-deficient male *mdx* mice (n=16). Both the heart weight to body weight ratio (**C**) and heart weight to tibia length ratio (**D**) are reduced in TG versus ntg female dystrophin-heterozygous *mdx* carriers (n=16) (*P<0.05, **P<0.01). (**E**) The percent area of the heart staining positive for collagen does not differ between ntg and TG male *mdx* mice (n=6) (Scale bar = 200µm). (**F**) The percent area of the heart staining positive for collagen does not differ between ntg and TG female *mdx* carrier mice (n=12) (Scale bar = 200µm).

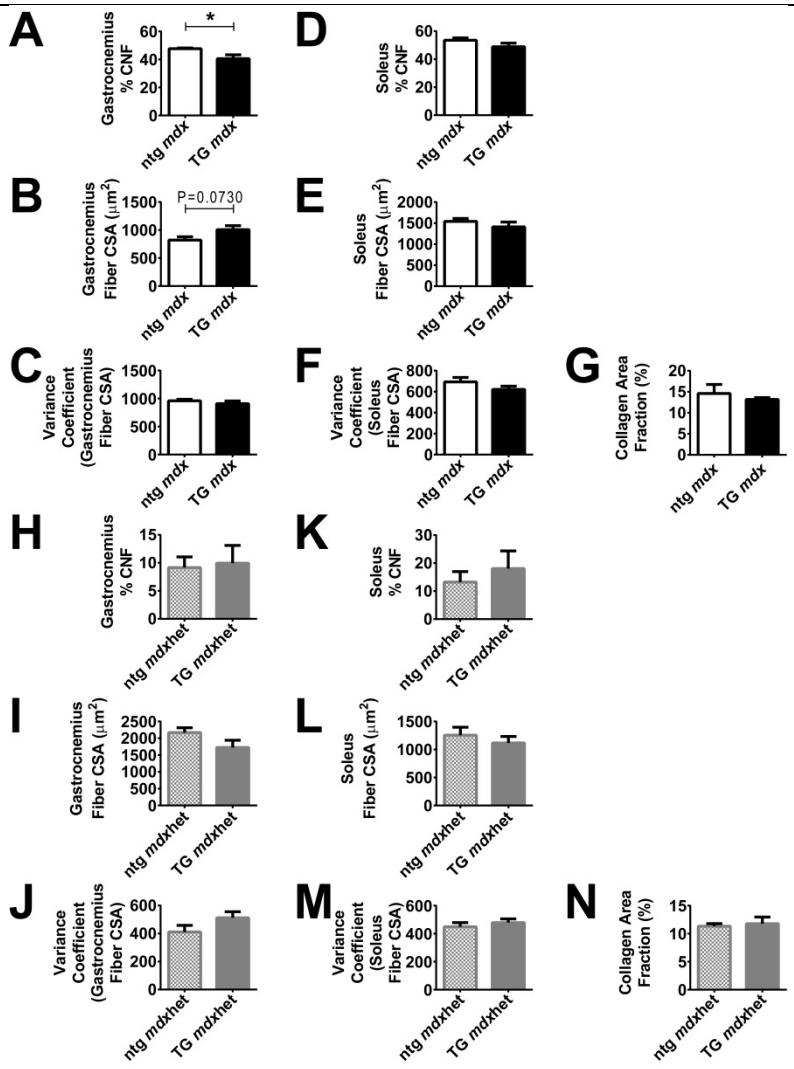

Figure 4.10. DDAH transgene expression does not alter skeletal muscle pathology in female dystrophin-heterozygous *mdx* carrier mice. (**A**) The percentage of centrally-nucleated fibers (CNF) is reduced in the gastrocnemius muscle of DDAH-transgenic (TG) versus non-transgenic (ntg) male dystrophin-deficient *mdx* mice (*P<0.05). Fiber cross-sectional area (CSA) (**B**) and fiber CSA variance (**C**) do not differ between the gastrocnemius of ntg and TG male *md*x mice. The percentage of CNF (**D**), fiber CSA (**E**), and fiber CSA variance (**F**) do not differ between the soleus muscle of ntg and TG male *mdx* mice. (**G**) The percent area of the gastrocnemius and soleus muscles staining positive for collagen does not differ between ntg and TG male *mdx* mice. The percentage of CNF, fiber CSA, and fiber CSA variance do not differ between the gastrocnemius muscle (**H-J**) or soleus muscle (**K-M**) of ntg and TG female dystrophin-heterozygous *mdx* carrier mice. (**N**) The percent area of the gastrocnemius and soleus muscles staining positive for collagen does not differ between ntg and TG female *mdx* carrier mice. (n=6).

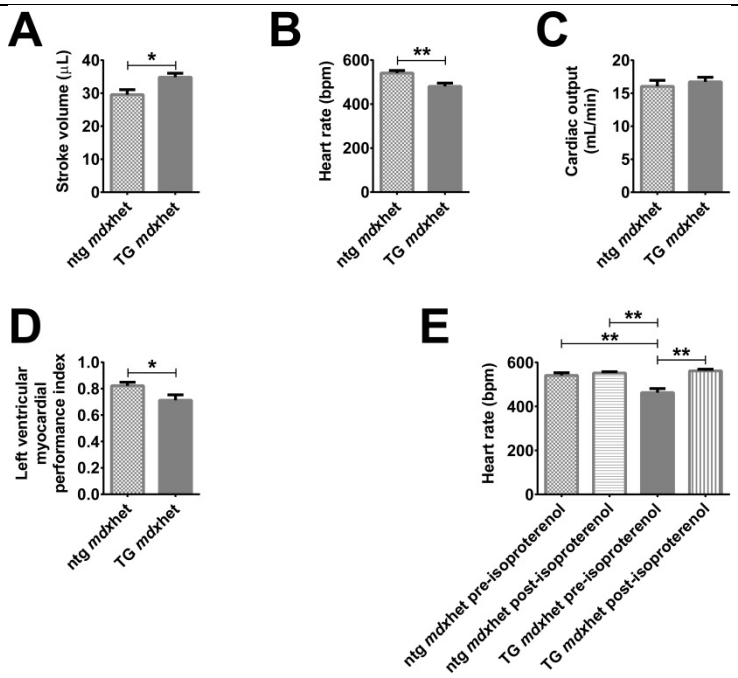

Figure 4.11. DDAH transgene expression improves contractile performance and restores cardiac functional reserve in female *mdx* carriers. (**A**) Stroke volume is increased in DDAH-transgenic (TG) compared to non-transgenic (ntg) female dystrophin-heterozygous *mdx* carriers (n=12) (*P<0.05). (**B**) Basal heart rate is reduced in TG *mdx* carriers (n=12) (**P<0.01). (**C**) Cardiac output is similar between ntg and TG *mdx* carriers (n=12). (**D**) The left ventricular myocardial performance index is reduced in TG versus ntg *mdx* carriers (n=12) (*P<0.05). (**E**) Acute isoproterenol challenge increases the heart rate of TG but not ntg *mdx* carriers (n=6) (**P<0.01).

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CHAPTER 5

Conclusions and future directions.

