Oxidative/Nitrosative Stress, Contractile Dysfunction and Gene Replacement Studies in Skeletal Muscles in Mouse Models of Neuromuscular Disease

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August, 2005

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of PhD.

Your file Votre référence
ISBN: 978-0-494-21642-2
Our file Notre référence
ISBN: 978-0-494-21642-2

Acknowledgments

I have many people to thank for making the fours years that I spent as a PhD student in at McGill University one of the best periods of my life.

First of all, I would like to express my profound gratitude to my supervisor Dr. Basil Petrof for his support, motivation, and tutelage. Dr. Petrof led by example, and instilled in me a questioning, thorough, hardworking approach to scientific research. I will certainly take these valuable traits with me in my future endeavours, and for that I am truly grateful.

I would like to thank all the wonderful lab-mates for their assistance and friendship, and for all the fun we had together. This includes the researchers, technicians and fellow students at the Royal Victoria Hospital and Meakins-Christie Laboratories and those in the labs of our collaborators at the Montreal Neurological Institute, the University of Montreal, and the Montreal Children's hospital.

From Dr. Petrof's labs, I thank Johanne Bourdon, Won-Kyung Cho, Satoru Ebihara, Ghiabe Guibinga, Gawiyou Danialou, Weisheng Bao, Maziar Divanghani, Duska Gvosdic, Linda Yahiaoui, Stefan Matecki, and Alexandre Demoules. These incredible friends made the things unbelievably fun both in and out of the lab. From the Montreal Neurological Institute, I thank Carol Allen, Steve Prescott, Mylène Bourget, Klara Rostworowski, and Yifan Lu. Special thanks to research directors Rénald Gilbert, Josephine Nalbantoglu, and George Karpati for their guidance and support, and especially for the invaluable research opportunities they provided me. From the University of Montreal, I thank Maya Khairallah for her friendship and her important contributions to my work, and Christine des Rosiers for her helpful suggestions. From the Montreal Childrens Hospital I thank my friend Shawn Mohammed for untiring technical assistance, and Larry Lands for his advice and expertise.

I thank Alain Comtois, Sabah Hussain, and Jim Martin for their direction inmy projects, and the Chair of my thesis committee, Katherine Cianflone, for her helpful guidance. I would like to express my appreciation for the funding provided by the Montreal Chest Hospital and The Canadian Lung Association-Canadian Institutes of Health Research Studentships.

I thank all my fellow students in the division of Experimental Medicine. In particular, I thank the friends that served with me on the Graduate Students Society, and the many

friends outside the lab, especially the members Ste-Anne-de-Bellevue Rugby Football Club. I can't begin to explain the how these wonderful people and the beautiful town of Ste-Anne-de-Bellevue have impacted my life. In particular, I thank Martin Silverstone for being a great friend.

I thank Margaret Thompson for her love and support, and for being there through the all the joys and struggles. Finally, I thank all my extraordinary family in St. John's, Newfoundland, whose love and encouragement have been behind my every accomplishment. This thesis is dedicated to them.

Contribution of Authors

I have chosen to present the current thesis as a dissertation which takes the format of a collection of research papers. The following individuals appear as co-authors, in addition to myself, on these papers. Their contributions are as follows:

Won-Kyung Cho, a former post-doctoral fellow in Dr. Basil Petrof's laboratory, assisted in the muscle contractility and immunohistochemistry experiments described in Chapter 2.

Gawiyou Danialou, a research assistant in Dr. Basil Petrof's laboratory, helped with the mouse hindlimb ischemia-reperfusion (I/R) experiments described in Chapter 3.

David E. Eidelman, a research director at the Meakins-Christie Laboratories of McGill University, provided experimental guidance for the NOx analysis described in Chapter 3.

Karuthapillai Govindaraju, a research assistant in the laboratory of Dr. David Eidelmann at the Meakins-Christie Laboratories, assisted in the NOx analysis described in Chapter 3.

Shawn Mohammed, a research assistant in the laboratory of Dr. Larry Lands at the Montreal General Hospital, performed the glutathione analyses and glutathione peroxidase (GPx) and glutathione reductase (GR) enzymatic activity analyses described in Chapter 4.

Larry Lands, a research director at the Montreal General Hospital, provided advice, experimental guidance, technical services and funding towards the analyses of glutathione status and glutathione-associated enzymes as described in Chapter 4.

Maya Khairallah, a PhD student in the laboratory of Dr. Christine des Rosiers of the Notre-Dame Hospital and co-supervised by Dr. Basil Petrof, performed the analyses of isocitrate

degydrogenase (ICDH) and aconitase enzymatic activities described in Chapter 4.

Christine des Rosiers, a research director at the Notre-Dame Hospital provided advice, and critical analysis pertaining to the work described in Chapter 4. In addition, the isocitrate dehydrogenase (ICDH) and aconitase enzymatic activity assays described in this chapter were performed in her laboratory.

An-Bang Liu, a former post-doctoral fellow in Dr. George Karpati's laboratory, assisted in the design and construction of the helper-dependent adenoviral (HDAd) vector used in Chapters 5 and 6.

Stefan Matecki, a former post-doctoral fellow in Dr. Basil Petrof's lab, assisted with the muscle contractility experiments described in Chapter 6.

Yifan Lu, a post-doctoral fellow in Dr. George Karpati's laboratory, performed the real-time PCR experiments described in Chapter 6.

Renald Gilbert, a former post-doctoral fellow in Dr. George Karpati's laboratory, designed and constructed the helper-dependent adenoviral (HDAd) vector used in Chapters 5 and 6; performed the immunoblotting experiments and statistical analyses described in Chapter 5; and supervised much of the work conducted in Chapter 6.

Josephine Nalbantoglu, a research director Neuromuscular Research Group of the Montreal Neurological Institute, was instrumental in the design and supervision of experiments described in Chapters 5 and 6; and contributed to the intellectual discussion of the results obtained in these chapters.

George Karpati, head of the Neuromuscular Research Group of the Montreal Neurological Institute, planned, supervised and directed the experiments described in Chapters 5 and 6; and contributed to intellectual discussion of the results obtained in these chapters.

Basil Petrof, a research director at the Meakins-Christie Laboratories and my PhD supervisor. Dr. Petrof contributed to the design, supervision, critical discussion of all the experiments described throughout this thesis.

Previously Published Papers Included in this Thesis

This thesis contains chapters adapted from the following previously published papers, of which I was the primary author or second author.

Chapter 5 adapted from:

GILBERT, R., **DUDLEY, R. W.**, LIU, A. B., PETROF, B. J., NALBANTOGLU, J., and KARPATI, G. (2003). Prolonged dystrophin expression and functional correction of mdx mouse muscle following gene transfer with a helper-dependent (gutted) adenovirus-encoding murine dystrophin. Hum. Mol. Genet. 12, 1287-1299.

Chapter 6 adapted from:

DUDLEY, R. W., LU, Y., GILBERT, R., MATECKI, S., NALBANTOGLU, J., PETROF, B. J., and KARPATI, G. (2004). Sustained improvement of muscle function one year after full-length dystrophin gene transfer into mdx mice by a gutter helper dependent adenoviral vector. Human Gene Therapy 15, 145-156.

The publishers of these journals have given written permission allowing the adapted chapters to be included in this thesis (see appendix 1).

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List of Abbreviations

Ad: adenovirus

AGEs: advanced glycation end products

ALS: amyotrophic lateral sclerosis

BMD: Becker muscular dystrophy

CAT: catalase

CK: creatine kinase

CMV: cytomegalovirus

COX: cytochrome c oxidase

CTL: cytotoxic T-lymphocyte

DGC: dystrophin-glycoprotein complex

DMD: Duchenne muscular dystrophy

DPC: dystrophin-associated protein complex

Dys+: dystrophin positive

Dys-: dystrophin negative

EBD: Evans blue dye

EDL: extensor digitorum longus

EMG: electromyography

eNOS: endothelial nitric oxide synthase

FGAd: first generation adenovirus

GCS: γ-glutamylcysteine synthase

GPx: glutathione peroxidase

GR: glutathione reductase

GSH: reduced glutathione

GSNO: S-nitrosoglutathione

GSSG: oxidized glutathione

H&E: hematoxylin and eosin

HDAd: helper-dependent adenovirus

HDCBDysM: helper-dependent CMV enhancer/β-actin promoter dystrophin (murine)

HNE: hydroxynonenal

hsp: heat shock protein

I/R: ischemia/reperfusion

ICDH: isocitrate dehydrogenase

iNOS: inducible nitric oxide synthase

ITR: inverted terminal repeat

LGMD: limb girdle muscular dystrophy

Lo: optimal length

MDA: malondialdehyde

MHC: myosin heavy chain

MPO:myeloperoxidase

NADH: reduced nicotinamide dihydrogen

NADPH: reduced nicotinamide dihydrogen phosphate

NF-κB: nuclear factor-kappa B

nNOS: neuronal nitric oxide synthase

NOS: nitric oxide synthase

NOx: nitric oxide derivatives (nitrite, nitrate, nitrosothiol)

PUFA: polyunsaturated fatty acid

rAAV: recombinant adeno-associated virus

RNS: reactive nitrogen species

ROS: reactive oxygen species

RyR1: ryanodine receptor Ca^{2+} release channel

SERCA: sarcoplasmic/endoplasmic reticulum Ca^{2+}-dependent ATPase

SMA: spinal muscular atrophy

SMN: survival motor neuron

SOD: superoxide dysmutase

TA: tibialis anterior

TBARS: thiobarbituric acid reactive substances

TCA: tricarboxylic acid

XDH: xanthine dehydrogenase

XO: xanthine oxidase

ABSTRACT

Amyotrophic lateral sclerosis (ALS) and Duchenne muscular dystrophy (DMD) are two fatal neuromuscular disorders for which there is no effective treatment. Furthermore, the exact pathogenic mechanisms of these diseases have not been precisely determined. Oxidative/nitrosative stress has been implicated in the skeletal muscle dysfunction in both cases. A subset of ALS patients (~2%) have mutations in the antioxidant enzyme SOD1, and transgenic mice overexpressing these mutant enzymes develop motor neuron disease. ALS primarily affects motor neurons, but recent evidence suggests skeletal muscle damage, in addition to denervation changes, may contribute to disease pathogenesis. The purpose of Part I of this thesis was to test contractile function, and to evaluate markers of oxidative and nitrosative stress, and indices of primary myopathy (myofiber necrosis, serum creatine kinase, central nucleation) in skeletal muscles of the mutant SOD1-G37R transgenic mouse model of ALS. Transgenic SOD1-G37R muscles demonstrated decreased isometric force production, altered contractile kinetics, and trends toward increased nitrosative stress relative to non-transgenic littermates, but displayed no overt myopathic process. DMD, an x-linked recessive disorder, caused by the absence of the cytoskeletal protein dystrophin primarily affects skeletal muscle. The exact mechanism by which the loss of dystrophin induces skeletal muscle destruction has not been resolved. Proposed mechanisms include mechanical stress, oxidative/nitrosative stress and functional ischemia. The purpose of the Part II of this thesis was to explore the possible relationships between these three mechanisms in mdx muscle, and to investigate antioxidant responses to oxidative/nitrosative stress in these dystrophin-deficient muscles. We subjected mdx and normal C57 muscles to ischemia-reperfusion (I/R) injury, a well-

established model of in vivo oxidative/nitrosative stress. We tested for markers of oxidative and nitrosative stress and compared susceptibility to mechanical stress-induced sarcolemmal injury following I/R in muscles in the two mouse strains. We found that mdx muscles display abnormal susceptibility to I/R-induced nitrotyrosine formation, lipid peroxidation and sarcolemmal fragility. We performed a detailed analysis of the glutathione antioxidant system to test whether antioxidant deficiencies contribute to this heightened vulnerability. At baseline, mdx muscles displayed an altered glutathione status, which confirmed the presence of oxidative/nitrosative stress, but on the contrary, we found an upregulation of the antioxidant enzymes, glutathione peroxidase (GPx) and glutathione reductase (GR). In part III of this thesis we turned our attention to the study of adenoviral vector-mediated gene transfer therapy for DMD. Specifically, we tested the ability of a fully-gutted adenoviral vector (HDCBDysM) carrying two full-length isogenic (murine) dystrophin cDNAs to correct functional deficits following injection in neonatal and juvenile (4- to 6-week-old) mdx muscles. In juvenile mdx muscles, HDCBDysM provided marked improvement of muscle histology and physiology up to 60 days post-injection. However, dystrophin expression in juvenile mdx mice was only transient and was associated with an inflammatory response. In neonatal mdx mice muscles, HDCBDysM provided high-level dystrophin expression and correction of dystrophic histopathology and functional deficits up to one year following treatment. It is hoped that these findings contribute to the understanding of the pathogenesis of ALS and DMD, and advance the pursuit of effective treatments for these devastating diseases.

ABSTRACT

La sclérose latérale amyotrophique (SLA) et la dystrophie musculaire de Duchenne (DMD) sont deux maladies neuromusculaires incurables don't la pathogénie exacte n'est pas connue avec précision. Dans ces deux maladies, le stress oxydatif contribue à la dysfonction des muscles squelettiques. Un sous-groupe de patients SLA (environ 2%) est porteur de mutations de l'enzyme SOD1, et les souris transgéniques qui hyperexpriment ces enzymes mutantes développent une SLA. Bien que la SLA affecte avant tout les motoneurones, des données récentes suggèrent qu'en plus des lésions de dénervation, des dommages musculaires pouraient contribuer à la pathogénie de cette maladie. L'objectif de la première partie de la présente thèse était d'évaluer la fonction contractile, les marqueurs de stress oxydatif, ainsi que les indices de myopathie primaire (nécrose des fibres musculaires, créatine kinase sérique et centro-nucléation) dans les muscles squelettiques d'un modèle murin de SLA, le mutant transgénique SOD1-G37R. Nos résultats montrent que, compare aux souris de phénotype sauvage, les muscles des souris SOD1-G37R présentent une diminution de la force isométrique, une altération de la cinétique contractile, et une tendance a l'augmentation du stress oxydatif. Néanmoins, ces souris ne présentaient aucun signe histologique spécifique de myopathie. La DMD, maladie récessive liée à l'X, est une myopathie dont la cause est l'absence de dystrophine, une protéine du cytosquelette. Le mécanisme exact par lequel la perte de dystrophine induit la dysfonction musculaire reste inconnu et pourrait relever de plusieurs mécanismes : stress mécanique, stress oxydatif et ischémie fonctionnelle. L'objectif de la seconde partie de cette thèse était d'explorer, dans les muscles de souris mdx, les possibles relations entre

ces trois mécanismes et d'étudier les réponses antioxydantes au stress oxydatif dans ces muscles dépourvus de dystrophine. Nous avons réalisé des lesions d'ischémie-reperfusion musculaire, un model in vivo bien établi de stress oxydatif, chez des souris mdx et C57/BL6 (animaux contrôles). Au décours de l'ischémie-reperfusion, nous avons évalué et comparé chez ces deux lignées de souris les marqueurs de stress oxydatif et la susceptibilité aux lésions sarcolemmales qu'induit un stress mécanique. Nos résultats montrent que les muscles de souris mdx sont anormalement susceptibles à la formation de nitrotyrosine, la peroxidation lipidique et la fragilité sarcolemmale qu'induit l'ischémie-reperfusion. Nous avons analysé en détail le système anti-oxydant lié au glutathion afin de savoir si une diminution de l'activité des systèmes anti-oxydants pouvait contribuer à cette susceptibilité plus élevée observée chez les souris mdx. A l'etat de base, les muscles de souris mdx présentaient des altérations du système glutathion, confirmant l'existence d'un stress oxtydatif ; mais, au lieu d'être diminuée, l'activité de deux enztymes anti-oxydantes, la glutathion peroxidase (GPx) et la glutathion réductase (GR) était accrue. Dans la troisième partie de cette thèse, nous nous sommes tourné vers la thérapie génique de la DMD par les vecteurs adenoviraux. De façon plus précise, nous avons évalué dans quelle mesure l'injection intramusculaire d'un vecteur adenoviral totalement "vide" transportant deux ADNc isogéniques (murins) codant pour la totalité du gène de la dystrophine (HDCBDysM) pouvait corriger les déficits fonctionnels musculaires de souriceaux mdx nouveau-nés et de souris mdx juvénile (âgées de 4 a 6 semaines). Chez les jeunes souris, l'injection d'HDCBDysM s'est accompagnée d'une une nette régression des anomalies histologiques et physiologiques qui persistait 60 jours après l'injection. Néanmoins, l'expression de la dystrophine chez ces jeunes souris mdx n'était que transitoire et était associée a une

importante réaction inflammatoire. Chez les souriceaux mdx nouveau-nés, l'injection intramusculaire d'HDCBDysM s'accompagnait d'une expression élevée de la dystrophine et d'une régression des anomalies histologiques et fonctionnelles, laquelle persistait un an après l'injection. Nous espérons que ces résultats contribueront á une meilleure compréhension de la pathogénie de la SLA et de la DMD, ainsi qu'aux progrès dont doit bénéficier le traitement de ces deux maladies.