SUMMARY OF THESIS WORK

The most striking phenotype of the muscular dystrophies is the characteristic progressive weakening and wasting of the skeletal muscles that first caught the attention of the physicians in the mid-1800s(1, 2). A defect in muscle plasma membrane integrity was described in these and other early works, and the eventual identification of causative mutations in components of the sarcolemmal dystrophin-glycoprotein complex (DGC)(3) support a fundamental role for this structure in preventing mechanical injury of the membrane. While a primary defect in sarcolemmal stability can explain the increased susceptibility of dystrophic muscle cells to damage when subjected to mechanical stress, sarcolemmal instability does not offer a ready explanation for all the phenotypes observed in muscular dystrophy patients. For example, muscular dystrophy patients are easily fatigued by even mild physical activity such as walking that does not place undue strain on the muscles, a phenomenon noted in early case studies by Duchenne(2). The discovery that the enzyme neuronal nitric oxide synthase (nNOS) is retained near the skeletal muscle sarcolemma by interactions with the DGC, but is mislocalized to the cytosol when the DGC is disrupted and is no longer activated appropriately during exercise(4-12), suggested an additional signaling function for the complex. The loss of DGC-dependent regulation of muscle nitric oxide (NO) production is thought to contribute to impaired exercise tolerance in muscular dystrophy patients because muscle NO production is critical for the local maintenance of blood flow to the muscle during exercise(13, 14). Despite the potential relevance of this signaling pathway to disability in Duchenne muscular dystrophy

and related diseases, the molecular mechanisms by which dystrophin and the DGC regulate nNOS activity are poorly understood. Therefore, the goal of this project was to dissect the molecular pathways leading from the loss of dystrophin to impaired striated muscle nitric oxide production.

A live-cell imaging assay was developed to monitor NO production directly while wild-type and dystrophin-deficient cardiomyocytes were stretched *in vitro*. The strength of this assay was the ability to monitor acute activation of NO synthesis in combination with standard biochemical analyses in order to explore the contribution of intracellular mechanosensitive signaling pathways to the regulation of nNOS activity, and how these pathways are affected by the loss of dystrophin (Chapter 2). These experiments identified dystrophin-dependent mechanoactivation of AMP-activated protein kinase (AMPK) as an upstream regulator of mechanosensitive NO production in muscle cells. Furthermore, these studies showed that direct pharmacologic activation of AMPK could restore acute nitric oxide production in dystrophin-deficient cardiomyocytes (Chapter 2). To expand these studies, further experiments outlined in Chapter 3 showed that acute AMPK activation could activate skeletal muscle NOS activity and improve exercise tolerance *in vivo* in the dystrophin-deficient *mdx* mouse model of Duchenne muscular dystrophy (DMD). In addition to changes in intrinsic signaling cascades within individual dystrophin-deficient muscle cells, muscular dystrophy patients display dramatic alterations in the composition of the extracellular fluid that reflect the high degree of cell damage and degeneration within their striated muscle(15, 16). Chapter 4 showed the significance of elevated circulating levels of endogenous nitric oxide synthase inhibitors to impaired NO production and exercise intolerance in dystrophin-deficient *mdx* mice and dystrophin-heterozygous *mdx* carrier mice, a model for female carriers of DMD mutations. Collectively, the experiments presented in the preceding chapters demonstrated the contributions of muscle cell-intrinsic and -extrinsic alterations on impaired striated muscle nitric oxide production and exercise intolerance observed in dystrophinopathies. Importantly, these experiments have identified new therapeutic targets to improve exercise tolerance in Duchenne and potentially other DGC-related muscular dystrophies. The findings described in the previous chapters are summarized in the following sections to demonstrate how they

support the hypothesis that **both the mechanosignaling and the structural, membrane-stabilizing functions of the DGC are essential for normal striated muscle nitric oxide signaling and a normal ability to withstand exercise**. The next sections will also discuss how the relative contribution of these two mechanisms to nitric oxide synthase impairment and exercise intolerance may vary between the fully dystrophin-deficient and dystrophin-heterozygous state (Fig. 5.1). It is important to note that the relative contribution of these mechanisms to NO production and exercise phenotypes may differ between species as well, a consideration that will be important for the planning and interpretation of future pre-clinical translational studies relying on animal models of muscular dystrophy.

DGC-dependent mechano-AMPK signaling stimulates striated muscle NO production.

Chapter 2 described the consequences of dystrophin deficiency for mechanically-activated signaling pathways that regulate nNOS activity within adult mouse cardiomyocytes. Whereas *in vitro* passive stretch stimulated NO production and a downstream increase in cellular cGMP content in wild-type (WT) cells, these effects were abrogated in dystrophin-deficient *mdx* cells, suggesting that impaired nNOS function in dystrophin-deficient muscle may be a direct result of disrupted mechanotransduction. nNOS-specific inhibition partially attenuated stretch-induced NO production in WT cardiomyocytes, verifying that this particular nitric oxide synthase isoform is activated by mechanical stimulation of this tissue. Western blotting revealed that this impaired mechano-NO production in *mdx* cardiomyocytes was associated with attenuation of mechanically-activated phosphorylation of nNOS at the stimulatory residue serine 1412, along with impaired activating phosphorylation of a kinase that targets this residue, AMP-activated protein kinase (AMPK). Pharmacologic AMPK inhibition inhibited the stretch-induced NO production observed in WT cardiomyocytes, and the endogenous level of nNOS serine 1412 phosphorylation was reduced *in vivo* in *mdx* mouse hearts. Together, these findings support the idea that dystrophin-dependent mechanoactivation of AMPK contributes to the mechanically-induced stimulation of nNOS enzymatic activity, and that impaired mechanoactivation of AMPK contributes to defective regulation of nNOS activity and phosphorylation state within the beating dystrophin-deficient

heart. Neither nNOS nor the catalytic α-subunit of AMPK were specifically enriched at the sarcolemma of WT or dystrophin-deficient cardiomyocytes under basal conditions, and were not recruited to the plasma membrane during acute mechanical stretch of the cells. This work identifies AMPK as a mediator of dystrophin-dependent nNOS regulation in striated muscle, and, importantly, demonstrates that dystrophin can influence striated muscle nNOS activity even in the absence of a direct physical interaction between nNOS and the DGC. Thus, scaffolding interactions between the DGC and nNOS in skeletal muscle may primarily be important for targeting nNOS to physiologically-important sites of action, rather than for direct regulation of nNOS activity.

Acute pharmacologic stimulation of AMPK improves exercise tolerance of dystrophin-deficient mice.

The final experiments described in Chapter 2 tested whether direct pharmacologic stimulation of AMPK-nNOS signaling could stimulate nitric oxide production in dystrophin-deficient cardiomyocytes. Indeed, acute treatment of *mdx* cardiomyocytes either with the AMP-analog AICAR or with the aspirin metabolite salicylate caused increased activating phosphorylation of AMPK, increased stimulatory phosphorylation of nNOS-serine 1412, and increased nNOS-dependent cellular NO production. The magnitude of these effects were similar between wild-type and dystrophin-deficient cardiomyocytes, suggesting that apart from uncoupling AMPK activation from mechanical stimuli, the loss of dystrophin does not otherwise negatively impact the functional capacity of downstream components of the AMPK-nNOS signaling pathway in cardiac muscle.

The investigations described in Chapter 3 started by testing if pharmacologic AMPK activation could also stimulate nitric oxide synthesis in dystrophin-deficient skeletal muscle cells. In these experiments, acute salicylate treatment increased cellular NO production in isolated WT and *mdx* flexor digitorum brevis fibers, but AICAR did not stimulate fiber NO production in fibers of either genotype. Taken with the findings from the analogous experiments in cardiomyocytes, this result suggests that AMPK activation is sufficient to stimulate NO production across both types of striated muscle, but that the AMPK heterotrimers

that are expressed and that functionally target NOS likely differ in their composition of AICAR-sensitive subunits between these two types of muscle. Importantly, the observation that salicylate treatment increased NO production even in dystrophin-deficient skeletal muscle fibers suggests that nNOS within these cells can still be activated and is not completely defective as a result of the loss of nNOS-DGC binding. Thus, this finding provides further support to the notion that physical interactions between nNOS and the DGC are not strictly required for striated muscle nNOS activity. Collectively, the *in vitro* drug experiments of Chapters 2 and 3 provide foundational evidence for acute pharmacologic AMPK activation as a therapeutic strategy to increase NO production by dystrophin-deficient striated muscle.

The second part of Chapter 3 expanded upon these *in vitro* results to test the hypothesis that acute pharmacologic AMPK activation improves exercise tolerance in dystrophin-deficient *mdx* mice. Two separate models of treadmill running were first validated for their ability to detect distinct components of exercise intolerance observed in muscular dystrophy: exaggerated exercise-induced fatigue and a diminished total exercise capacity. In control experiments, treatment of *mdx* mice with the phosphodiesterase 5 (PDE5) inhibitor sildenafil, a drug that has been previously shown to increase muscle blood flow and alleviate exercise-induced fatigue in this model, attenuated the decrease in the animals' voluntary ambulatory activity following exercise. This result indicates that PDE5 inhibition does indeed alleviate post-exercise fatigue in dystrophic animals, as has been reported by others(17), and verified that the fatigue assay used in this study was sensitive enough to detect an improvement in fatigue resistance following pharmacologic intervention. In contrast, sildenafil treatment did not affect the time or distance that *mdx* mice were able to run before reaching exhaustion, suggesting that inhibiting the breakdown of cGMP and improving muscle blood flow does not significantly affect the maximal exercise capacity of dystrophin-deficient mice. Acute treatment of *mdx* mice with pharmacologic AMPK activators yielded effects that were distinct from those of PDE5 inhibition. Both AICAR and salicylate increased the time and distance that *mdx* mice ran before reaching exhaustion, demonstrating that AMPK activation increases the exercise capacity of dystrophin-deficient animals. These data represent intriguing proof-of-concept evidence for the

therapeutic potential of acute pharmacologic AMPK activation to improve exercise tolerance in Duchenne muscular dystrophy.

Endogenous nitric oxide inhibitors released following sarcolemmal injury of dystrophin-deficient cells may contribute to exercise intolerance in subjects with mosaic dystrophin expression.

Finally, Chapter 4 discusses experiments designed to understand the contribution of endogenous nitric oxide synthase inhibitors to impaired exercise tolerance in DMD. Initial studies showed that circulating concentrations of the methylated arginines asymmetric dimethylarginine (ADMA) and monomethylarginine (L-NMMA) are elevated in dystrophin-deficient *mdx* mice, a finding consistent with the observation of excessive urinary ADMA concentrations in human muscular dystrophy patients(18, 19). Induction of acute degenerative muscle injury with cardiotoxin increased circulating methylated arginine concentrations in wild-type mice, and infusion of exogenous ADMA into WT mice was sufficient to impair exercise performance on voluntary running wheels. Collectively, these findings suggest that the elevated circulating methylated arginine concentrations in DMD results from the increased amount of cell damage and necrotic cell death in dystrophin-deficient muscle, and that excessive ADMA may contribute to reduced exercise tolerance in dystrophin-deficient subjects. Therefore, dystrophin-deficient male *mdx* and dystrophin-heterozygous female *mdx* carrier mice expressing a transgene encoding dimethylarginine dimethylaminohydrolase (DDAH), the enzyme that degrades ADMA, were generated in order to test the effects of excessive ADMA on exercise tolerance in DMD and DMD carriers.

DDAH transgene expression attenuated the drop in voluntary ambulatory activity following treadmill exercise in dystrophin-heterozygous female mice, but not in dystrophin-deficient male mice. The beneficial effect of DDAH transgene expression on the exercise phenotype of female *mdx* carriers was associated with reduced heart mass, a lower resting heart rate, and an increased stroke volume in transgenic *mdx* carriers. Echocardiography revealed that DDAH transgene expression lowered the left ventricular myocardial performance index in female *mdx* carriers, indicating that it improved the heart's systolic and/or diastolic

function. Finally, the chronotropic response to acute β-adrenergic stimulation was lost in female *mdx* carriers, but was restored with expression of the DDAH transgene. Together, these data support the idea that endogenous methylated arginines promote pathological remodeling and impair the function of the dystrophin-heterozygous heart. They further suggest that dystrophin-heterozygous subjects adapt to impaired contractile function with a compensatory increase in basal heart rate. A diminished chronotropic reserve may mean that dystrophin-heterozygous hearts have less functional reserve available to increase cardiac output during exercise, possibly contributing to exercise-induced fatigue in *mdx* carriers.

The findings presented in this thesis expand our understanding of the mechanisms by which disruption of the dystrophin-glycoprotein complex alters physiologically-relevant signaling pathways within striated muscle and thus may contribute to disability in the muscular dystrophies. Because these results support a role for both the signaling and structural functions of the DGC in the regulation of muscle nitric oxide production and exercise performance, they have important implications for both for muscular dystrophies in which sarcolemmal integrity is compromised, as well in instances where DGC-dependent signaling is perturbed even though sarcolemmal stability is maintained(9, 10, 20). The implications of these findings and their relevance to muscular dystrophy therapies will be addressed in more depth in the remainder of this chapter. The rest of the chapter will also outline future experiments to determine the mechanisms by which the DGC regulates AMPK activity, as well as the role of ADMA in cardiac remodeling.

IMPLICATIONS AND FUTURE DIRECTIONS

Towards an integrated model for the mechanosignaling and structural functions of the dystrophin-glycoprotein complex in the regulation of striated muscle nitric oxide production.

The dystrophin-glycoprotein complex is critical for the protection of the sarcolemma from mechanical injury during muscle contraction. This membrane-stabilizing function of the DGC is mediated by its structural linkage of the muscle cell cytoskeleton and the extracellular

matrix, and disruption of this physical connection between the interior and exterior of the muscle cell is a primary factor contributing to muscle cell injury, muscle death, and the progressive weakening and disability that is seen in many forms of muscular dystrophy. Recent studies have suggested an additional role for the DGC in regulating cell signaling(4, 5, 21-31), although how disruption of this signaling capacity affects muscle function and contributes to the overall disease phenotype in muscular dystrophy patients is not fully understood. Considered together, the results discussed above support a model in which both the signaling and structural functions of the DGC have important consequences for the mechanical regulation of nitric oxide production by striated muscle cells (Fig. 5.1).