Chapter 1

INTRODUCTION

1.2 OVERVIEW

All living cells produce potentially damaging reactive oxygen species (ROS). As such, living organisms have evolved elaborate antioxidant systems for removing these potentially harmful molecules. If the production of ROS increases beyond the capacity of the antioxidant system to cope, or if antioxidant defences are for some reason diminished, cellular redox balance is shifted toward the often deleterious condition known as oxidative stress. The formation of ROS is often accompanied by the production of so-called reactive nitrogen species (RNS), the adverse effects of which include oxidation, nitration (addition of NO_2^+ to aromatic groups) and nitrosation (addition of NO^+ to amine, thiol, hydroxy aromatic groups) reactions. An abnormal increase in the rate of such RNS-mediated reactions in biological systems is now commonly referred to simply as nitrosative stress. The combination of oxidative/nitrosative stress has been implicated in a wide variety of pathophysiological conditions from cancer to cardiovascular disease and from Alzheimer's disease to AIDS (1). In some cases reactive oxygen and nitrogen species appear to play a primary causative role in pathogenesis, in other cases the formation of these species is a secondary, albeit important, consequence of underlying pathological processes. All living cells produce free-radicals and as such no organ is free from their potentially damaging effects. Skeletal muscle is no exception. Indeed, oxidative/nitrosative stress has been shown to play a role in skeletal muscle injury in settings such as ischemia-reperfusion (I/R), sepsis, and exhaustive exercise. The role of oxidative/nitrosative stress in skeletal muscle pathology and dysfunction due neuromuscular disorders, however, is less clear.

Substantial evidence suggests that free-radicals contribute to the pathogenesis of amyotrophic lateral sclerosis (ALS), a neuromuscular disease of neurogenic origin. The

finding of mutations in the antioxidant enzyme superoxide dismutase (SOD) in some ALS patients suggest that oxidative stress may actually be the primary cause of motor neuron degeneration in a subgroup of this disease. Skeletal muscle weakness is the major cause of disability and death in ALS patients. SOD1 is expressed in all mammalian tissues, and recently it has been suggested that primary skeletal muscle defects, in addition to denervation atrophy, may contribute to ALS pathogenesis (2-11). However, it remains unresolved whether mutant SOD1 expression causes oxidative/nitrosative stress-mediated injury and contractile dysfunction in skeletal muscles. The first study (Chapter 2) of this thesis will examine muscle contractility parameters and markers of oxidative/nitrosative stress in a mutant SOD1 mouse model this disease.

Duchenne muscular dystrophy (DMD), on the other hand, is a neuromuscular disorder of myogenic origin resulting from the absence of the dystrophin protein in skeletal muscles. Again abundant evidence suggests oxidative stress plays a role in DMD, however, much debate has revolved around whether ROS are primary mediators of muscle pathology or whether they merely arise as a result of the muscle damage and inflammation in this disease. In either case, these potentially damaging species may be important, as recent evidence suggests that dystrophin-deficient myotubes are abnormally susceptible to oxidative/nitrosative stress (12). Sarcolemmal (muscle cell membrane) injury is an early characteristic feature of skeletal muscle pathology in DMD patients and the mdx mouse model of this disease. Furthermore, mdx mouse muscles are abnormally susceptible to membrane lesions due to contraction-induced mechanical stress (13). Recent evidence from normal muscle cells suggests oxidative/nitrosative stress and mechanical stress work synergistically to induce sarcolemmal disruptions (14). Dystrophin-deficient muscle has also

been shown to suffer functional ischemia due to the secondary loss of neuronal nitric oxide synthase (nNOS) from the sarcolemma; and I/R is perhaps the most well established condition of free-radical mediated skeletal muscle damage. It remains to be determined whether abnormal susceptibility to I/R plays a role in predisposing dystrophin-deficient muscle to membrane damage during contraction. Chapters 3 of this thesis will examine how I/R-induced oxidative/nitrosative stress contributes to sarcolemmal fragility in mdx skeletal muscle; and Chapter 4 will examine the glutathione antioxidant status of dystrophin-deficient muscles and how this system responds to acute I/R insults.

The absence of dystrophin is fundamentally responsible for all cellular disturbances in skeletal muscles of DMD patients and mdx mice, including those involving oxidative/nitrosative stress. Therefore, restoration of dystrophin expression, via gene replacement with viral vectors carrying the dystrophin cDNA is a promising approach to treat DMD. An ideal viral vector for treating DMD in this manner should be non-toxic and able to produce sufficient, long-term dystrophin expression in the targeted muscles. First generation, replication-deficient adenoviral vectors can efficiently transduce muscle fibers in vivo (15-18), but have two major disadvantages. Firstly, gene expression is quickly lost as protein products of viral genes induce immune-mediated destruction of transduced fibers and/or silencing of transgene expression (19). Secondly, the small insert capacity (~8 kb) is insufficient to carry a full-length dystrophin cDNA (~12 kb).

In an attempt to overcome these problems, gutted or helper-dependent adenoviral vectors (HDAd) devoid of all viral sequences (except for the inverted terminal repeats and DNA packaging sequences) with insert capacities of up to ~36 kb have been developed (20-24). These HDAd vectors are much less immunogenic than the first generation

adenoviral vectors, allowing for greater longevity of transgene expression (25-29). Despite these potential advantages, relatively few preclinical studies using HDAd carrying dystrophin exist. Gilbert et al. (30) observed only weak dystrophin expression after treatment of mdx mouse and dystrophic golden retriever dog muscles with HDAd carrying dystrophin regulated by the cytomegalovirus (CMV) promoter. Subsequently, a modified HDAd vector containing two tandem human dystrophin cDNAs regulated by the powerful hybrid CMV enhancer/β-actin (CB) promoter was found to confer high level dystrophin expression. Despite these improvements, the use of this vector still only resulted in transient dystrophin expression in mdx mouse muscle (31). These disappointing results may have been due to the fact that the dystrophin cDNAs used were human, not murine (i.e., non-isogenic) in origin. In chapters 5 and 6 of this thesis we will examine the efficacy and long-term stability of gene transfer in mdx skeletal muscle using an HDAd vector carrying two full-length murine dystrophin cDNAs.

1.2 FREE RADICALS AND OTHER ROS/RNS IN LIVING SYSTEMS

A free radical is any species that has one or more unpaired electrons. Biologically relevant free radicals (Fig. 1) include superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), alkoxyl radical ($LO^{\bullet}$), and peroxyl radical ($LOO^{\bullet}$). The partially reduced form of oxygen, $O_2^{\bullet -}$, is formed in all aerobic cells, mostly from the leak of electrons onto oxygen as they pass along the mitochondrial electron transport chain (1). $O_2^{\bullet -}$ is only moderately reactive and cannot directly attack most proteins, lipids, or DNA. It does, however, have the capacity to inactivate certain iron-sulfur containing enzymes,

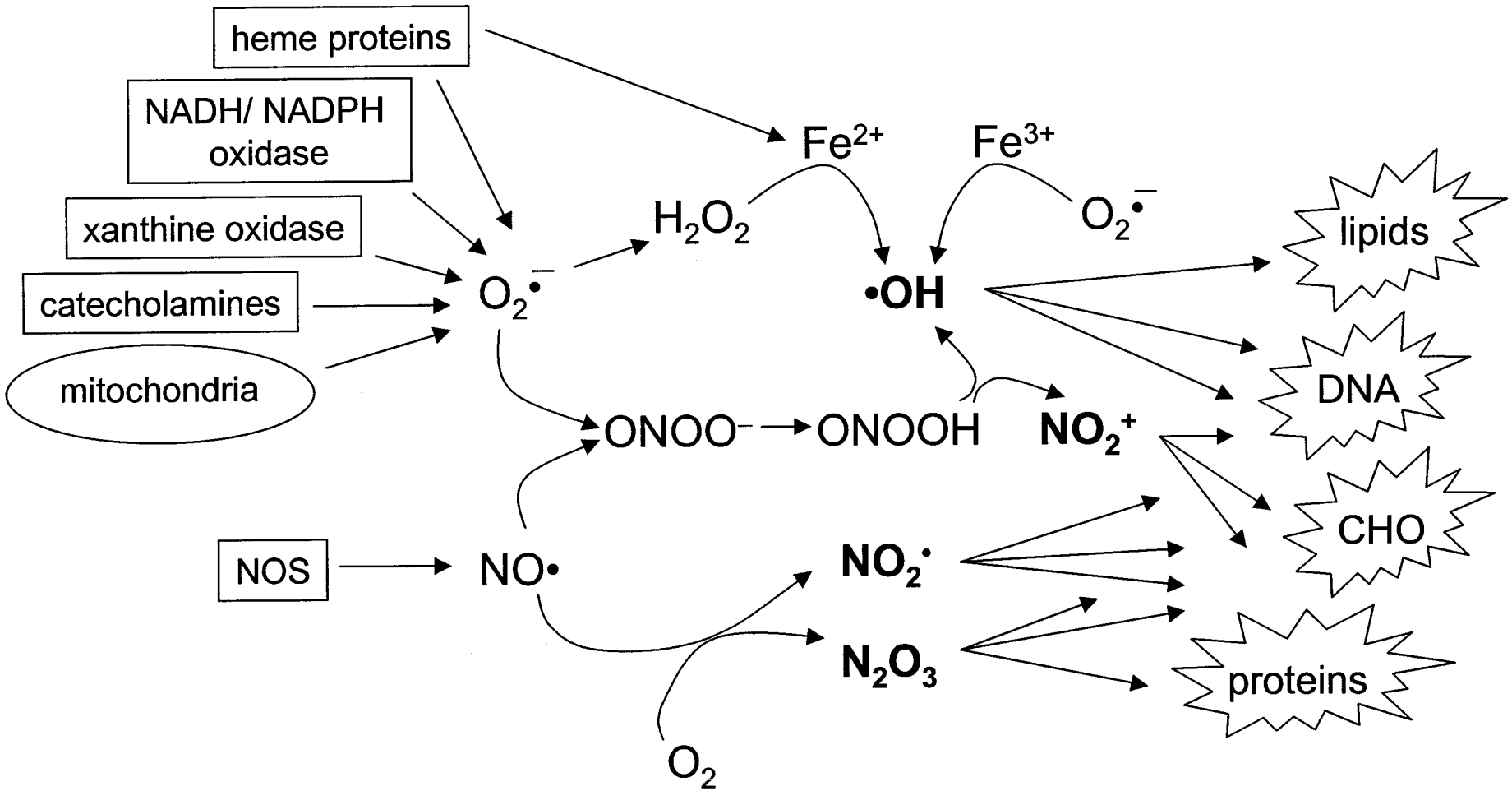

Figure 1. Pathways of ROS and RNS production and damage in biological systems. superoxide ($O2^{\bullet -}$), nitric oxide (NO•), hydrogen peroxide (H_2O_2), oxygen (O_2), ferrous iron (Fe^{2+}), ferric iron (Fe^{3+}), hydroxyl radical (•OH), peroxynitrite ($ONOO^-$), peroxynitrous acid (ONOOH), nitronium ion (NO_2^+), nitrogen dioxide ($NO_2^{\bullet}$), dinitrogen trioxide (N_2O_3), carbohydrates (CHO).

including those essential to the TCA cycle, such as aconitase (32). In addition , the importance of $O_2^{\bullet -}$ is underscored by the fact that it gives rise to many other reactive species.

Physiological removal (spontaneous and/or enzyme-catalysed dismutation) of $O_2^{\bullet -}$ gives rise to the non-radical hydrogen peroxide (H_2O_2). Certain enzymes, such as xanthine oxidase; auto-oxidizable molecules, like catecholamines; and heme proteins, such as haemoglobin and myoglobin, also contribute to the cellular production of both H_2O_2 and $O_2^{\bullet -}$ (33). Recent reports suggest skeletal muscles contain NADH oxidase and NADPH oxidase enzymes which also produce $O_2^{\bullet -}$ (34,35). H_2O_2 is even less reactive than $O_2^{\bullet -}$, but abnormally high levels of H_2O_2 can cause the release of iron from heme proteins, including myoglobin. Most importantly, in the presence of transition metals, H_2O_2 can be converted to the most reactive radical of all - the hydroxyl radical ($^{\bullet}OH$). Reduced forms of transition metals, such as ferrous (Fe^{2+}) iron, catalyse this conversion, in what is known as the Fenton reaction. With the assistance of $O_2^{\bullet -}$, ferric (Fe^{3+}) iron can also promote the production of $^{\bullet}OH$, in what is known as the Haber-Weiss reaction (1). In skeletal muscle, $^{\bullet}OH$ levels increase during strenuous exercise, and this has been implicated in the development of muscle fatigue (36). One of the many damaging effects of $^{\bullet}OH$ is lipid peroxidation. This results in the formation of lipid peroxides (LOOH), which can be degraded in the presence transition metals to form alkoxyl radicals ($LO^{\bullet}$) and peroxyl radicals ($LOO^{\bullet}$). Collectively, $O_2^{\bullet -}$ and its derivatives, radicals and non-radicals alike, are often referred to as reactive oxygen species (ROS).