The findings from Chapter 2 indicate that the loss of dystrophin disrupts mechanically-induced activation of AMPK-nNOS signaling, resulting in impaired mechanical activation of cellular NO production. These data demonstrate that a direct function for dystrophin and the DGC, possibly as a mechanotransducer, is critical for the activation of intrinsic, biochemical signaling cascades that stimulate the enzymatic activity of nNOS. It is important to note that for technical reasons relating to the ease with which they can be passively stretched in bulk in tissue culture systems, these experiments were conducted using cardiomyocytes as a general model for striated muscle mechano-NO signaling. Thus, it remains to be determined whether DGC-dependent activation of AMPK similarly regulates nNOS activity within skeletal muscle. Previous investigations have shown that skeletal muscle contraction is associated with the concurrent activation of nNOS and AMPK(32); that genetic disruption of AMPK activity is associated with reduced nNOS phosphorylation *in vivo*(33); and that pharmacologic stimulation of AMPK with AICAR is sufficient to stimulate nNOS phosphorylation and NO production in skeletal muscle myotubes *in vitro*(33, 34). These data suggest that AMPK does play some role in the regulation of nNOS activity in skeletal muscle. Future studies could modify the approach used in Chapter 2 and use electrical field stimulation to stimulate contraction or carbon micro-fibers(35, 36) to stretch isolated mature skeletal muscle fibers *in vitro* in combination with genetic knockout or pharmacologic inhibition of AMPK in order to determine the specific contribution of AMPK to mechanically-activated NO production in this tissue.

The research presented in Chapter 4 indicates that the structural function of the DGC to stabilize the sarcolemma may also play an indirect role in regulating striated muscle NO production. The finding that acute degenerative muscle injury with cardiotoxin was sufficient to cause elevated circulating levels of methylated arginines in wild-type mice suggests that in dystrophin-deficient *mdx* mice and DMD patients, elevated circulating levels of the endogenous NOS inhibitors are a direct result of increased sarcolemmal instability and necrosis of injured dystrophin-deficient muscle cells. In the model proposed here, the lack of an effect of DDAH expression and subsequent lowering of ADMA levels in male *mdx* mice to significantly alter exercise-induced fatigue, a phenotype that is related to impaired striated muscle NO production, can be explained by the observation from Chapter 2 that the normal activation of muscle nNOS activity is already disrupted due to the impairment of mechanosensitive AMPK signaling within dystrophin-deficient muscle cells (Fig. 5.1 A and B). In other words, relieving any ADMA-dependent inhibition of nNOS may do little to increase striated muscle NO production in dystrophin-deficient subjects, because this process is still impaired due to cell-intrinsic defects in DGC-dependent mechanosignaling. Because monomethylarginine (L-NMMA) can also inhibit NOS(37) and its circulating concentration was elevated in dystrophin-deficient animals but not significantly reduced by DDAH transgene expression in the studies described in Chapter 4, it is also possible that this compound exerts an additional layer of nNOS inhibition that could help to explain the lack of improved NO production and fatigue resistance in DDAH-transgenic dystrophin-deficient animals.

Furthermore, the proposed model would predict that in dystrophin-deficient subjects, *in vivo* pharmacologic stimulation of AMPK in an attempt to stimulate NOS activity within the striated muscle may ultimately do little on its own to increase cellular NO production because nNOS would still be subject to inhibition by excessive methylated arginines. Indeed, attenuated nNOS activity as combined result of impaired mechanosignaling and inhibition by methylated arginines could help to explain why cellular NO production is only slightly increased (~0.2-fold) in nNOS-transgenic dystrophin-deficient skeletal and cardiac muscle despite robust overexpression of nNOS in these tissues(38, 39). Experiments measuring indices of *in vivo* muscle cell NO production, like protein nitrosylation or tissue nitrite + nitrate (NO_x) content, in

mdx mice treated with AMPK-activating drugs that stimulate nNOS *in vitro* could shed light on whether nNOS inhibition by excessive methylated arginines may override normal endogenous stimulation of muscle NO production. Another way to test this question could be to use *in situ* fluorescence imaging of *mdx* skeletal muscles loaded with NO-sensitive dyes in order to measure their NO production following acute administration of AMPK-activating drugs. If elevated concentrations of methylated arginines do inhibit NOS in intact dystrophin-deficient striated muscle, pharmacologic AMPK activation should fail to stimulate muscle NO production in non-transgenic *mdx* mice. However, pharmacologic AMPK activation may be sufficient to stimulate muscle NO production in DDAH-transgenic *mdx* mice in which any NOS inhibition by AMDA should be minimized. Studies like these would also help to clarify whether the beneficial effects of acute pharmacologic AMPK activation on the exercise tolerance of dystrophin-deficient animals are mediated by increased striated muscle NO production.

A model incorporating both the cell-intrinsic functions of the DGC in mechanosignaling and its structural role to prevent membrane damage and the excessive release of methylated arginines to the extracellular fluid on striated muscle NO production also has interesting implications for cases of mosaic dystrophin expression. This pattern of expression occurs in the cardiac muscle of female DMD carriers, and could also arise in male DMD patients treated with gene-delivery, exon skipping, or stem-cell based therapies that may restore dystrophin expression to only a subset of skeletal muscle fibers or cardiomyocytes(40-43). In these instances, populations of dystrophin-positive or wild-type cells and dystrophin-mutant or -null cells would be distributed throughout the muscle tissue (Fig. 5.1C). In dystrophin-positive cells, intact mechano-DGC-AMPK signaling would allow for normal phosphorylation of nNOS by AMPK under periods of increased mechanical stress. However, when placed under mechanical stress, the population of dystrophin-null cells would be susceptible to sarcolemmal lesion and subsequent necrotic cell death, resulting in the local release of methylated arginines. These compounds could then inhibit NO production by nNOS in neighboring dystrophin-positive cells, despite intact mechanically-regulated phosphorylation of nNOS by AMPK within that population (Fig. 5.1C). This model may explain why DDAH transgene expression had a beneficial effect in the hearts of *mdx* carriers: in non-transgenic carriers, there were dystrophin-positive

cardiomyocytes with intact mechano-AMPK signaling, but whose nNOS activity was inhibited by ADMA released from nearby damaged, dystrophin-null cardiomyocytes. Expression of the DDAH transgene would likely have reduced ADMA concentration in the local microenvironment, thus relieving nNOS inhibition and restoring the capacity for NO production in dystrophin-expressing cells, ultimately leading to positive effects on cardiac function. The unexpected finding that *mdx* and *mdx* carrier mice show increased urinary NO_x levels consistent with increased total body iNOS activity suggests that any effects of extracellular ADMA to inhibit muscle nNOS likely occur on a very localized level within muscle tissue, with NOS inhibition by ADMA being the most pronounced within intact cells in the immediate vicinity of degenerating cells (Fig. 5.1C). Thus, the structural function of the DGC to stabilize the plasma membrane seems to play an important, indirect role in maintaining a local extracellular milieu that is permissive for striated muscle nitric oxide signaling.

The proposed model predicts that nNOS activity would be impaired in both dystrophin-positive and dystrophin-null cell populations within mosaic muscle, albeit by distinct mechanisms. Accordingly, nNOS activity in muscle with mosaic dystrophin expression should be impaired as it is in fully dystrophin-deficient muscle, although the extent of impairment would likely vary with the degree of muscle damage and local methylated arginine release, and thus may be more pronounced following physical activity involving eccentric exercise. While restoration of dystrophin expression can restore nNOS sarcolemmal localization in DMD skeletal muscle fibers(41, 44), the effect of mosaic dystrophin expression on NO production in the hearts of DMD carriers or in partially-corrected DMD skeletal muscle has not yet been reported in the literature. Therefore, it would be interesting for future investigations to explore the effect of mosaic dystrophin expression on muscle nNOS activity. Mechanosensitive NO production in dystrophin-mosaic tissue could be assessed by increasing the frequency of electrical pacing of working hearts isolated from dystrophin-heterozygous female *mdx* carrier mice or by stimulating contraction *in situ* in skeletal muscles of wild-type/*mdx* chimeric mice generated by the injection of wild-type embryonic stem cells into *mdx* blastocysts(45). If both dystrophin-positive and dystrophin-null cells in these dystrophin-mosaic tissues have impaired NO production due to the combined effects of disrupted mechanosignaling and inhibition of

nNOS by the local release of ADMA from damaged cells, then the total mechanosensitive NO production by these intact preparations should be reduced compared to wild-type to an extent approaching the degree of impairment observed in fully dystrophin-deficient muscle. Critically, if the proposed model is correct, *in vitro* examination of heterogeneous populations of enzymatically dissociated cardiomyocytes or skeletal muscle fibers that are surrounded by a relatively large volume of media should minimize the local accumulation of methylated arginines at high concentrations surrounding any damaged cells. In this setup, measurement of cellular NO production during mechanical stimulation by passive stretch or electrical field stimulation of contraction should, in contrast to studies of intact muscle tissue where methylated arginines can accumulate locally, reveal discrete populations of cells that respond to mechanical perturbation with increased NO production and cells that do not respond to mechanical perturbation with increased NO production. Fixing the cells and performing immunofluorescence staining for dystrophin expression following completion of the mechanical stimulation protocol could verify that the mechano-responsive population was indeed comprised of dystrophin-positive cells, and that the non-responsive population contained the dystrophin-null cells.

The mechanical regulation of AMPK by the dystrophin-glycoprotein complex.

In addition to direct allosteric regulation by adenine nucleotides and glycogen that allow AMPK to sense cellular energy and nutrient status(46-48), several studies suggest that AMPK is also subject to regulation by the mechanical activity of a given cell. Experiments in contracting skeletal muscle or beating hearts support the idea for mechanical activation of AMPK that can even be graded according to the mechanical load against which the tissue must work(32, 49). However, interpretation of the direct consequences of mechanical stimulation as opposed to metabolic stress on AMPK activation in this context is confounded by the potential for increased contractile activity of muscle to accelerate cellular turnover of ATP. Models of passive muscle stretch, which impose less metabolic demand than muscle contraction, indicate that this mode of mechanical stimulation also activates AMPK(50, 51), supporting the idea that there may be unique mechanisms for mechanical regulation of this enzyme separate from its

metabolic regulation. The activation of AMPK by passive stretch also occurs in non-muscle tissue, and in lung epithelial cells has been shown to depend on the expression of dystroglycan, a core component of the dystrophin-glycoprotein complex within muscle(52). Taken with the findings presented in Chapter 2, this observation supports a role for the DGC in the mechanical regulation of AMPK.

How, then, might the DGC regulate mechanically-induced AMPK activation? A number of targets known to regulate AMPK activity across a wide range of tissues could be activated downstream of the intact DGC and couple DGC-dependent mechanosensation to the stimulation of striated muscle AMPK activity (Fig. 5.2). The upstream kinases liver kinase B1 (LKB1) and Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ) phosphorylate the catalytic alpha subunit of AMPK at threonine 172 and thereby increase its enzymatic activity(53-57). Whether either of these two kinases is specifically activated by mechanical stimulation, and whether dystrophin plays any direct role in this process, remains to be determined. However, since CaMKKβ can be activated by calcium, the cytosolic levels of which are increased by muscle contraction or stretch(58-60), this protein is a promising candidate for mechanically-induced AMPK activation. The phosphatases PP2A and PP2C oppose the actions of LKB1 and CaMKKβ and dephosphorylate AMPKα-Thr172(61-63), and thus represent another signaling pathway regulating AMPK activity. The observation that PP2A activity is increased by calcium(61) could help to explain why increased cytosolic calcium flux with stretch or eccentric contraction of dystrophin-deficient muscle cells(36, 64) is not associated with increased AMPK activation, as might be expected based on the ability for calcium to stimulate CaMKKβ. In this scenario, dystrophin and the DGC may serve a permissive rather than a direct role in AMPK regulation, by preventing stretch-induced calcium overload and excessive phosphatase activity in the muscle cell. Activation of phosphatases in dystrophin-deficient muscle cells that occurs specifically under periods of mechanical stress may also help to explain why application of AICAR or salicylate to non-stretched *mdx* cardiomyocytes increased AMPKα-Thr172 phosphorylation to an extent similar to that observed in treated WT cardiomyocytes. Alternatively, the discrepancy between the efficacy of mechanical versus AICAR- or salicylate-induced AMPKα-Thr172 phosphorylation and stimulation of kinase activity in dystrophin-

deficient cells may reflect distinct roles for the different upstream AMPK kinases in phosphorylating AMPK under these different conditions.

In addition to stimulatory phosphorylation of AMPKα-Thr172, a number of other post-translational modifications of AMPK may be mechanically regulated and altered by dystrophin expression. Phosphorylation of AMPKα-Thr479 by glycogen synthase kinase (GSK) increases the access of phosphatases to AMPKα-Thr172, thus favoring Thr172 dephosphorylation and tending to decrease AMPK activity(65). GSK's kinase activity towards AMPK can be activated by AKT, which can also phosphorylate AMPKα-Ser485 leading to reduced Thr172 phosphorylation(65, 66). Notably, the mechanical activation of AKT is increased in dystrophin-deficient muscle(23). This effect may be mediated by increased mechanosensitive production of reactive oxygen species (ROS) in the absence of dystrophin(58), as increased ROS can activate AKT(67). Thus, a second permissive role for the DGC in the appropriate mechanoregulation of AMPK may be the prevention of excessive ROS generation and subsequent activation of AKT and GSK during periods of mechanical stress. Furthermore, excess mechano-ROS production could also oppose mechano-AMPK activation in dystrophin-deficient muscle cells directly via the inhibitory oxidation of cysteine residues in the alpha subunits of AMPK(68).