Another extremely important free radical is nitric oxide ($NO^{\bullet}$). This free radical is produced enzymatically from L-arginine and O_2 by nitric oxide synthases (NOS).

Interestingly, when L-arginine availability is diminished, these enzymes can catalyse the production of $O_2^{\bullet -}$ (1). $NO^{\bullet}$ is continually produced at low levels in skeletal muscle, but increases dramatically during muscle contraction. $NO^{\bullet}$ itself, however, is not extremely reactive, but like $O_2^{\bullet -}$ and H_2O_2, it can give rise to more cytotoxic species. The most well studied of these is peroxynitrite ($ONOO^-$), which is formed by the diffusion-limiting reaction of $NO^{\bullet}$ with $O_2^{\bullet -}$, or its dismutation product H_2O_2 (37,38). $ONOO^-$ is easily protonated at physiological pH to form ONOOH. Under certain circumstances ONOOH can undergo heterolytic fission to form nitronium ion (NO_2^+), a potent nitrating species. Some evidence also suggests that ONOOH may also decompose to form $^{\bullet}OH$ (1). $NO^{\bullet}$ can also react with O_2 to form nitrogen dioxide ($NO_2^{\bullet}$) and dinitrogen trioxide (N_2O_3), both of which are harmful to biological tissues (39). $NO^{\bullet}$, and its derivatives, are often referred to as reactive nitrogen species (RNS). Often the formation of ROS and RNS goes hand in hand.

Inflammatory cells including macrophages, neutrophils, mast cells, eosinophils and microglial cells of the brain use ROS to kill invading microorganisms by the so-called respiratory burst. This process, so-named because of the associated increase in O_2 uptake, has been most well studied in neutrophils and macrophages. These phagocytic cells use a membrane-bound NADPH oxidase complex to reduce the elevated levels of O_2 to $O_2^{\bullet -}$ [(1)]. Due to its low reactivity and the fact that this charged molecule cannot cross membranes, it is unlikely that $O_2^{\bullet -}$ kills bacteria directly. Non-enzymatic conversion to H_2O_2 and subsequent $^{\bullet}OH$ production is most likely responsible. The anti-microbial activity of neutrophils, but not macrophages, also involves myeloperoxidase, a haem-containing enzyme, which in the presence of H_2O_2 (from $O_2^{\bullet -}$ dismutation) and Cl^- ions produces the highly reactive oxidant hypochlorous acid (HOCl). This species can also react with $O_2^{\bullet -}$ to

form $^{\bullet}OH$. Under certain conditions, neutrophils and macrophages can also produce large quantities $NO^{\bullet}$ from the inducible isoform of NOS (iNOS). High concentrations (uM) of $NO^{\bullet}$ can kill bacteria directly, or by reacting with $O_2^{\bullet -}$ to form $ONOO^-$ (40). The adverse effects of ROS/RNS are not limited to invading microbes. Host tissue damage, including skeletal muscle injury, is often associated with the production of these mediators from inflammatory cells (41).

1.3 ANTIOXIDANT SYSTEMS

The production of cytotoxic ROS/RNS is a natural consequence of the use of oxygen by aerobic organisms to produce energy. Through evolution, oxygen-dependant organisms developed elaborate antioxidant defence systems which enabled their survival. These antioxidants include enzymatic as well as non-enzymatic systems which prevent the formation of ROS/RNS and/or scavenge these species once they have been formed (Fig. 2). Metal chelators also play an important role in preventing $^{\bullet}OH$ production, decomposition of lipid peroxides to peroxyl and alkoxyl radicals, as well as autoxidation reactions. Despite their great importance, antioxidants are not equally distributed among all tissues in living systems. For example, the liver has the highest concentrations of many antioxidants, while the brain and skeletal muscle appear to have the lowest (1). This unequal distribution is also the case within skeletal muscle. Antioxidant enzyme activity is greater in slow-twitch (type I) and fast-twitch oxidative (IIa) muscle fibers, than in fast-twitch glycolytic (type IIb) fibers (42). Interestingly, fast-twitch glycolytic fibers are predominantly effected in myopathic conditions associated with free radical damage, such Duchenne muscular dystrophy and ischemia-reperfusion injury. The following is an overview of important antioxidants skeletal

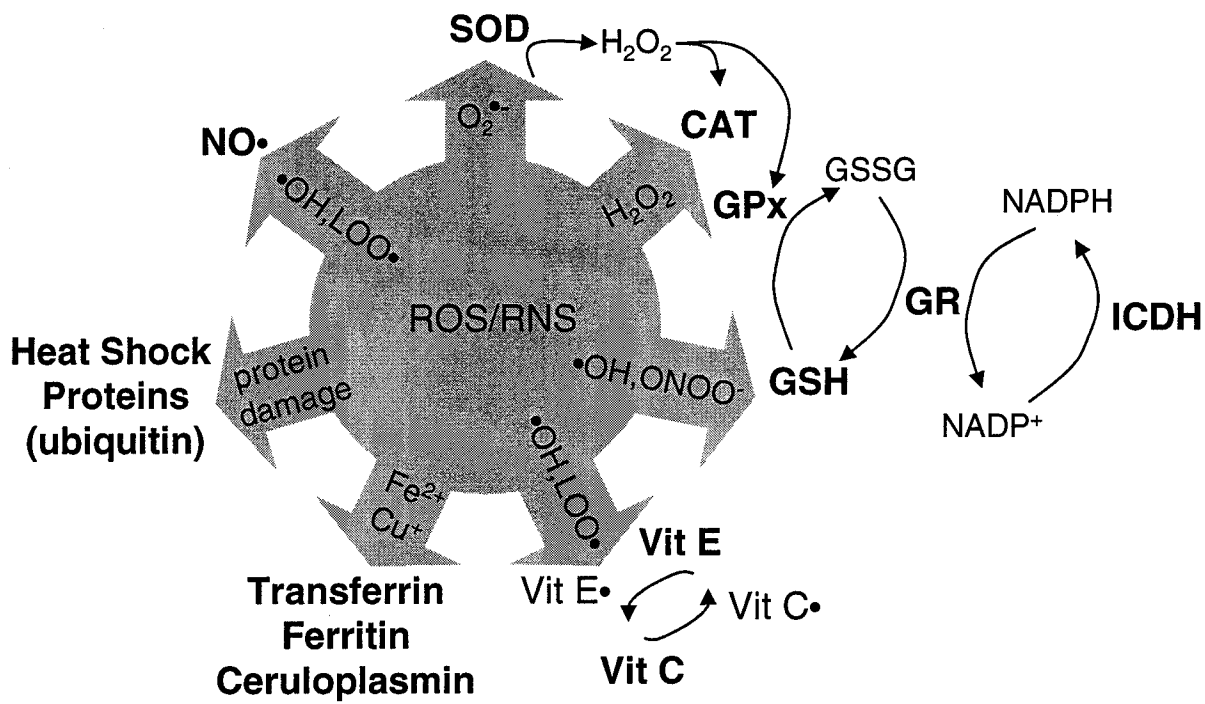

Figure 2. A wide variety of antioxidants (bolded) protect skeletal muscle from damage due to reactive oxygen species (ROS) and reactive nitrogen species (RNS). These include (clockwise from top) superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), isocitrate dehydrogenase (ICDH), glutathione (GSH), vitamin E (Vit E), vitamin C (Vit C), metal binding proteins (transferrin, ferritin, and ceruloplasmin), heat shock proteins, and nitric oxide (NO•). Additional abbreviations: cupprous copper (Cu^+), ferrous iron (Fe^{2+}), hydrogen peroxide (H2O2), hydroxyl radical (•OH), oxidized glutathione (GSSG), oxidized nicotinamide dihydrogen phosphate ($NADP^+$), peroxynitrite ($ONOO^-$), peroxyl radical (LOO•), reduced nicotinamide dihydrogen phosphate (NADPH), superoxide dismutase ($O2^{\bullet-}$), vitamin E radical (Vit E•), vitamin C radical (Vit C•).

muscle. Other antioxidants such as the thioredoxin, glutaredoxin enzyme systems, α-lipoic acid, and uric acid are less well characterized in skeletal muscle and thus will not be discussed here.

1.3.1 Antioxidant Enzymes.

Superoxide dismutase (SOD) is the main means of removing $O_2^{\bullet -}$ from biological systems (42). There are three isoforms of SOD: the copper and zinc-containing enzyme (Cu/Zn SOD) of the cytoplasm and nucleus, which is also known as SOD1; the manganese-containing enzyme of mitochondria (MnSOD), or SOD2; and a glycosylated Cu/Zn SOD enzyme found in the extracellular space, SOD3 (1). SOD oxidizes one molecule of $O_2^{\bullet -}$ to O_2, and reduces another to H_2O_2. If it is not subsequently removed, the H_2O_2 produced by SOD can also be problematic, especially in the presence of transition metals. Thus, the removal of H_2O_2 is catalysed by two enzymes, glutathione peroxidase (GPx) and catalase (CAT). Overexpression of Cu/Zn SOD can be damaging if the expression/activity of these antioxidant enzymes responsible for removing H_2O_2 are not sufficiently high. For example, young mice overexpressing human Cu/Zn SOD suffer more damage due to cerebral ischemia-reperfusion than do wild-type mice, while adult mice overexpressing the enzyme show some resistance to such injury (43,44). This believed to be due to the fact that CAT and GPx systems are not sufficiently developed in the younger mice. In addition, muscle specific overexpression of wild-type SOD1 has been shown to cause a muscular dystrophy-like phenotype in mice (45). This again may be due to increased H_2O_2 formation and conversion to $^{\bullet}OH$.

CAT is an iron-dependant enzyme found in highest concentrations in peroxisomes and mitochondria where it converts H_2O_2 to water and oxygen (42). GPx is a selenium (Se)-dependent enzyme which converts, not just H_2O_2 to water, but also organic hydroperoxides to alcohols (29). The fact that it can remove organic hydroperoxides, such as fatty acid hydroperoxides, makes it an important defense against membrane damage due to lipid peroxidation reactions (42). For the most part, removal of lipid hydroperoxides requires their release from membranes via lipase activity, however several isoforms of GPx exist, including a specific phospholipid hydroperoxide GPx, which can independently act on such substrates (1,46). A GPx-like enzyme, which does not require selenium is glutathione-S-transferase (previously referred to as non-Se GPx). This enzyme is actually specific for organic peroxides, and cannot use H_2O_2 as a substrate.

All GPx enzymes use reduced glutathione (GSH) as an electron donor (1). As H_2O_2, or organic hydroperoxide substrates are reduced, GSH is oxidized to GSSG. Another enzyme, glutathione reductase (GR) converts GSSG back to GSH. GR uses NADPH as the reducing equivalent in this reaction. NADPH is produced mostly by glucose-6-phosphate dehydrogenase in many cells. However, in skeletal muscle this appears to be accomplished mainly by the TCA cycle enzyme isocitrate dehydrogenase (ICDH), which catalyses the oxidative decarboxylation of isocitrate to α-ketoglutarate. This reaction consumes NAD+ or NADP+ thus producing NADH and NADPH. The NADPH which is formed is not only used by GR. CAT also requires NADPH binding for its antioxidant activity. ICDH is therefore considered another essential component of cellular antioxidant systems (47-49).

Oxidative/nitrosative stress causes an upregulation/activation of antioxidant enzymes (42). Therefore, assessments of mRNA transcription, protein expression and enzyme

activity are often used as indirect measures of oxidative/nitrosative stress. On the other hand, some antioxidant enzymes, including ICDH can be inactivated by oxidative/nitrosative stress (48,50). One of the few direct harmful reactions attributed to $O_2^{\bullet -}$ and H2O2 are enzyme inactivations. H2O2 can inactivate SOD enzymes (51), while $O_2^{\bullet -}$ can inactivate CAT and GPx (52). Therefore, SOD and CAT/GPx work together to protect each other from oxidative inactivation (48).

1.3.2 Non-Enzymatic Antioxidants.

GSH, a thiol-containing tri-peptide (γ-glutamyl-cysteinyl-glycine), is the most abundant and most important antioxidant in skeletal muscle (53). As discussed above, GSH is necessary for the antioxidant functions of GPx, but it also has the ability to scavenge ROS and RNS directly. GSH has a high affinity for $^{\bullet}OH$, $ONOO^-$ and N_2O_3 (54). In fact, $NO^{\bullet}$ related toxicity is very much dependent on GSH concentrations. Several studies have shown that if GSH is depleted, toxicity due to $NO^{\bullet}$ is increased several fold (55-58). Finally, GSH has been shown to be involved in recycling of non-enzymatic antioxidant free radicals, including those of vitamin E and vitamin C (42). All the antioxidant functions of GSH result in its conversion to GSSG, and as such the ratio of GSSG to GSH is a widely used method of examining oxidative stress in cells and tissues. The importance of GSH in skeletal muscle is highlighted by the fact that systemic GSH depletion in mice results in severe skeletal muscle pathology (59).

Vitamin E (α-tocopherol and its structural isomers) is a lipid soluble anti-oxidant which converts free radicals, such as $O_2^{\bullet -}$, $^{\bullet}OH$, and $LOO^{\bullet}$, to less reactive forms. Because

of its lipid solubility, and the fact that it can break lipid peroxidation chain reactions, it is considered the most important antioxidant of biological membranes. In muscle it is found in the sarcolemma as well as membranes of mitochondria and the sarcoplasmic reticulum. Like GSH depletion, one of the most common and severe symptoms of vitamin E-deficiency is skeletal muscle degeneration (60-62). Vitamin C (ascorbic acid) is hydrophilic and found most abundantly in the cytosol. It also has the ability to directly scavenge free radicals and, like GSH, is also important in recycling vitamin E radicals. While vitamin C is an important antioxidant, in the presence of transition metals, high concentrations of vitamin C may induce oxidative stress. This is because vitamin C can reduce Fe^{3+} to Fe^{2+}, which can then catalyse the Fenton reaction resulting in the production of $^{\bullet}OH$ from H_2O_2, or $LO^{\bullet}$ from lipid hydroperoxides (63).

Carotenoids, such as β-carotene, like vitamin E, are hydrophobic antioxidants found in biological membranes (42). Carotenoids contain long chains of conjugated dienes, which are very susceptible to free radical attack. These are potent scavengers of free radicals and inhibitors of lipid peroxidation reactions. Ubiquinones, such as coenzyme Q10, and their reduced forms, ubiquinols, are also lipid soluble antioxidants. These are found most abundantly in mitochondrial membranes and their anti-oxidant activity is due to their phenol ring structure. These too act to prevent lipid peroxidation and recycle vitamin E during periods of oxidative/nitrosative stress.