Additional levels of allosteric control may also play direct or permissive roles in DGC-dependent AMPK regulation. Glycogen inhibits AMPK through interaction with the glycogen-binding domain of the beta subunits(69). Increased muscle glycogen content reported in dystrophin-deficient *mdx* mice(70) and in some human DMD patients(71) may therefore attenuate any mechanically-induced activation of AMPK. The cytoskeleton of dystrophin-deficient muscle cells could also affect the allosteric regulation of AMPK. The cytoskeletal protein plectin binds to AMPKγ1 subunits in differentiated muscle and has been shown to reduce the AMP-sensitive activity of γ1-containing AMPK heterotrimers, possibly by stabilizing the expression of the less responsive α1 subunit over the more responsive α2 subunit(72). Several reports indicate that plectin expression is increased in dystrophin-deficient skeletal muscle(73, 74), and, consistent with this model, a recent study demonstrated a decrease in AMPKα2 expression in the gastrocnemius muscle of dystrophin-deficient dogs(75). Thus, alteration of the cytoskeleton in dystrophin-deficient muscle cells may influence the subunit

composition of AMPK heterotrimers in a manner that ultimately reduces the sensitivity of their kinase activity to regulation by normal allosteric regulators, such as increased cellular AMP content during stretch or contraction.

Distinct from such a permissive role of the DGC in allowing mechanosensitive AMPK activation is the particularly intriguing idea that dystrophin or the DGC directly binds to and mechanically regulates conformational changes in the regulatory γ- or β-AMPK subunits, which are then translated to the α-subunit to favor the phosphorylation of AMPKα-Thr172 and promote AMPK kinase activity. A recent study demonstrated that inhibition of contraction with myosin inhibitors attenuated the increase in phosphorylation of AMPKα-Thr172 in electrically-stimulated isolated muscles(76). This observation could be explained either by attenuation of the mechanical forces normally exerted on the sarcolemma by contraction of the muscle, resulting in less mechanical stimulation of DGC-dependent signaling; or by the fact that inhibiting cross-bridge cycling will result in reduced cellular consumption of ATP, thus placing less metabolic stress on the electrically-stimulated muscle cells. Interestingly, though, the attenuation of AMPKα phosphorylation was associated with a specific and potent inhibition of the kinase activity of AMPK heterotrimers composed of α2β2γ3 subunits. The authors also reported a distinct increase in the kinase activity of AMPK heterotrimers containing γ1 subunits, which presumably was correlated to the observed net increase in AMPKα phosphorylation in stimulated muscles(76). The different AMPK γ-subunits do differ in their overall sensitivity to regulation by adenine nucleotides, with AMPK trimers containing the γ-3 subunit being the least sensitive to activation by AMP(77).The observations by Jensen et al.(76) would further indicate that the γ-AMPK subunits also differ in their sensitivity to mechanical stimulation, suggesting that different AMPK subunits may play specific roles in distinct metabolically- or mechanically-regulated signaling pathways. While striated muscle expresses a variety of AMPK subunits (Fig. 5.2), whether any of the γ-AMPK subunits, or even the β-AMPK subunits, are specifically associated with the DGC in skeletal or cardiac muscle, and whether disruption of the DGC affects their function, is currently unknown.

Answering this question would help to clarify the mechanism by which loss of dystrophin-dependent mechanosensation perturbs striated muscle AMPK signaling. Studies to

this end could use cardiomyocytes or skeletal muscle fibers from mice with subunit-specific knockout of AMPK, starting with γ3(78). In such experiments, it would be important to quantitatively assess how the loss of specific individual AMPK subunits affects stretch-induced increases in AMPK activation, measured by stimulatory phosphorylation of AMPKα-Thr172 in addition to AMPK kinase activity, as indicated by phosphorylation of validated endogenous AMPK targets like acetyl-CoA carboxylase within cells or *in vitro* phosphorylation of the artificial SAMS peptide by AMPK heterotrimers isolated from mechanically-stimulated muscle cells or tissue. It would also be interesting to monitor changes in cellular respiration and ATP content under these conditions, in order to understand how specific AMPK subunits contribute to metabolic responses to mechanical stress. This general approach could also be modified to measure mechanically-induced nNOS activation in order to identify the subunit composition of AMPK trimeric complexes that participate in the mechanical regulation of nNOS. Finally, the effect of dystrophin deficiency on the localization of AMPK heterotrimers of different subunit composition could be assessed using immunofluorescence labelling of wild-type and *mdx* muscle fibers and cardiomyocytes, and its effect on mechanoactivation of different AMPK heterotrimers could be measured biochemically following immunoprecipitation of specific AMPK complexes from lysates of stretched wild-type and *mdx* cells. If these experiments provided evidence of direct, dystrophin-dependent mechanical regulation of a particular subset of AMPK subunits, they could help establish a role for dystrophin and the DGC in the coupling of mechanosensation and metabolic regulation within striated muscle.

ADMA and cardiac hypertrophy in DMD carriers.

Increased circulating levels of asymmetric dimethylarginine have been correlated with cardiovascular disease and cardiac hypertrophy for some time(79, 80). However, since excessive ADMA inhibits endothelial cell nitric oxide production and can increase blood pressure(81, 82), it is difficult to know whether the association between ADMA levels and cardiac hypertrophy is a direct result of ADMA actions within the cardiomyocytes, or rather is a secondary consequence of hypertension. As DDAH transgene expression was associated with reduced cardiac size and improved cardiac performance in dystrophin-heterozygous *mdx* carrier

mice in Chapter 4, a deeper understanding of the mechanisms by which ADMA affects cardiac function and remodeling could have important implications for attempts to mitigate the risk of cardiomyopathy in carriers of DMD mutations. A recent study reported elevated systolic blood pressure in Duchenne muscular dystrophy carriers who exhibit late gadolinium enhancement on cardiac MRI, indicative of increased cardiac fibrosis(83). This association between cardiac fibrosis and increased blood pressure could perhaps reflect increased angiotensin II signaling in DMD carriers(84), but an interesting alternative explanation is that the DMD carriers who present with the most cardiac fibrosis are those who have had the most focal cardiomyocyte damage and therefore the greatest release of methylated arginines from dying cells. This excessive methylated arginine release from the heart could impair NO production in the vascular endothelium and lead to elevated blood pressure that may exacerbate cardiac remodeling. It could also have direct effects on cardiac function by altering nitic oxide signaling within dystrophin-positive cardiomyocytes (Fig. 5.1C).

The results presented in Chapter 4 support the notion that local methylated arginine signaling affects cardiac remodeling and heart function. However, whether excessive methylated arginine levels directly affect cardiomyocyte remodeling or contractile function, and the interplay between cellular remodeling and contractile function *in vivo*, remains unclear. The observation that ADMA can stimulate hypertrophic growth of cultured adipocytes(85) supports the idea that this compound may promote cardiac hypertrophy, even independent of effects on hemodynamic load. The hypothesis that local elevation of ADMA has direct effects on both hypertrophic remodeling and the contractile function of cardiomyocytes could be tested using *in vitro* approaches. For instance, isolated rat cardiomyocytes or human induced pluripotent stem cell-derived cardiomyocytes could be maintained in culture in media with or without exogenous ADMA, and after several days the cardiomyocyte size and protein content of these two groups could be compared. If these initial experiments suggested an effect of ADMA to promote cell hypertrophy, gene expression could be assessed using microarrays in order to identify candidate genes responsible for this effect. Furthermore, comparison of changes in gene expression between wild-type cardiomyocytes treated with ADMA versus alternative nitric oxide synthase inhibitors, or between ADMA-treated wild-type and ADMA-treated NOS-

deficient cardiomyocytes, could help to distinguish any NO-dependent versus NO-independent effects of ADMA(86) on hypertrophic gene expression. To establish a causative role for any identified genes in ADMA-induced hypertrophy, candidate genes could be individually disrupted in cultured cells using the CRISPR/Cas9 system(87). The ability of sustained ADMA exposure to induce hypertrophy could then be compared in modified cells and unmodified controls.