1.3.3 Metal binding proteins.

As we have seen transition metals (iron and copper, in particular) can participate in many of the reactions associated with oxidative/nitrosative stress. Slight disturbances of iron homeostasis, either toward deficiency or overload, can have deleterious consequences. Iron is therefore tightly regulated in living systems (64). This regulation includes proteins which bind iron and prevent it from taking part in harmful reactions. The plasma glycoprotein transferrin binds circulating iron with great efficiency such that free iron is virtually non-existent in the blood, and iron is transferred to the cells which require it through specific transferrin receptors. Once inside the cell, iron is, for the most part, stored in another iron binding protein called ferritin (65). This ubiquitous protein forms a hollow shell and can store up 4500 iron atoms as well as trace amounts of other metals, including copper. Copper can also be cytotoxic if not bound tightly to proteins (66). Therefore, a copper binding protein known as ceruloplasmin binds this metal and carries it in the blood (1). Inadequate binding of copper to ceruloplasmin has been implicated in various diseases including mutant SOD1-mediated familial ALS (67,68,69).

1.3.4 Heat-shock/stress proteins and the ubiquitin-proteasome pathway

Cells respond to oxidative/nitrosative stress by upregulating, not just antioxidant enzymes, but also heat-shock proteins (70,71). These are usually not considered as classic antioxidants, because they are involved in the cellular response to a diverse range of stresses including thermal insults, alcohol and heavy metals. They do, however, play an important role in repairing damage done by ROS/RNS. A common feature of many cellular insults including oxidative/nitrosative stress is the ability to damage proteins (to be

discussed in more detail below) often resulting denaturation or misfolding. The job of many heat-shock proteins, including some of the hsp70 family, is to facilitate the refolding or removal of damaged proteins. If proteins are damaged beyond repair, they become bound by small heat-shock proteins commonly referred to as ubiquitin proteins. This marks these aberrant proteins for final degradation by multicatalytic protease complex known as the proteasome (1). Aggregates of misfolded proteins and dysfunction of ubiquitin-proteasome pathway have been implicated in mutant SOD1-linked ALS (72-75). Interestingly, hsp70 activity has been shown to be significantly diminished in a transgenic mutant SOD1 mouse model of ALS (74).

1.3.5 The special case of NO•.

Nitric oxide (NO•) is a highly diffusible free-radical signalling molecule, which usually acts through its ability to bind heme-centers of proteins, such as guanylate cyclase, to exert a diverse range of physiological functions. In skeletal muscle, NO• is involved in muscle development and repair (76,77), contractile function (78), glucose metabolism (79), calcium release from the SR (80,81), mechanical-mediated signal transduction (82), inhibition of leukocyte adhesion (83), and most recently the regulation of blood flow during muscle activity (84-86). NO• is produced by three isoforms of nitric oxide synthase (NOS). Two of these, neuronal NOS (nNOS) and endothelial NOS (eNOS), are calcium/calmodulin-dependent, constitutive forms. The third isoform is inducible and is independent of calcium/calmodulin regulation. This so-called iNOS is expressed at highest levels in immune cells such as activated macrophages, but can be expressed in skeletal muscle during periods of inflammation and muscle injury (41,81). nNOS and eNOS are

constitutively expressed in myofibers, but nNOS is most abundant. The fact that eNOS knock-out mice are indistinguishable from control mice in terms of skeletal muscle NO• production and contractile function, suggests that nNOS is the main regulator of NO• homeostasis in this particular tissue (87).

Derivatives of NO•, such as $ONOO^-$ and $NO_2^{\bullet}$, contribute to oxidative/nitrosative stress in various pathophysiological conditions of skeletal muscle such as sepsis, skeletal muscle fatigue and muscle wasting (36,81,88-90). Some evidence, however, also suggests that NO•, itself, may act as an antioxidant (80,91-94). The proposed antioxidant activities of NO• include: (1) direct scavenging of •OH; (2) the ability to react with lipid peroxyl and alkoxyl radicals to terminate lipid peroxidation reactions (39); (3) inhibition of •OH production from heme-containing proteins (63); and (4) the ability to antagonize neutrophil-mediated ROS production by preventing adherence to endothelial cells and directly inhibiting $O_2^{\bullet -}$ production from NADPH oxidase (95-98). The antioxidant versus pro-oxidant effects of NO• appear to depend on the concentration. It seems that low (<1μM) levels are protective against oxidants, while high (>1μM) levels are damaging (39).

1.4 OXIDATIVE/NITROSATIVE STRESS

Despite the general acronyms, ROS/RNS have varying degrees of reactivity. The moderate reactivity of some of these species makes them suitable for useful purposes, such as inter- and intracellular signalling. NO• is the most well established free-radical signalling molecule playing roles in many diverse physiological processes. Similarly, H_2O_2 is involved in regulation of contractile function, and is a potent second messenger for the activation of the transciption factor NF-κB (99). The more reactive derivatives of oxygen and

nitrogen, on the other hand, are potentially harmful to biological systems. When ROS/RNS production increases beyond the capacity of antioxidants to cope, or if antioxidants are diminished, cellular redox homeostasis is lost and cellular damage can ensue. $^{\bullet}OH$ can react and modify any macromolecule it encounters including lipids, proteins, DNA, and carbohydrates. The primary reaction of ROS is oxidation, while those of RNS are nitrosations and nitrations (39). Depending on whether ROS or RNS are involved, these situations produce what is referred to as oxidative stress or nitrosative stress, respectively. The close relationship between oxidative and nitrosative stress implies that both may occur simultaneously. $ONOO^-$, for example, is formed from the interaction of ROS and RNS and is a potent mediator of both forms of cellular stress. Oxidative/nitrosative stress adversely affects cellular/organ function and survival, and has been implicated in a wide array of pathophysiological conditions.

1.4.1 General Targets of Oxidative/Nitrosative Stress

DNA Modifications.

The ribose backbone of DNA as well as both pyrimidine and purine bases are susceptible to attack by $^{\bullet}OH$, resulting in a wide array of reaction products (100). Modifications to bases can cause mutations either directly or during DNA replication and repair processes (1). Modifications to deoxyribose sugars can result in single- and double-strand breaks. In addition, if both DNA and nearby nuclear proteins are attacked, resulting protein radicals can cross-link with DNA-radicals (101). Such DNA-protein cross links can interfere with replication, transcription and genome repair processes. RNS are slightly more selective when it comes to their DNA targets. $ONOO^-$, $NO_2^{\bullet}$, and N_2O_3 usually attack bases resulting

in nitrations, nitrosations, or deaminations, all of which can result in genetic mutations (102). Mitochondrial DNA (mtDNA) is also subject to modifications by ROS. In fact, the incidence of oxidative damage to mtDNA is much higher than that of the nuclear genome (103). This is presumed to be related to the close proximity between the mtDNA and the mitochondrial electron transport chain, and the fact that, unlike nuclear DNA, mtDNA does not have histone proteins to provide some defense against oxidative attack (1,103).

Lipid peroxidation.

ROS/RNS of sufficient reactivity can also induce lipid peroxidation reactions. Cell membranes and the membranes of cellular organelles are comprised primarily of lipid bilayers, and a varying degree of proteins depending on the function of the particular membrane subdomain. Accordingly, oxidative damage initiated in lipid components of membranes can also result in protein modifications (104). Lipid peroxidation causes decreased membrane fluidity, increased permeability to molecules which normally do not pass, such as calcium, and inactivation of membrane associated enzymes (1). In addition, oxidatively modified lipids can have secondary detrimental consequences (including additional oxidation reactions) on other non-membrane cellular components (105). Therefore, lipid peroxidation reactions are among the most damaging consequences of oxidative/nitrosative stress.

Lipid peroxidation can be initiated by any species which can abstract a hydrogen from an allylic or bis-allylic (a carbon adjacent to a carbon bearing a double bond, or adjacent to two carbons each of which is bearing a double bond, respectively) carbon, which in the presence of O_2 results in the formation of lipid peroxyl radicals (LOO $^{\bullet}$).

Several species, including $^{\bullet}OH$, $ONOO^{-}$, $NO_2^{\bullet}$, and $LOO^{\bullet}$ themselves can initiate lipid peroxidation reactions (1). The fact that $LOO^{\bullet}$ can induce such reactions underlies a major injurious consequence of lipid peroxidation reactions. That is, once initiated, these become self-propagating chain-reactions. Owing to their large number of double-bonds, polyunsaturated fatty acid (PUFA) side-chains, are very susceptible to lipid peroxidations and the more double bonds a PUFA has, the more susceptible it will be. For example, eicosapentaenoic acid and docosaheaenoic acid which are most abundant in cell membranes of neurons and skeletal muscle cells have 5 and 6 double bonds, respectively (33,106). These fatty acids are several times more susceptible to lipid peroxidation than linoleic acid which has only one double bond. Not only are PUFA with more double bonds more suceptible, but they will also give rise to more secondary $LOO^{\bullet}$ derivatives (107).

Decomposition of lipid peroxides gives rise hydrocarbons and carbonyl derivatives, including ketones and unsaturated aldehydes, in particular. Unsaturated aldehydes have received the most attention, both because these compounds can have further deleterious effects in living systems and because they have been used in assays to examine oxidative stress. Malondialdehyde (MDA) and hydroxynonenal (HNE) are two such unsaturated aldehyde breakdown products of lipid peroxidations reactions. MDA modifies DNA and proteins in vivo (1). It also reacts with thiobarbituric acid to produce a coloured product and as such forms the basis of the thiobarbituric acid reactive substances (TBARS) test for lipid peroxidation. This assay is not very specific for MDA and as such its use for this purpose is waning. HNE, formed by the metal-induced cleavage of lipid hydroperoxides, can attack GSH and protein thiol groups, as well as amino groups of DNA, proteins, and phospholipids. For instance, HNE can modify the glutamate transporter protein known as

excitatory amino acid transporter-2 of astrocytes which has been implicated in ALS (108). General assays for HNE-proteins adducts have partially replaced the TBARS test as a standard measure of lipid peroxidation.

Glycoxidations & Glycations.

Carbohydrates are also subject to oxidation reactions (glycoxidation). In the presence of trace quantities of transition metals, glycoxidation gives rise to more ROS, such as $^{\bullet}OH$, as well as reactive carbonyls (aldehydes and ketones) (1). Carbonyls can modify proteins by binding amino acid side chains. Alternatively, carbohydrates can first behave like aldehydes and directly bind proteins, DNA, and lipids (glycation reactions), and then, secondarily, become oxidized (109) . Whichever route is taken, these final bound and oxidized derivatives of carbohydrates are referred to as advanced glycation end products (AGEs). AGEs themselves may contribute to oxidative/nitrosative stress by releasing $O_2^{\bullet-}$ and H_2O_2, and stimulating phagocytes (110).

Protein Modifications.

Proteins can be directly attacked by ROS/RNS, or by end products of lipid peroxidation reactions such as HNE and MDA (1). In addition, glycation reactions can damage proteins. Thiol groups of cysteine and methionine are particularly susceptible to direct attack resulting in thiyl radicals and disulphide bonds (111). In addition, proteins can bind metals, which when exposed to H_2O_2 catalyse the formation of $^{\bullet}OH$. Reactions with $^{\bullet}OH$ can modify many amino acids, break peptide bonds or promote protein-protein cross-linkage. Like the formation of lipid-peroxides during lipid peroxidation reactions, amino-acid peroxides can

form during free radical attack on proteins. Tryptophan residues are particularly sensitive to $^{\bullet}OH$ attack, resulting in additional peroxide and peroxyl radical formation (111).

RNS ($ONOO^-$ and $NO_2^{\bullet}$) cause oxidation of methionine as well as nitrations of tyrosine, tryptophan and phenylalanine residues. Of these products, 3-nitrotyrosine has received the most attention and assays designed to detect this product are widely used as indicators of nitrosative stress (112,113). HOCl can chlorinate tyrosines; and the reaction of various free radicals with tyrosine can produce tyrosine radicals. The importance of tyrosine phosphorylation in signal transduction pathways makes all of these aberrant tyrosine-related products a major concern in biological systems. Direct oxidation of certain amino acids including arginine, proline and lysine results in protein carbonyl formation. Some peptide bond cleavage reactions such as addition of lipid peroxidation bi-products, including HNE, as well as glycoxidation/glycation reactions also result in protein-carbonyl formation. As such, assays for protein-bound carbonyls are used, not only to detect protein oxidation, but also as indicators of overall oxidative stress (114).

1.5 SKELETAL MUSCLE AND OXIDATIVE/NITROSATIVE STRESS

1.5.1 Skeletal muscle may be highly susceptible to oxidative stress.

All human tissues are subject to oxidative damage, yet some tissues may be more susceptible that others. For example, certain mitochondrial DNA (mtDNA) deletions associated with oxidative stress occur at a higher rate in nervous tissue and skeletal muscle than in all other human tissues (115). Indeed, the brain is believed to be highly susceptible to oxidative/nitrosative stress for a variety of reasons (33). Skeletal muscle may also be particularly vulnerable for the following reasons:

(1). Skeletal muscle is subject to large transient increases in oxygen consumption (116). Under basal conditions, approximately 3 % of the oxygen which enters the mitochondrial electron transport leaks away from the inner mitochondrial membrane as the partially reduced $O_2^{\bullet -}$. During exercise, oxygen-uptake by contracting muscle may increase as much as 100-fold (1), resulting in large scale leak of $O_2^{\bullet -}$ and the subsequent production of more potent radicals including $^{\bullet}OH$. These ROS can damage the electron transport chain itself leading to a vicious cycle of even greater $O_2^{\bullet -}$ release. The fact that skeletal muscle constitutively produces $NO^{\bullet}$, which could lead to $ONOO^-$ generation in the presence of elevated $O_2^{\bullet -}$, may only compound these dangers.

(2). Skeletal muscle has low amounts of antioxidants relative to other tissues. Skeletal muscle of the mouse has the lowest SOD concentration of any organ tested in this species (117). This is also generally the case for catalase and glutathione peroxidase activity in humans, except that the grey matter of the brain has slightly lower catalase activity (1). Glutathione reductase is also low in rat muscle relative to other organs and, while glutathione is considered to be the most important antioxidant in skeletal muscle, again all organs of the rat including the brain have higher concentrations of this low molecular weight antioxidant than does skeletal muscle (118). Since ROS are known to increase force production in non-fatigued skeletal muscle (119), it may be that excessive removal of ROS by high levels of antioxidants would be detrimental to contractile function.

(3). Skeletal muscles have high levels of myoglobin, which itself can act as an pro-oxidant by slow oxidation to form $O_2^{\bullet -}$. In addition, if myoglobin is exposed to excess H_2O_2 in vitro, both heme and iron are released (120). The iron will react with H_2O_2 to form $^{\bullet}OH$, which can stimulate lipid peroxidation. If muscle is damaged (due to excessive exercise,

eccentric contraction, disease) myoglobin outside its normal site is potentially damaging. Myoglobin released as a result of muscle crush injury is believed to cause kidney failure by a mechanism involving the release of iron and oxidative stress (121).

(4). Non-dividing, excitable tissues, such as skeletal muscle have the highest concentrations of polyunsaturated fatty acids (PUFAs) of any tissues in the body (106,122,123). Therefore, skeletal muscle may be abnormally susceptible to oxidative stress-lipid peroxidation and membrane damage. Indirect support for this comes from the observation that muscle disease is the major pathological consequence of deficiency in the major antioxidant vitamin of biological membranes, tocopherol, or vitamin E (124,125).