The contractile function of isolated cardiomyocytes treated acutely with ADMA *in vitro* could also be assessed using measurements of sarcomere shortening and calcium transients in electrically-stimulated cells(88). Such experiments could indicate whether ADMA has direct effects on cardiac function that are independent of changes in cardiac remodeling. Assessment of effects of acute ADMA exposure on mouse nNOS- or eNOS- knockout cardiomyocytes(89-91) in these studies could clarify whether any effects of ADMA on contractile function depend on its function as a NO synthase inhibitor, or might act through NO-independent mechanisms.

DGC-dependent regulation of muscle nitric oxide production: therapeutic implications.

The restoration of NO/cGMP signaling in dystrophin-deficient striated muscle has received considerable attention as a therapeutic goal for the improvement of muscle blood flow during exercise, cardiac function, and exercise tolerance in Duchenne and Becker muscular dystrophy. Although phosphodiesterase 5 inhibition, currently the only pharmacologic strategy available for this purpose, yielded promising results in studies of *mdx* mice(17, 92), translation to human patients so far has had only limited success(93-96). The *in vitro* data presented in Chapters 2 and 3 of this thesis suggest that an alternative strategy, direct pharmacologic stimulation of AMPK activity, is effective at stimulating nNOS activity and boosting NO production in dystrophin-deficient striated muscle. This represents the first pharmacologic approach that has the potential to restore both the cGMP-dependent and cGMP-independent downstream effects of nNOS within dystrophic muscle. The demonstration that pharmacologic AMPK activation can stimulate NO production in dystrophin-deficient striated muscle also offers a new mechanistic explanation for how chronic treatment with AMPK activators improved muscle pathology in *mdx* mice in previous pre-clinical studies(97-99). Indeed, several of the improved phenotypes in these studies, including reduced muscle fiber damage and

improved force generation(97-99), could be explained by improved perfusion of the dystrophin-deficient muscle with increased local NO signaling or by improved NO-dependent control of calcium handling within the muscle fibers(100-102). The *in vivo* experiments discussed in Chapter 3 further indicate that pharmacologic AMPK activation has acute beneficial effects on exercise capacity in the *mdx* mouse model of Duchenne muscular dystrophy. This work represents an important advance from earlier translational studies in which chronic AMPK activation showed little long-acting improvements in the exercise performance of dystrophin-deficient animals(103). However, the overall model for the regulation of striated muscle NO production supported by the work in Chapters 2 and 4 of this thesis (Fig. 5.1) raises a puzzling question: if nNOS activity in dystrophin-deficient muscle is still subject to inhibition by elevated levels of endogenous methylated arginines, how does acute treatment with AICAR or salicylate improve exercise tolerance in dystrophin-deficient mice?

The discrepancy between the *in vitro* findings of Chapters 2 and 3 and the predictions of the model likely reflect the fact that in *in vitro* experiments, cardiomyocytes and skeletal muscle fibers are dissociated from neighboring cells and surrounded by a volume of media that is infinitely greater than that of the extracellular fluid immediately surrounding each cell *in vivo.* Thus, any methylated arginines released by damaged muscle cells in culture are likely diluted to a concentration much lower than in the microenvironment surrounding a necrotic cell in intact tissue. This would tend to minimize any inhibitory effect of methylated arginines on nNOS activity, allowing for AMPK-stimulated cellular NO production when dystrophin-deficient cells are treated with AICAR or salicylate *in vitro*. If correct, this reasoning would imply that a substantial component of the effect of acute pharmacologic AMPK activation on the exercise capacity of *mdx* mice is attributable to AMPK targets other than striated muscle nNOS, because nNOS would still be subject to inhibition by methylated arginines in intact dystrophin-deficient muscle. Generation of dystrophin-deficient mice lacking nNOS specifically within the heart and skeletal muscle would allow for direct testing of whether the beneficial effects of pharmacologic AMPK activation on exercise capacity require striated muscle nNOS activation. Interestingly, improved muscle blood flow may still contribute to the observed effects of AMPK activation on exercise, even independent of increased muscle nNOS activity, since eNOS is a

known AMPK target and has been shown to function in sympatholysis(14, 104). A contribution of vascular eNOS to sympatholysis may help to reconcile the reported benefits of PDE5 inhibition on muscle blood flow and exercise tolerance in *mdx* mice and Duchenne/Becker muscular dystrophy patients and the effects of AMPK activation on exercise tolerance in *mdx* mice reported here with the proposed working model's inhibition of muscle nNOS activity in dystrophin-deficient muscle. Thus, the findings of this thesis still support the therapeutic potential for acute AMPK activation to improve exercise tolerance in Duchenne muscular dystrophy, although it may ultimately do so through mechanisms independent of the restoration of striated muscle nNOS activity.

CONCLUSIONS

The ability for striated muscle to withstand the mechanical and metabolic stress of contraction requires endogenous mechanisms to protect the integrity of the sarcolemma, and to sense changes in mechanical activity and couple them to the regulation of local oxygen and nutrient delivery. Both of these mechanisms can be disrupted in muscular dystrophy, resulting in progressive loss of muscle cells as well as impaired vasoregulation within the muscle tissue. The goal of this thesis was to understand the mechanisms by which dystrophin and the dystrophin-glycoprotein complex, a structure that is essential for the maintenance of sarcolemmal integrity, regulate striated muscle nitric oxide production, a phenomenon that is critical for contraction-induced regulation of the muscle vasculature.

The findings presented in this thesis demonstrate that dystrophin expression is required for the normal mechanical activation of AMPK and nNOS within cardiomyocytes, and that direct pharmacologic stimulation of AMPK activity can rescue nitric oxide production in dystrophin-deficient striated muscle cells *in vitro* and improve exercise tolerance in dystrophin-deficient mice. Additional work suggests that methylated arginines released following sarcolemmal injury of dystrophin-deficient muscle cells also impair muscle nNOS activity, an effect whose functional consequences are best appreciated in muscle with mosaic dystrophin expression. Collectively, these findings support a model in which both the structural and signaling functions

of the DGC are indispensable for the normal mechanical regulation of striated muscle NO production. As nitric oxide production is critical for the contractile function of the striated muscle and is necessary for its functional adaptations to increased work demands during exercise, the model proposed here will be useful in guiding the development of new therapies to improve exercise performance and tolerance in Duchenne muscular dystrophy and related muscle diseases.