(5). Skeletal muscle is highly dependent on calcium utilization. Oxidative stress/nitrosative stress is known to deleteriously effect calcium homeostasis by damaging mitochondria and calcium regulatory proteins of the sarcoplasmic reticulum such the ryanodine-sensitive calcium release channel and the calcium-dependent ATPase (126-128). In addition, rises in intracellular calcium can activate enzymes which are known to contribute to oxidative/nitrosative stress such as xanthine oxidase, the endothelial and neuronal nitric oxide synthases (eNOS, nNOS), and phospholipase A_2 (1).

(6). The turnover rate of skeletal muscle proteins is slower than that seen for proteins in most other tissues (129). Therefore, skeletal muscle proteins may be partially susceptible to accumulations of free-radical mediated modifications, damage, and dysfunction.

1.5.2 Specific skeletal muscle targets of ROS/RNS

ROS/RNS regulate force production in unfatigued skeletal muscle. Low basal levels of ROS and $NO^{\bullet}$ are essential to normal contractile function. Antioxidants, such as SOD and CAT, can decrease force production, while moderate ROS supplementation causes force to rise (119). On the other hand, high concentrations of ROS and RNS such as that experienced during periods of fatiguing exercise, ischemia-reperfusion, and sepsis decrease force production (36). As we have seen, ROS/RNS can modify or damage all major categories of cellular macromolecules. Several specific targets of these species have been identified in skeletal muscle (Fig. 3).

Membrane Cell Surface Membrane (Sarcolemma).

Membrane disruption is a characteristic feature of several pathophysiological conditions of skeletal muscle including dystrophic pathology, sepsis-related muscle injury, and exhaustive exercise (130-132). Sarcolemmal lesions also occur in normal muscle as a result of mechanical stress, such as that imposed during forceful lengthening (eccentric) contractions (133). Due to a high proportion of PUFAs, the sarcolemma is extremely susceptible to lipid peroxidation reactions (1).

A good example of sarcolemmal damage which is primarily radical-mediated is ischemia/reperfusion (I/R) injury. It is well established that skeletal muscle I/R results from the production of ROS (134-142). The major source of free radical production in skeletal muscle subjected to I/R is believed to be xanthine oxidase (XO) (134,138,143). Normally this enzyme exists as xanthine dehydrogenase (XDH), but during ischemia it is converted to xanthine oxidase by proteolytic cleavage or thiol oxidation (144,145). XO produces $O_2^{\bullet-}$

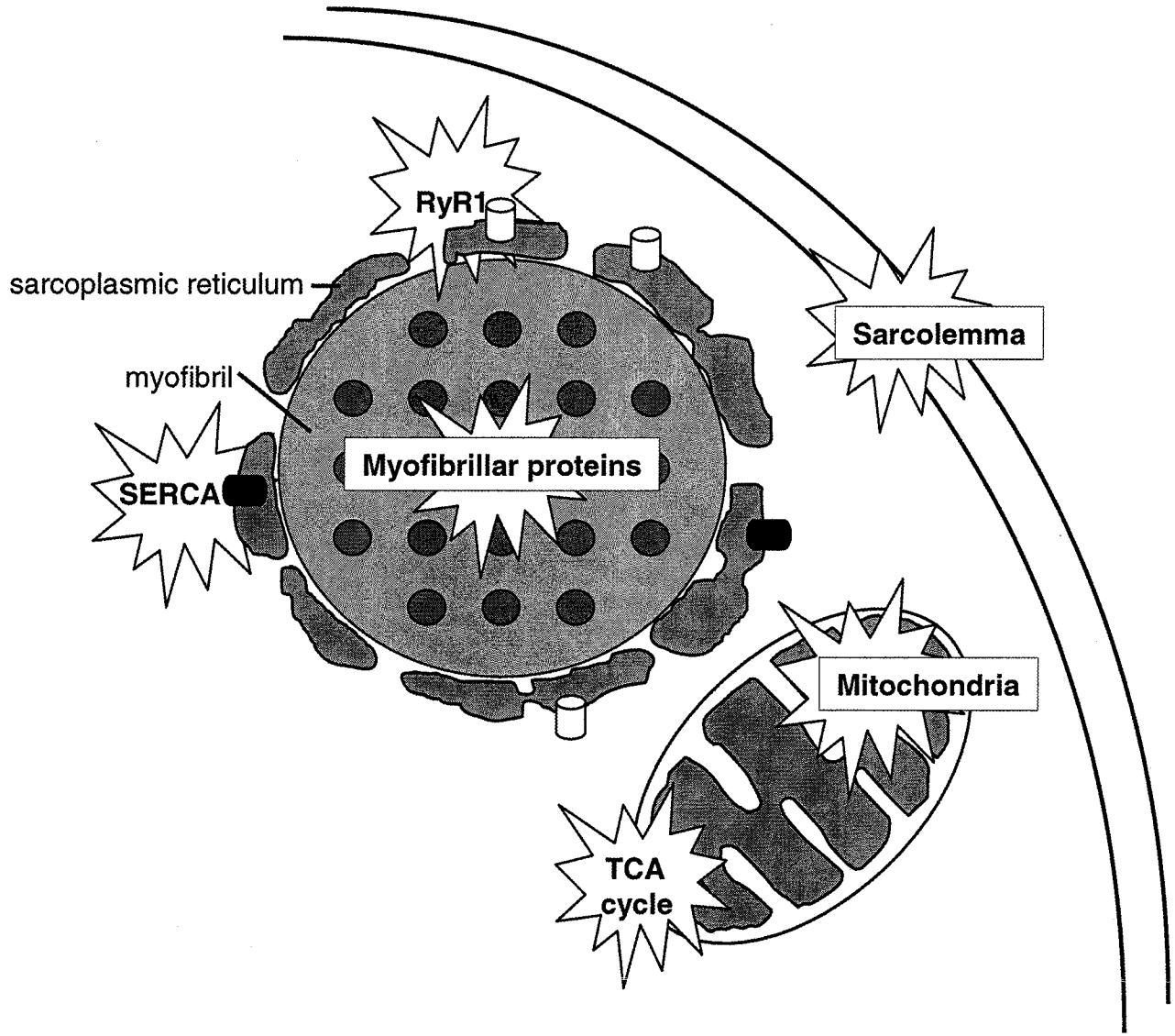

Figure 3. Illustration of a myofiber in cross-section showing potential targets (bold) of damage due to oxidative/nitrosative stress in skeletal muscle. RyR1, ryanodine receptor Ca^{2+} release channel; SERCA, sarcoplasmic/endoplasmic reticulum calcium-dependent ATPase; TCA cycle, tricarboxylic acid cycle.

and H_2O_2 upon reperfusion, and these moderately reactive species are then believed to give rise to $^{\bullet}OH$, the primary damaging radical in skeletal muscle I/R injury (134). Immunolocalization techniques have revealed that XO is present at the sarcolemma and in mitochondria of myofibers, as well as in the skeletal muscle capillary endothelium (146-148). The major source of XO during I/R may be vascular endothelial cells, as these cells possess several fold more XO expression than do the myofibers (149,150). Another consequence of ischemia is a rise in intracellular calcium levels (151). Excess intracellular calcium may also promote $NO^{\bullet}$ production from NOS (152). $NO^{\bullet}$ could react with $O_2^{\bullet -}$ to produce $ONOO^-$, or with the returning O_2 to produce other RNS. An additional insult comes from inflammatory cells (153). The initial reactive species produced in the endothelium and myocytes may act as signals for leukocytes, which then produce their own round of ROS. In this regard, neutrophils have also been shown to induce sarcolemmal damage in vitro by ROS-dependent mechanisms (98).

Choudhury et al (135) were among the first to study the effects of I/R on skeletal muscle membrane integrity. Using an isolated canine gracilis muscle model, these authors showed that I/R caused significant increases in serum CK and in the uptake of the low molecular weight tracer compound Evans blue dye, both well established indicators of sarcolemmal injury. Furthermore, these authors found that the lipid soluble antioxidant coenzyme Q10 reduced this membrane damage. More recently, Nanobashvili et al (142) made a detailed investigation of sarcolemmal injury in skeletal muscle of rabbits subjected to hindlimb I/R. In this study, electron microscopy revealed severe sarcolemmal injury, which was associated with myofiber swelling, increased serum CK and MDA, and decreased isometric force production.

Sarcoplasmic Reticulum

It is well established that calcium-regulatory proteins of the sarcoplasmic reticulum (SR) are subject to modulation by ROS and NO• (36). The ryanodine receptor Ca^{2+} release channel (RyR1), a principal excitation-contraction coupling component is one of the major targets of these interactions. Excitation-contraction coupling has been defined as the process of coupling electro-chemical events at the sarcolemma to the release of Ca^{2+} from the SR (155) . RyR1 regulates the release of Ca^{2+} into the cytoplasm leading to muscle contraction. Several authors have described the modulation of RyR1 activity by ROS and NO• (80, 81,156,157). Briefly, ROS increase the probability of RyR1 channel opening by inducing disulfide cross-links between its sulfhydryl-containing subunits, and these effects are opposed by NO•.

The sarcoplasmic/endoplasmic reticulum Ca^{2+}-dependent ATPase, (SERCA), which is responsible for scavenging Ca^{2+} from the cytosol following contraction resulting in muscle relaxation, is another potential SR protein target of ROS/RNS (36). Several sulfhydryls in the vicinity of the SERCA active site modulate its activity. These sulfhydryls must be oxidized in order for Ca^{2+} uptake to take place. However, greatly increased levels of ROS (oxidative stress) inhibit SERCA activity (146,158-160). Exaggerated levels of RNS (nitrosative stress) induce protein nitrations and thiol oxidations, which also inhibit Ca^{2+} uptake by SERCA (161-163). I/R-injury in the heart results in ROS/RNS-mediated damage to the cardiac isoform of SERCA (SERCA2a), while the chronic oxidative stress associated with aging damages the skeletal muscle isoforms of the enzyme (124,163-165). Furthermore, oxidation of SERCA as well as RyR1 result in increased cytosolic calcium

levels. Therefore, oxidative/nitrosative stress may cause a loss of calcium homeostasis. The ability of elevated Ca^{2+} levels to promote further oxidative/nitrosative stress through XO, NOS, and phospholipase activation may intensify these deleterious processes by promoting a vicious cycle.

Myofibrillar Proteins.

In a similar fashion to the Ca^{2+}-regulatory proteins of the SR, myofibrillar proteins respond to ROS in a biphasic manner. In single skeletal muscle fibers, low physiological levels of ROS increase myofilament force production, while higher levels (oxidative stress) induce contractile dysfunction by damaging myofibrillar proteins (36,166-168). The exact myofibrillar targets of ROS regulation/damage are not well defined. Myosin heavy chains and troponin contain sulfhydryls and as such these are prime candidates. In addition, the myofibrillar isoform of creatine kinase (MM-CK) has been shown be highly susceptible to attack by $ONOO^-$ (169,170).

Sepsis is a well characterized pathophysiological condition in which oxidative/nitrosative induces contractile dysfunction (171). To specifically examine how ROS/RNS affects contractile protein function in sepsis, Callahan et al (171) used single Triton-skinned diaphragmatic fibers from rats given endotoxin alone or with various anti-oxidant agents. As in whole muscles, endotoxin reduced maximal force production and these effects were reversed by SOD and NOS inhibitors. Furthermore, polyacrylamide gel electrophoresis (PAGE) revealed a loss of contractile proteins from skinned fibers of endotoxin treated animals. Importantly, both SOD and the NOS inhibitors protected against

loss of contractile proteins, suggesting that $ONOO^-$ was responsible. Along these same lines, Supinski et al (172) showed that $ONOO^-$ impairs contractile protein function in vitro.

Tricarboxylic Acid (TCA) Cycle Enzymes and Mitochondria

Certain enzymes of the TCA cycle, including aconitase and isocitrate dehydrogenase (ICDH) are particularly susceptible to inactivation by ROS. Andersson et al (32) showed that $O_2^{\bullet -}$, or $NO^{\bullet}$ donors, or a combination of both, could significantly reduce aconitase activity in strips of rat soleus muscle. Aconitase is believed to be vulnerable to ROS/RNS-mediated inactivation due to its easily oxidizable active-site iron-sulfur cluster (27-31). ICDH does not contain such a site, but in vitro studies of this enzyme have shown that it is susceptible to ROS-mediated protein carbonyl formation, unfolding, peptide cleavage, and thiol nitrosylation resulting in inactivation (48,173). As discussed earlier ICDH plays an antioxidant role by providing reducing equivalents of NADH/NADPH to GR, CAT and thioredoxin. Therefore, inactivation of this enzyme by ROS may have significant deleterious effects on cellular redox status. Indeed, the pathophysiology of several forms of mitochondrial disease has been attributed to oxidative damage (103). Friedreich's ataxia is a neuromuscular disease caused by mutations in the mitochondrial protein called frataxin (174). This disease is associated with increased levels of mitochondrial iron, as well as aconitase and ICHD inactivation due oxidative damage (175). Antioxidant treatment with a combination of vitamin E and coenzyme Q was found to beneficial to skeletal muscle ATP production in Friedreich's ataxia patients (174). Mitochondrial MnSOD (SOD2) mutant mice display dilated cardiomyopathy associated with oxidative mtDNA damage and

aconitase inactivation which is believed to result from increased mitochondrial ROS production (176). In humans, dilated cardiomyopathy has been associated with aconitase inactivation (177). Mitochondrial defects related to oxidative/nitrosative stress, including decreased cytochrome c oxidase and NADH:CoQ oxidoreductase activities, and diminished SOD2 levels have also have also been found in skeletal muscles of ALS patients (4,6).

1.6 OXIDATIVE/NITROSATIVE STRESS IN NEUROMUSCULAR DISEASE

Amyotrophic lateral sclerosis (ALS) and Duchenne muscular dystrophy (DMD) are two fatal neuromuscular diseases for which oxidative/nitrosative stress has been implicated in skeletal muscle dysfunction and damage. ALS and DMD patients die due to respiratory insufficiency because of involvement of the diaphragm and other respiratory muscles. Muscle weakness in ALS is, for the most part, believed to be due to secondary denervation atrophy following motor neuron degeneration. DMD, on the other hand, is a primary myopathic process. Contractile dysfunction and loss of strength in this disease is a direct result of myofiber destruction. The exact pathogenic mechanisms of both diseases have not been fully elucidated and no effective treatments exist.

1.6.1 Neurogenic Disease: Amyotrophic Lateral Sclerosis

ALS is the most common (prevalence of 4-6 per 100 000 people) motor neuron disease of adults. ALS is characterized by selective degeneration of large motor neurons of the cortex, brainstem and spinal cord leading to progressive muscle weakness, atrophy and eventual paralysis (178). Patients usually die within 2-5 years of disease onset due to

paralysis of the diaphragm and other respiratory muscles (179). Clinically indistinguishable familial and sporadic forms of ALS exist, with the latter representing approximately 90% of all cases. There is no cure and no effective treatment for ALS and the causes of most occurrences remain unresolved. Current evidence suggests that a complex interaction of genetic and environmental factors contribute to ALS pathogenesis, including oxidative/nitrosative stress, the formation of aberrant protein aggregates, glutamate excitotoxicity, and mitochondrial dysfunction (180). However, a first major breakthrough in understanding the pathogensis of ALS came with the discovery that approximately 20% of familial ALS cases are due mutations in the SOD1 gene (181).

1.6.1.1 SOD1 mutations result in a cytotoxic gain-of-function

More than 100 unique SOD1 mutations have been found familial ALS patients (180). The great majority of these mutations are dominantly inherited conversions of single amino acids. Initially, it was suspected that familial ALS-linked SOD1 mutations caused disease by a loss of dismutase activity. However, contrary to what would be expected of a loss-of-function mutation, age of disease onset and severity of progression do not correlate with mutant enzyme activity (178). In addition, many ALS-causing mutant enzymes maintain considerable activity. For instance, SOD1-G37R (conversion of glycine to arginine at amino acid position 37) maintains full dismutase activity (182). Furthermore, several lines of transgenic mice overexpressing various mutant enzymes developed an ALS-like motor neuron disease, while SOD1 knock-out and wild-type SOD1 overexpressing mice do not (183-187). Together these results suggest ALS-linked SOD1 mutations result in a toxic gain-of-function. Two hypotheses to explain how this may occur have been proposed. First,

the oxidative damage hypothesis suggests that less-tightly bound copper or zinc in mutant SOD1 results in aberrant oxidative chemistry, in which the enzymes use abnormal substrates such as H_2O_2 and $ONOO^-$, thereby resulting in hydroxyl radical production and nitration of protein tyrosine residues (67,68). Secondly, an oligomerization hypothesis states that SOD1 mutations result in misfolded proteins, which form aggregates that overwhelm heat-shock proteins, choke the ubiquitin-proteasome pathway and interfere with the regulation of important proteins related to cell survival (72,73,75,188). It should be noted that these two types of hypotheses are not mutually exclusive.

1.6.1.2 Mutant SOD1-mediated oxidative/nitrosative stress

Findings of elevated protein carbonyl formation, nitration, and glycation in ALS patients and mutant SOD1 transgenic mice have strengthened the proposal that mutant SOD1-mediated oxidative/nitrosative stress plays a role in this disease (189). The main role of wild-type SOD1 is dismutation of $O_2^{\bullet -}$. However, under rare circumstances SOD1 can catalyse other reactions. These include peroxidation reactions (the conversion of H_2O_2 to $^{\bullet}OH$), and the heterolytic cleavage of protonated peroxynitrite (ONOOH) to form the nitronium ion, a potent nitrating species (1,190-192). Early x-ray crystallography studies comparing mutant ALS-linked SOD1 with the wild-type enzymes revealed that the active-site channel of mutant enzymes is slightly larger than that of the wild-type (193). This suggested that less-tightly folded enzymes might have a reduced ability to bind copper or zinc and might use unusual substrates, such as H_2O_2 and ONOOH, to promote the production of deleterious ROS and RNS (67,68, 69). All ALS-linked SOD1 mutant enzymes bind copper to some degree (194) and copper chelators have been found to provide

protection against mutant SOD1-mediated toxicity (195,196). Wiedau-Pazos et al (197) reported that SOD1 mutant enzymes display a Cu-dependent increase in the use H_2O_2 as a substrate, and that peroxidase activity of these mutant enzymes was more susceptible to inhibition by copper chelators than was this reaction of the wild-type SOD1. These findings were supported by two independent reports of increased free radical production by SOD1 mutant enzymes (68,198). Furthermore, Crow et al (69) showed that some SOD1 mutants bind zinc less tightly than the wild-type enzyme. Subsequent work revealed that when introduced into primary motor neuron cultures, mutant or wild-type SOD1 enzymes depleted of zinc caused rapid cell death, which was dependent nNOS activity and caused increased nitrotyrosine formation (199).The toxicity associated with these enzymes also required bound copper. The most obvious histopathological feature of motor neurons in mutant SOD1 transgenic mice is vacuolized degenerating mitochondria (185,187,200,201). Abnormal mitochondria have also been found in anterior horn cells of ALS patients (202). This lends support to the oxidative/nitrosative stress theory, as it is well established that mitochondria are both vulnerable targets of oxidative stress and potent sources of reactive oxygen species (ROS) (203). Recent evidence also suggests mitochondrial defects and oxidative/nitrosative stress may not be restricted to motor neurons in ALS (4,6-8).

1.6.1.3 Skeletal muscle and ALS.

ALS patients die due to skeletal muscle weakness, particularly of the diaphragm and other respiratory muscles (179,204). Muscles of these patients show typical histopathological signs of denervation including small angular myofibers and fiber-type grouping. However, several lines of evidence suggest that skeletal muscles also display additional primary

defects, other than denervation, which might to contribute to disease pathogenesis. Weidemann et al (4) examined mitochondrial function in skeletal muscle homogenates and saponin-permeabilized muscle fibers from biopsy samples of ALS and spinal muscular atrophy (SMA) patients and compared these to healthy control subjects. These authors found decreased activity of NADH:CoQ oxidoreductase, a mitochondrial DNA (mtDNA) encoded enzyme, as well as decreased respiratory chain activity in ALS. These changes did not appear to be related to muscle denervation because they did not occur in the samples from patients with SMA, another denervating disease. This group later confirmed their results in another group of ALS patients and also found decreased activity of cytochrome c oxidase (COX), another mtDNA encoded enzyme, as well as mtDNA defects, and diminished levels of MnSOD in skeletal muscle samples from ALS patients (6). A COX microdeletion in skeletal muscle of a ALS patient had previously been reported (205). Siliciano et al (8) investigated oxidative metabolism in exercising muscles of ALS patients, healthy subjects and non-ALS chronically denervated controls. These authors assessed venous lactate levels, a marker of in vivo mitochondrial function in contracting muscles, and found significantly greater increases in the ALS group than in the control groups. Most recently, these same authors examined blood levels of lipid peroxides and found significant increases in ALS patients during exercise and at rest. This indicator of lipid peroxidation also correlated with blood lactate levels during exercise (8). Collectively, these results suggest that ROS-induced mitochondrial damage and respiratory chain dysfunction in skeletal muscle may contribute to the pathophysiology of ALS.

SOD1 is a ubiquitously expressed enzyme and transgenic mice overexpressing mutant SOD1 show very high levels of this enzyme in skeletal muscle (185). Leclerc et al

(7) studied skeletal muscle mitochondrial respiration properties in the mutant SOD1-G93A transgenic mouse model of ALS. First of all, these authors found greater total SOD1 activity in skeletal muscles than in the spinal cord or brain. Maximal oxygen consumption was decreased in saponin-permeabilized fibers from transgenic soleus (an oxidative muscle), but not in the transgenic EDL (a glycolytic muscle) relative to controls. Fibers from the soleus also displayed a decreased Km of oxidative phosphorylation for ADP, suggesting decreased oxidative metabolism in this muscle group.

Transgenic mice which overexpress the wild-type enzyme do not develop motor neuron disease, but have some subclinical features similar to that seen in the mutant SOD1 mice. For instance, the neuromuscular junctions of wild-type SOD1 transgenic mice display abnormalities reminiscent of pathology seen in ALS-like motor neuron disease. In addition, two studies of wild type SOD1 transgenic mice have described skeletal muscle abnormalities ranging from increased ROS production to full-blown dystrophic pathology. Using electron paramagnetic resonance spectroscopy, Peled-Kamar et al (206) found that free radical production in skeletal muscles of wild type SOD1 transgenic mice was significantly higher than in normal mice. Electromyography (EMG) of these muscles, however, showed signs of muscle denervation rather than a primary myopathy. In another investigation of wild type SOD1 transgenic mice, Rando et al (45) found a severe age-dependent skeletal muscle pathology very reminiscent of muscular dystrophy. Selected muscle groups displayed atrophy, necrosis, and central nucleation, with fatty cell and connective tissue infiltration (45). Serum creatine kinase was significantly increased over levels seen in non-transgenic controls. Finally, the TBARS assay revealed greater lipid peroxidation bi-products in skeletal muscles of the transgenic animals relative to controls.

Together these results suggest overexpression of wild-type SOD1, at least in this particular strain, causes a primary myopathy.

In the wild-type SOD1 transgenic model, it is speculated that muscle damage is caused by the increased SOD1-mediated conversion of $O_2^{\bullet -}$ to H_2O_2, without concomitant increases in GPx or CAT to remove excess H_2O_2. Several reports from other cell types, including neurons, support this argument (207,208). However, muscle pathology in SOD1 overexpressing mice is not a consistent finding (209). Such discrepancies may involve the genetic background of the mice in question (210). Interestingly, one patient with Down's syndrome, a condition associated with increased SOD1 activity, was found to have a muscular dystrophy-like phenotype (211). These reports are relevant to the various mutant SOD1 models of ALS, because in these models the mutant SOD1 enzymes being overexpressed often maintain a high level of activity. In particular, the mutant enzyme which is overexpressed in the SOD1-G37R transgenic mice is fully active. Hence when combined with the endogenous mouse SOD1, these animals have higher than normal SOD1 activity. Therefore, it is possible that some mutant SOD1 transgenic mice and some SOD1-linked ALS patients suffer primary skeletal muscle damage in addition to denervation atrophy, which might contribute to muscle weakness.

1.6.1.4 Lessons from animal models of SMA and DMD

Examples of dual myogenic and neurogenic components in neuromuscular disease and their potential interactions, come from findings in animal models of spinal muscular atrophy (SMA) and Duchenne muscular dystrophy (DMD). SMA is an autosomal recessive disease and one of the most common genetic causes of death in children (212). SMA is caused by

mutations in the gene known as survival motor neuron (SMN) and is characterized by degeneration of motor neurons of the spinal cord with associated muscle weakness, atrophy and paralysis (213). The SMN gene exists in duplicate in humans and a truncation 5 nucleotides distinguishes SMN from its copy, SMN^c (214). The full-length protein is produced mostly from SMN, while SMNc gives rise to a smaller form. The protein products of these genes are ubiquitous and localize to the cytosol and to a multi-protein nuclear complex known as a gem (gemini of coiled bodies), which plays a role in spliceosome assembly and the recycling of splicing factors following pre-mRNA processing (20,215,216).

In mice, the SMN gene is not duplicated and this may explain why targeted deletions of SMN are embryonic lethal (217-219). Therefore, conditional knock-out approaches have been employed in order to generate a viable mouse model of SMA (218-221). In one such approach, a deletion of exon 7 of SMN was made in neurons, but not in skeletal muscle (218). These mice displayed tremors at 2 weeks of age and had a mean life span of only 25 days. Cellular abnormalities found in the spinal cord included vacuolation of cytoplasm and deformation/fragmentation of motor neuron nuclei. In addition, gems were not detected in motor neurons of these mice. Instead, large nuclear aggregates of coiled-body specific proteins were found. Detailed examination of skeletal muscles revealed typical signs of denervation atrophy including small angular fibers and upregulation of extrajunctional acetylcholine receptors.

However, in a similar study by the same group, the conditional knock-out was made specifically in skeletal muscle, in order to determine whether the gene defect in skeletal muscle plays a role in SMA pathogenesis (221). Similar to the motor neuron-specific SMN

knock-out mice, these animals exhibited an average life span of only 33 days. At 3 weeks of age, these mice displayed reduced spontaneous and induced motor activity and exhibited only rare necrotic myofibers. At 4 weeks of age, however, many of the features of muscular dystrophy were found, including necrosis, central nucleation, variation in fiber size, and muscle infiltration by connective tissue and inflammatory cells. In addition, these mice had serum creatine kinase levels six times higher than normal, and Evans blue dye uptake into myofibers was significantly increased even at 3 weeks of age. The latter findings indicate sarcolemmal injury, suggesting that skeletal muscle pathology is an early feature in these mice. Interestingly, these muscles also displayed a decrease in dystrophin, the cytoskeletal protein which is absent in Duchenne muscular dystrophy, and the decrease in dystrophin correlated with Evans blue dye uptake. Morphology or number of motor neurons did not change in these mice.

Since, skeletal muscles of SMA patients do not show the kind of overt myopathic changes described in the skeletal muscle-specific SMN knock-out mouse, it may be that denervation masks an underlying myopathic potential, especially if the latter is based on muscle fiber activity. Support for this idea comes from genetic crosses mice with primary neurogenic and myogenic defects. The most widely used animal model of DMD is the mdx mouse. To examine the effect of denervation on the dystrophic process, Suh et al (1994) crossed mdx mice with gad mice, which exhibit a peripheral neuropathy. Gad/mdx mice were found to have lower serum creatine kinase levels and fewer centrally nucleated fibers than mdx mice. These data suggest that denervation may actually mask an underlying primary myopathic process. Therefore, it is possible that a similar situation exists in ALS,

particularly given the evidence for muscle changes which are not characteristic of denervation outlined earlier.

1.6.2 Myogenic Disease: Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is a fatal X-linked recessive disorder affecting 1 in 3500 male births. This disease is caused by genetic mutations which result in the absence of a large (427 kDa) cytoskeletal protein known as dystrophin, and is characterized by progressive muscle destruction, fibrosis, and fatty cell infiltration leading to muscle weakness. Patients die in their early 20s, usually due to respiratory insufficiency. The mdx mouse, the most widely used animal model of DMD, has a premature stop codon in the dystrophin gene and consequently lacks the protein product (222,223) . One of the most prominent features of dystrophic muscle pathology in both DMD patients and mdx mice is increased membrane fragility (130,224).