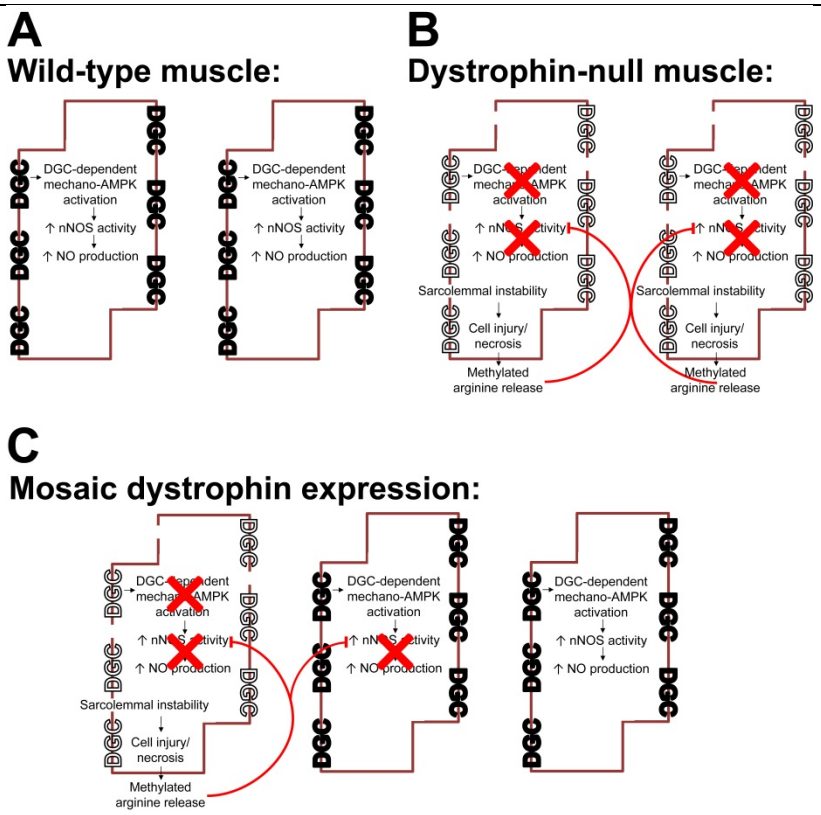
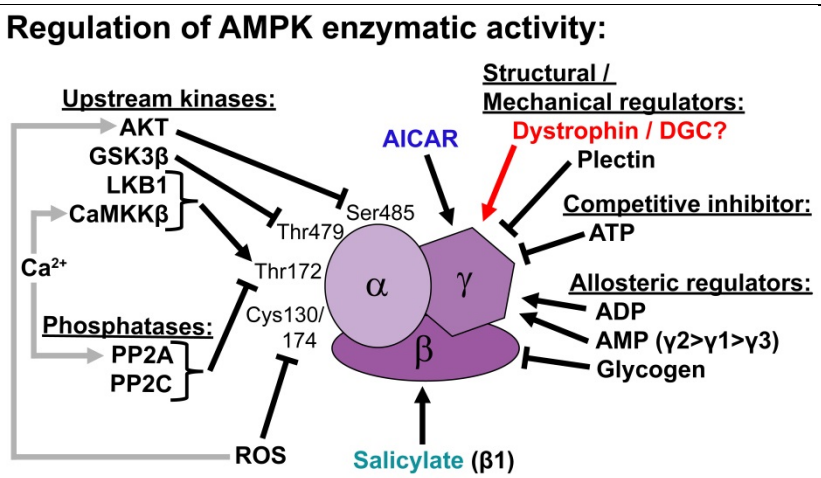


Figure 5.1. Proposed model integrating the signaling and structural functions of the dystrophin-glycoprotein complex in the mechanical regulation of striated muscle nitric oxide production. (**A**) In wild-type striated muscle, the dystrophin-glycoprotein complex is intact (indicated by filled text) and maintains the structural integrity of the sarcolemma while also mediating the mechanical activation of AMPK-nNOS signaling that allows cells to respond to mechanical stress with an increase in nitric oxide (NO) production. (**B**) In dystrophin-deficient muscle, the DGC is disrupted (indicated by open text). This results in loss of DGC-dependent mechanoactivation of AMPK and subsequent loss of the ability for cells to increase nNOS activity in response to mechanical stress. DGC disruption also results in sarcolemmal instability, leading to increased injury of the muscle cells under periods of mechanical stress, and the release of methylated arginines such as ADMA from necrotic muscle cells. Locally-elevated concentrations of methylated arginines can further inhibit nNOS activity within the muscle cells, distinct from disruptions in AMPK signaling. (**C**) In muscle displaying mosaic dystrophin expression, both dystrophin-deficient and dystrophin-positive cells exist side by side. In this instance, methylated arginines released from damaged, dystrophin-deficient cells may inhibit nNOS activity in neighboring dystrophin-positive cells, even though DGC-dependent mechanoregulation of AMPK remains intact within this population. Inhibitory effects of methylated arginines released by necrotic muscle cells may be spatially confined and have little influence on NO production in distant dystrophin-positive cells. While this model is largely informed by experiments conducted in cardiac muscle, it may hold true for the regulation of NO production in skeletal muscle as well.



Striated muscle AMPK subunit expression:

Ubiquitous:	Cardiac muscle:	Skeletal muscle:
α1	α1, α2	α1, α2
β1	β1, β2	β1, β2
γ1	γ1, γ2	γ1, γ3

Figure 5.2. Regulation and subunit expression of AMPK in striated muscle. AMPK is subject to multiple layers of regulation by kinases, phosphatases, reactive oxygen species (ROS), allosteric regulators, and components of the cytoskeleton. Arrows depict the subunit at which regulators exert their effects, with pointed arrowheads representing regulation that stimulates AMPK enzymatic activity, and blunt arrowheads representing regulation that inhibits AMPK activity. Phosphorylation at AMPKα-Thr172 increases kinase activity(53-57), and allosteric activators such as AMP and ADP induce conformational changes that are translated to the alpha subunit, protecting this site from dephosphorylation by phosphatases(105). Phosphorylation at AMPKα-Thr479 or AMPKα-Ser485 or the binding of allosteric inhibitors such as glycogen increases the accessibility of Thr172 to phosphatases, resulting in decreased AMPK activity(65, 66, 69). Oxidation of AMPKα-Cys130/174 causes aggregation of AMPK that prevents stimulatory phosphorylation of Thr172(68). Possible crosstalk between these different regulatory mechanisms is highlighted with grey arrows. ATP can also inhibit AMPK activity by competing with AMP and ADP for binding to regulatory Bateman domains in the gamma subunit(106). The cytoskeletal protein plectin binds to AMPKγ subunits and attenuates maximal nucleotide-sensitive AMPK catalytic activity by selectively stabilizing the expression of α1 subunits over α2 subunits(72). Any direct role for dystrophin or the dystrophin-glycoprotein complex (DGC) in regulating AMPK activity or subunit expression remains to be determined. Potential mechanisms by which dystrophin or the DGC may affect established AMPK-regulatory pathways to control the mechanical activation of AMPK are discussed in the text. The unique array of AMPK subunits that are expressed in cardiac and in skeletal muscle(105, 107) raises the possibility of distinct mechanisms mediating DGC-dependent AMPK activation in these different tissues.

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