In normal skeletal muscle, dystrophin exists as part of a multimolecular complex, referred to as the dystrophin-glycoprotein complex (DGC), which spans the sarcolemma and thereby permits a physical linkage to be established between the intracellular cytoskeleton and the extracellular matrix of myofibers (225,226). Dystrophin itself is a submembranous rod-shaped protein, which attaches to the cytoskeletal network of F-actin filaments via its N-terminus and portions of its rod-like domain. Binding to the associated members of the DGC occurs via its cysteine-rich domain and the first portion its C-terminus. In addition to dystrophin, the DGC is comprised of three distinct subgroups of proteins referred to as the dystroglycan, sarcoglycan-sarcospan and syntrophin subcomplexes. Also, a protein with considerable structural homology to dystrophin, known

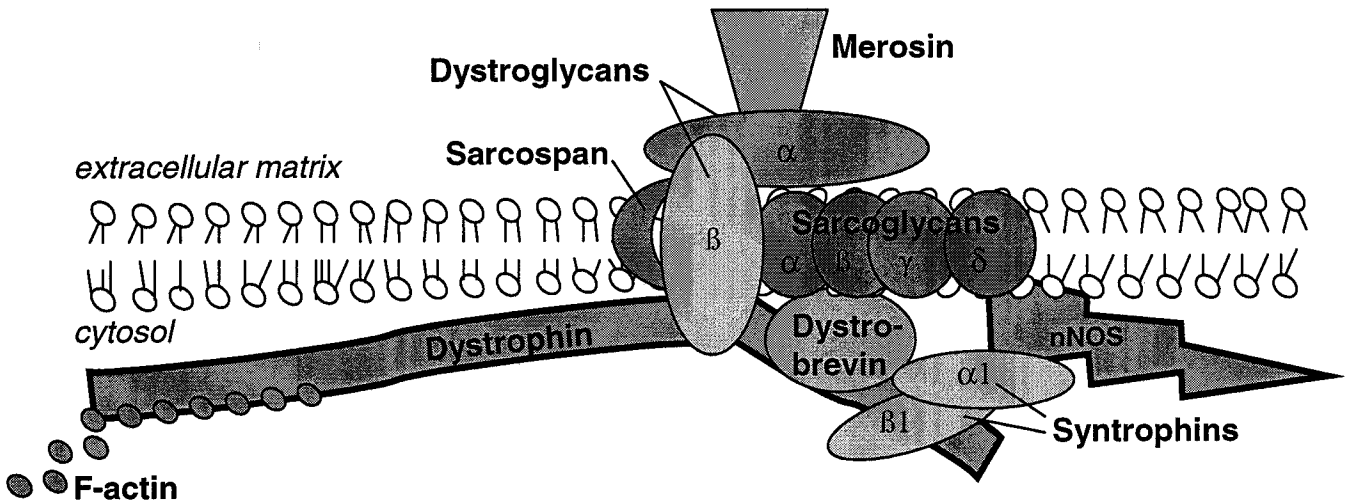

Figure 4. The dystrophin-glycoprotein complex.

as dystrobrevin, and potential signalling molecules, including neuronal NOS (nNOS) are found to be part of the DGC (227-230). In DMD patients and mdx mice, the primary deficiency of dystrophin destabilizes the entire multimolecular complex, thereby producing a secondary loss of other members of the DGC and a mislocalization of nNOS from the sarcolemma to the cytosol, where the activity level of the enzyme is significantly reduced (231,232).

Despite enormous progress in our understanding of the molecular basis of DMD, the precise function of dystrophin and the DGC and, hence, the mechanisms by which its absence causes progressive muscle pathology, remain unclear. Structural similarity between dystrophin and the erythrocyte membrane-stabilizing protein, spectrin, as well as evidence of abnormal membrane fragility in dystrophin-deficient muscles, suggest that the absence of dystrophin causes a mechanical weakening of the sarcolemma. In addition to this membrane fragility hypothesis, other candidate pathogenic mechanisms implicated in the primary initiation of myofiber injury in dystrophin-deficiency include inappropriate calcium influx, aberrant cell signalling, increased oxidative stress, and, most recently, recurrent muscle ischemia during muscle activity as a result of the secondary loss of nNOS from the sarcolemma. These potential mechanisms are not mutually exclusive. For instance, it is well established that I/R causes severe muscle damage via oxidative stress. In addition, oxidative stress has been shown to cause membrane fragility and to modify with cellular signalling events (89,104).

1.6.2.1 Oxidative/nitrosative stress in dystrophic muscle

Pathological similarities between inherited muscular dystrophies and oxidative stress-induced myopathic conditions, such as that resulting from ischemia-reperfusion, exhaustive exercise, as well as vitamin E and GSH deficiencies, led to the oxidative-stress hypothesis of DMD. Omaye and Tappel (233), who were among the first to test this hypothesis, studied dystrophic chickens and mice (strain 129/Re, prior to the discovery of the mdx line), and found that GPx and GR activities as well as TBARS were increased in these animals. In addition, increases in total GSH, the GSSG/GSH ratio and protein carbonyl formation have been found in some dystrophic animals (234-239). Although these animal models do not have the same genetic basis (i.e. dystrophin deficiency) as the human disease, similar changes were found in skeletal muscles of DMD patients (237,240-244).

1.6.2.2 Oxidative stress in the mdx mouse

More recently, the role of oxidative/nitrosative stress in DMD has been studied using the mdx mouse, which is genetically orthologous to DMD. Ragusa et al (245,246) compared antioxidant enzymes and TBARS in skeletal muscle of mdx and normal C57 mice in two separate studies. In their first study, these authors reported increased GPx activity in the mdx gastrocnemius, as well as increased SOD1, GR, and non-SeGPx activities in the mdx diaphragm. GPx activity on the other hand, was decreased in the diaphragm and no differences were seen in MnSOD activity between the two mouse strains (245). In contrast, these authors later reported increased MnSOD activity in both the diaphragm and gastrocnemius of mdx mice, and decreased SOD1 and GR activity, but increased GPx

activity in the mdx diaphragm (246). Only the reported increase in GPx activity in the gastrocnemius was in agreement between these two studies. The diaphragms were excised from slightly older animals in the earlier study, which may account for some of the discrepancies. In the latter study, the authors also reported increased CAT enzyme activity in the mdx gastrocnemius and increased TBARS in all mdx muscles studied including the diaphragm, gastrocnemius, soleus and extraocular muscles. Finally, this study also examined the effect of hyperoxia on antioxidant upregulation and muscle TBARs content in mdx muscle. Normal and mdx mice were subjected to 80-85% oxygen for 3 days. Greater antioxidant upregulation (for CAT and MnSOD) was seen in the normal mice than in the mdx. In addition, with the exception of the extraocular muscles from the mdx mice, TBARs only increased in the normal mice exposed to hyperoxia. These results seem to suggest that the upregulation of antioxidant enzymes at baseline in the mdx mice is sufficient to protect against further lipid peroxidation due to oxidative stress, at least, in terms of that due to hyperoxia.

As we have seen, mitochondria are a major source of oxygen-derived free radicals. It is believed that mitochondria-derived free radicals contribute substantially to the aging process by damaging components of the electron transport chain, thus decreasing the efficiency of oxidative phosphorylation and interfering with the production of ATP (247). Faist et al (248) studied mitochondrial oxygen metabolism and antioxidant capacity in mdx mice and examined the effect of old age on these parameters. Adult (52 wks old) and elderly (80 wks old) mdx animals were included in this study. Mitochondrial oxygen consumption was found to be lower in adult mdx than in normal adult C57 mice, and dropped to greater extent with old-age in the dystrophin-deficient animals. Tissue levels

of GPx and α-tocopherol increased in C57 mice, but decreased significantly in mdx mice with old age. This study suggested that the efficiency of mdx mitochondrial electron transport decreases abnormally with age and that normal cellular antioxidant adaptation to aging is impaired or exhausted in these mice. Mitochondrial deficits in mdx could lead to a reduction in the ATP:ADP ratio. Excess ADP is believed to form complexes with ferrous-iron (Fe^{2+}) leading to the production of hydroxyl radicals (243). Indeed, it had previously been reported that skeletal muscles from DMD patients and mdx mice have decreased levels of ATP and an abnormally low ATP:ADP ratio (249,250). In addition, Chen et al. (251) used a 5600 gene microarray and found large scale downregulation of mitochondrial-expressed genes in skeletal muscles from DMD patients.

Increased production of free radicals by mitochondria also occurs during exercise. Healthy muscle adapts to regular exercise by increasing electron transport proteins and thus mitochondrial oxygen consumption, and by upregulating antioxidant enzymes (252). Faist et al (253) examined the effect of low intensity exercise on oxygen consumption and antioxidant status in mdx mice. Normal C57 and mdx mice were either kept sedentary or subjected to 240 m of treadmill running twice a day for 6 weeks. The authors reported that unlike normal muscles, mdx muscles subjected to exercise displayed abnormal mitochondrial oxygen consumption as well as large increases in mitochondrial TBARS and serum creatine kinase levels. In addition, exercise caused an increase in GPx activity in C57, but a decrease in the activity of this enzyme mdx muscles. This study suggests a deficiency in the ability of mdx mitochondria and antioxidant systems to adapt to exercise.

1.6.2.3 Oxidative/nitrosative stress before the onset of mdx dystrophic histopathology.

Muscle fiber necrosis does not occur in mdx mice until approximately 3 weeks of age. Studies of this so-called pre-necrotic stage are attractive because they can potentially provide important information regarding the factors which trigger the onset of degeneration in dystrophin-deficient muscles. Such studies have revealed that apoptosis (254) and contractile dysfunction (255) are characteristics of pre-necrotic mdx skeletal muscle. Studying the role of oxidative/nitrosative stress in the pre-necrotic stage is additionally advantageous because such investigations are not complicated by the fact that necrotic tissue, itself, as well as invading inflammatory cells can act as sources of free radicals. Disatnik et al. (12) examined the expression of antioxidant genes, TBARS, and nitrotyrosine content in pre-necrotic mdx skeletal muscles. As early as 15 days of age, mdx limb muscles showed increased transcription of genes encoding SOD1, SOD2, GPx, and CAT, as well as greater TBARS content relative to C57 controls. Nitrotyrosine was only increased in mdx muscle relative to controls after the onset of necrosis. In light of the fact that membrane injury does not occur in the pre-necrotic phase (130), this suggests that oxidative stress and damage to membrane lipids may be a primary pathogenic mechanism in dystrophic pathology which predisposes the sarcolemma to abnormal fragility.

1.6.2.4 mdx myotubes are abnormally susceptible to oxidative/nitrosative stress

Another means of studying the role of oxidative stress in mdx muscle pathology without the contribution of cell death and inflammation is by using primary cell cultures to study

pure populations of mdx myoblasts or myotubes. Rando et al (256) examined the susceptibility of mdx cells to oxidative stress and found that mdx myotubes were more susceptible than C57 myotubes to cell death due to various pro-oxidants including H_2O_2, paraquat (which generates $O_2^{\bullet -}$ (257)), menadione (a free radical producing redox cycling compound (258)), AAPH (2,2'-amino azo bis amidopropane hydrochloride, an initiator of lipid peroxidation (259)), and SNP (sodium nitroprusside, generates $NO^{\bullet}$, which reacts with endogenous $O_2^{\bullet -}$ to produce $ONOO^-$ (37,260)). No differences were seen between myotubes from the two strains when exposed to non-oxidant cellular toxins, such as staurosporine. Furthermore, myoblasts, which do not express dystrophin even in the normal muscle cells, died to the same extent when exposed to the pro-oxidant toxins. These results suggest that dystrophin-deficient mdx myotubes are abnormally vulnerable to cell death specifically due to oxidative stress. In a later study, these same authors further examined this in vitro susceptibility to oxidative stress by comparing primary cultures from transgenic mice expressing truncated forms of dystrophin associated with dystrophies of varying severity (261). They found that the relative susceptibility of the myotube cultures to oxidative stress correlated with severity of the myopathy in the transgenic strain from which the cultures were derived.

1.6.2.5 Contraction-induced sarcolemmal injury and oxidative/nitrosative stress

Several authors have shown that skeletal muscles of mdx mice are abnormally susceptible to damage caused by mechanical stress (13,262-265). Petrof et al (13) showed that dystrophin-deficient muscles of the mdx mouse exhibit an increased susceptibility to contraction-induced force deficits and sarcolemmal rupture. While membrane fragility

appears to play a major role in dystrophic pathology, the question remains as to whether this is the primary factor leading to muscle cell degeneration. A recent study in pre-necrotic muscle of mdx mice suggests this is not the case. Grange et al. (255) showed that muscles of young (9-12 day old) mdx mice are not more susceptible to contraction-induced sarcolemmal damage than muscle of age-matched normal mice. Although this protection against damage in young mdx mice might be attributed to other factors (eg. expression of utrophin, a functional homologue dystrophin), it is also possible that the absence of dystrophin by itself does not automatically make membranes more fragile. Instead, dystrophin-deficiency may act together with some other stress factor to predispose skeletal muscle fibers to membrane damage. In this regard, Ebihara et al (14) used normal rat (L6) myotube cultures growing on flexible surfaces which could be stretched and/or exposed to oxidative stress-producing compounds. These authors found that a relatively small concentration of SIN-1 (a $ONOO^-$-generating compound), or a low level of stretch, by themselves, caused only minimal membrane damage. However, when these same levels of oxidative stress and mechanical stress were combined, a significant synergistic effect was observed. The studies discussed above, which showed that oxidative/nitrosative stress occurs in mdx muscle and that dystrophin-deficient muscle cells are abnormally susceptible to this form of insult, combined with the results of Ebihara et al (14) suggest that oxidative/nitrosative stress may make the dystrophin-deficient sarcolemma abnormally fragile and more prone to contraction-induced injury. This hypothesis, which posits that oxidative/nitrosative stress and mechanical stress combine to damage dystrophin-deficient muscle fiber membranes thus leading to dystrophic pathology, has yet to be tested.

1.6.2.6 Revisiting the vascular hypothesis of DMD: The role of nNOS.

Prior to the knowledge that dystrophin deficiency forms the basis of DMD, it was postulated that dystrophic pathology was the result of some vascular abnormality resulting in skeletal muscle ischemia. This so-called "vascular theory" was based on the fact that a histopathological hallmark of dystrophic muscle, known as "grouped necrosis", resembles lesions seen in skeletal muscle following a prolonged cessation of blood flow. In support of this hypothesis, Mendell et al (266) found that experimentally-induced ischemia in rat skeletal muscle could reproduce these DMD-like features. Interestingly, it had been previously demonstrated that transient limb muscle ischemia by blood-pressure cuff application caused an increase in serum creatine kinase levels in DMD patients (267). Moreover, as discussed earlier, transient ischemia followed by reperfusion (I/R injury) causes free radical-mediated sarcolemmal damage. In spite of these reports, the vascular hypothesis fell out of favour when no vascular abnormalities or dysfunction could be linked to dystrophin deficiency. However, the recent discovery that DMD and mdx skeletal muscle suffer functional ischemia due to the loss of nNOS from the sarcolemma has rejuvenated interest in this proposal, especially in light of the fact that I/R injury might be associated with oxidative/nitrosative stress in these muscles.

1.6.2.7 nNOS is associated with the DGC in normal skeletal muscle

nNOS was so-named because it was first localized in neurons, but it is, in fact, expressed as highly in skeletal muscle as in the brain (268). Enzyme activity and mRNA levels are actually higher in skeletal muscle than in nervous tissue (268,269). A splice variant,

termed nNOSμ, containing an extra 34 amino acids, is also expressed in skeletal muscle and it appears that this slightly larger protein is the predominant form in mature myofibers (270,271). nNOS is present both in the cytosol and at the inner aspect of the sarcolemma in normal skeletal muscle (268). It is, however, most abundant at the sarcolemma, where it associates with the DGC (227) and it appears to be particularly enriched at neuromuscular (271) and myotendinous junctions (272). In rodents, nNOS is more abundant in fast-twitch type II than in slow-twitch type I fibers (269). In humans, the distribution of nNOS between slow and fast myofibers seems to be more uniform and some evidence suggests that, in fact, type I fibers may have slightly more nNOS (247). The main means of nNOS localization to the sarcolemma is believed to be binding of nNOS to α1-syntrophin through PDZ domains on both proteins (273). However, Chang et al (272) have shown that nNOS may be targeted to the membrane in the absence of dystrophin and syntrophins; and Kameya et al (274) demonstrated that membrane-associated nNOS activity is reduced 5-fold in skeletal muscle of α1-syntrophin knock-out mice, but remains 3-fold higher than in mdx mouse muscle. In addition, overexpression of nNOS in mdx myotubes resulted in some membrane bound enzyme even in the complete absence of the entire DGC. Because nNOS is also able to bind caveolin-3, the remaining membrane-associated nNOS activity in the absence of α1-syntrophin may be due to caveolin-3 binding (275,276), but this remains to be determined.

1.6.2.8 nNOS is displaced from the sarcolemma of dystrophin-deficient skeletal muscle

Using cellular fractionation, immunohistochemistry and enzyme activity assays, Brenman et al (227) first reported that nNOS was absent from the sarcolemma, but increased in the cytosol, of mdx skeletal muscle fibers. DMD patient biopsy samples, on the other hand, displayed both decreased sarcolemmal and cytosolic protein expression. The results concerning DMD patients were confirmed by Grozdanovic et al. (277) and Chang et al. (272), who reported that nNOS expression was absent or greatly reduced in the sarcolemmal fractions of DMD skeletal muscle. Contrary to the report of Brenman et al (227), however, these authors found that nNOS expression was reduced not only at the membrane, but also in the cytosol of mdx skeletal muscle. Furthermore, Chang et al., (272) reported that nNOS mRNA was also decreased in mdx skeletal muscle. While there is some disagreement as to the absolute level of expression and activity of nNOS in the cytosol of mdx mice, it is now well established that nNOS is displaced from the sarcolemma of dystrophin-deficient muscle. This has stimulated much interest in the potential contributory role of aberrant nNOS expression in dystrophic-pathology.

1.6.2.9 The role of mislocalized nNOS in dystrophic pathology

If mislocalization of nNOS plays a role in DMD, this could be due to a toxic gain-of-function in the cytosol or due a adverse loss-of-function at the sarcolemma. Brenman et al. (227) proposed that mislocalized nNOS could exacerbate muscle damage in DMD by producing nitrosative stress in the cytosol of dystrophin-deficient muscle. In this regard, regulation of mislocalized nNOS activity by calcium-calmodulin may be altered in the setting

dystrophin-deficiency, as elevated intracellular calcium is a characteristic feature of dystrophic pathology (278). As discussed earlier, the proposed roles of $NO^{\bullet}$ in skeletal muscle include antioxidant (80,91-94), anti-inflammatory (83,97), and vasoregulatory (75;47;68;130) functions. The loss of one or more of these physiological properties of $NO^{\bullet}$ at the sarcolemma, might contribute to the pathophysiology of DMD. The literature contains conflicting reports, some of which support and some of which argue against the potential deleterious roles for mislocalized $NO^{\bullet}$ in dystrophin-deficient muscle.

Chao et al (228) studied muscle biopsy samples from patients with Becker muscular dystrophy, a less severe variant of DMD in which the dystrophin protein is present in a truncated form. These authors found that the severity of muscle pathology correlated with the level of nNOS deficiency at the sarcolemma. Likewise, Crosbie et al (279) studied 7 strains of transgenic mdx mice expressing various truncated dystrophins, and found that the severity of the dystrophic phenotype correlated with the displacement of nNOS from the sarcolemma. In particular, the so-called Dp71 mdx mice, which express a truncated dystrophin missing all but the last 71 kD of the C-terminus, displayed a complete loss of nNOS from the sarcolemma and a severe dystrophic phenotype, eventhough the rest of DGC remained intact (279). These results parallel those of Disatnik et al. (261) discussed above, in which the susceptibility of myotubes to oxidative stress correlated with the degree of myopathy in the mdx transgenic strain from which the myotubes were derived. Finally, Grady et al. (280) generated α-dystrobrevin mice which retained the DGC, but displayed slightly lower levels of α1-syntrophin and a drastic reduction in nNOS at the sarcolemma. These mice developed a dystrophic phenotype very similar to that of mdx mice despite the fact that dystrophin levels were normal. Together,

these reports suggest that displacement of nNOS from the sarcolemma contributes to dystrophic pathogenesis and that oxidative stress plays a role in this process. Again, this proposed contribution could be due to the presence of nNOS in the cytosol, or the loss of the enzyme from the sarcolemma.

Several reports have refuted the concept that nNOS present in cytosol might contribute to dystrophic pathology. Two independent groups genetically removed nNOS from mdx mice by crossing them with nNOS knock-out mice and no improvement in muscle pathology was found (279,281). These results are supported by the study of Zhuang et al (141), which showed that susceptibility of cultured mdx myotubes to oxidative stress was independent of nNOS expression. These authors inhibited nNOS genetically or pharmacologically, or increased its expression using an adenovirus, and subjected the cells to various pro-oxidants. Reducing or increasing nNOS expression/activity did not affect cell death due to oxidative stress. Importantly, nNOS was also overexpressed in mdx myotubes and again this did not affect susceptibility to oxidative stress. These reports strongly disagree with the notion of displaced nNOS contributing to DMD via toxic gain-of-function in the cytosol. However, it can be argued that the experimental systems used did not accurately reproduce the specific situation of mislocalized nNOS in dystrophin-deficient muscles, or the forms of stress that these muscles are exposed to in vivo, such as exercise. It is well established that muscle damage in dystrophin-deficient muscles is greatly increased in the setting of in vitro or in vivo muscle activity (13,282,283). Therefore, to adequately test if the genetic removal of nNOS can protect against the dystrophic phenotype, these muscles would have to be subjected to some form of muscle activity. Such experiments were not performed in the double nNOS/dystrophin deficient mice.

Similarly, it is unlikely that the in vitro study of Zhuang et al (141) reproduced the kind of oxidative/nitrosative stress that muscle fibers are actually subjected to in living animals. In addition, since nNOS has many important physiological functions in skeletal muscle, the portion that remains in mdx myofibers, despite being mislocalized, may continue to play some beneficial role. If so, it is unlikely that total genetic removal of nNOS in dystrophin-deficent mice would actually improve muscle pathology, when these beneficial functions would also be lost.

1.6.2.10 The loss of nNOS from the sarcolemma causes functional ischemia

The studies of Crosbie et al. (279) and Disatnik et al. (261) suggest that the lack of NO• production at the sarcolemma contribute to dystrophic pathology because some normal NO•-mediated physiological process is lost. Thomas et al (84) found that NO• produced in normal C57 skeletal muscle attenuates norepinephrine-induced (adrenergic) vasocontriction during muscle contraction and that this effect could be reversed by NOS inhibition. On the other hand, mdx and $nNOS^{-/-}$ mice showed no such NO•-mediated modulation of the adrenergic vasoconstriction during muscle activity. In these two mouse strains, norepinephrine had the same vasoconstrictor effect whether the muscle was active or not, and L-NAME had no effect. Together these results suggest that NO• from nNOS plays a protective role in normal muscle by opposing sympathetic vasoconstriction during muscle contraction and that this response is impaired in mdx muscle, resulting in functional ischemia. This was the first evidence that an NO•-mediated physiological function was impaired in dystrophin-deficient skeletal muscle. Using near-infrared spectroscopy to measure muscle oxygenation, Sander et al. (284) extended these findings

to DMD patients, showing that children with the disease were unable to blunt the sympathetic vasocontrictor response during repetitive handgrip exercise. This functional ischemia was also a characteristic of children with spinal muscle atrophy (SMA), which also leads to a loss of nNOS from skeletal muscles. Healthy children and patients with polymyositis and limb-girdle muscular dystrophy (LGMD) all had abundant skeletal muscle nNOS and none of these subjects displayed the abnormal attenuation in muscle oxygenation during exercise. These results in DMD patients and the mdx mouse suggest a mechanism to explain how the severity of the dystrophic phenotype may correlate with the absence of nNOS from the sarcolemma. The absence of nNOS from the membrane causes functional ischemia, which when combined with the loss of dystrophin and the DGC from the membrane, could worsen the disease. Indeed, one of the most well-established conditions in which sarcolemmal damage occurs due to oxidative stress is, in fact, muscle ischemia/reperfusion. This is consistent with the results of Disatnik et al (261), who found that the severity of the dystrophic phenotype correlates with susceptibility to oxidative stress. Finally this concept of functional ischemia may also explain the longstanding observation that lesions of dystrophic muscles, such as grouped necrosis, greatly resemble histopathological markings induced by muscle ischemia.

If functional ischemia due to the loss of nNOS at the sarcolemma contributes to dystrophic pathology, one might expect that by enhancing the expression of the enzyme, particularly at the membrane, would be therapeutic. In this regard, Wehling et al (97) generated nNOS-transgenic mdx mice which had levels of $NO^{\bullet}$ production similar to that of normal C57 mice. The authors did not examine the localization of the newly introduced nNOS, but an in vitro study of Zhuang et al (141) suggested that if overexpression of the

enzyme is great enough, some will localize to the membrane fraction. Importantly, overexpression of the nNOS transgene greatly ameliorated the dystrophic phenotype in mdx mice. Central nucleation, fiber size variability, membrane damage, and serum creatine kinase all decreased in the nNOS-transgenic mdx mice. In particular, invading inflammatory cells were greatly reduced, leading the authors to propose that $NO^{\bullet}$ from the nNOS transgene prevented muscular dystrophy through its known anti-inflammatory effects, such as inhibition of leucocyte adhesion. However, the possibility that overexpression of the nNOS transgene ameliorated dystrophic pathology by preventing ischemia during muscle activity cannot be dismissed.

1.7 THERAPY FOR DMD

At present, no effective treatment is available for DMD. Corticosteroids (prednisone, prednisolone, deflazacort) provide only short-lived benefits and are associated with numerous side-effects including weight gain, cataracts, hypertension, diabetes, and behavioural changes (285-288). Therefore, the search for a safe and effective intervention for DMD continues. The wide range of potential therapeutic options under investigation include other pharmacological treatments, as well as cell-based, and gene-based therapies (289). In addition to corticosteroids, pharmacological efforts have included the use of (1) aminoglycoside antibiotics, which target ribosomes and allow translation to continue through premature stop codons in DMD mRNAs (290,291); (2) calcium channel blockers, in an effort to maintain calcium homeostasis in dystrophin-deficient muscle (292); (3) attempts to improve muscle strength by increasing muscle mass (293); and (4) antioxidant supplementation, due to the large amounts evidence implicating oxidative

stress in DMD. Cell-based therapies being investigated include myoblast (294-296) and stem cell (286,297) transplantation to repopulate dystrophin-deficient muscle with normal myofibers. Gene-based approaches include dystrophin or utrophin gene transfer using viral vectors (18,24,298,299) or naked DNA (300-303). In the final sections of this introduction we will focus on what has been done thus far in terms of two of these therapeutic strategies: (1) antioxidant supplementation and (2) dystrophin gene transfer.

1.7.1 Antioxidant therapy for DMD

Several studies and clinical trials using antioxidants have been undertaken in dystrophic animal models and DMD patients. Sefarin et al (304) described the therapeutic benefits provided by the combination of vitamin E and the sulfhydryl-containing reducing agent, penicillamine, in dystrophic chickens. Bornman et al (305) deprived mdx mice of dietary iron for six weeks, in an attempt to prevent metal catalysed free-radical production. These authors found that iron-deprivation reduced the number of invading macrophages and decreased the expression of the heat shock protein, Hsp70, in mdx muscle, but had no effect on central nucleation, serum creatine levels, or markers of lipid peroxidation. Most recently, Buetler et al (306) fed mdx mice green tea extract, for 4 weeks beginning at birth. Green tea extract is believed to possess antioxidant properties, such as the ability to trap hydroxyl and peroxyl radicals. This treatment reduced necrosis in the fast-twitch extensor digitorum longus (EDL), but had no effect in the slow-twitch soleus. Despite some degree of success in animal models, all antioxidant-based interventions attempted thus far, included trials of vitamin E, vitamin C, and SOD, have proved unsuccessful in DMD patients. Based on the promising results in dystrophic chickens, Fenichel et al (307) tested

a combination of vitamin E and penicillamine in an 18 month-long, double-blind controlled trial in 106 boys with DMD. Like its predecessors, antioxidant supplementation in this trial provided no therapeutic benefit.

One possible reason for the lack of effectiveness of these antioxidant treatments is that various antioxidants work together in elaborate systems, which allow for the detoxification of deleterious intermediate species produced as a result of the scavenging activity of the parent compounds (42). For instance, SOD works in collaboration with GPx and CAT so that H_2O_2 will not accumulate. Likewise, through its antioxidant function, vitamin E is converted to a harmful radical which must be converted back to α-tocopherol by vitamin C. As such, the use of vitamin E or SOD alone may not be very effective forms of antioxidant therapy. Recent positive trials of antioxidant vitamins for the treatment of other disorders such as cardiovascular disease (308), diabetes mellitus (309), and cystic fibrosis (310) have employed combinations of vitamins C and E. Similar combinations may prove beneficial for DMD either by themselves, or in conjunction with other potential therapeutic interventions currently under investigation, such a dystrophin/utrophin gene transfer therapy.

1.7.2 Dystrophin gene transfer using adenoviral vectors.

A promising approach to the treatment of DMD is to restore dystrophin expression in affected skeletal muscles using vectors carrying the dystrophin gene or the gene for its autosomal homolog, utrophin. An ideal vector for such therapeutic interventions should be non-toxic and produce sufficient, long-term dystrophin expression in the targeted muscles. While several potential vectors including adenoviruses, adeno-associated viruses, herpes

simplex viruses, retroviruses, and naked DNA plasmids have been studied in this regard, the majority of promising results have come from studies using adenoviral (Ad) vectors .

The Ad genome has two distinct sets of genes, the so-called early and late genes. The early genes (E1, E2, E3, and E4) are involved in viral replication, while the late genes encode the structural components of the adenovirus. Replication-deficient adenoviral vectors generated for gene transfer experiments were produced by replacing the E1 region with an exogenous transgene. These first generation adenoviral vectors efficiently transduce muscle fibers in vivo, but are associated with some disadvantages in terms of their use for gene replacement therapy. First of all, the remaining adenoviral genes and the transgene itself have harmful effects in host muscles, which are believed to compromise transduction efficiency by eliminating transduced fibers or by silencing transgene expression (18,19). Some of these detrimental effects occur early following delivery and are non-antigen-specific in nature. It was reported that transcriptionally active Ad particles caused a 20% drop in force-generating capacity in transduced muscles (311). This adverse reaction was not related to $CD8^+$ cytotoxic T-lymphocyte activity and occurred far too early to be due to antigen-specific adaptive immunity. Several mechanisms may explain these early, non-antigen specific negative effects.

First of all, Ad capsid proteins may be directly toxic. Petrof et al (312) showed that UV-inactivated Ad, which is infective but transcriptional inactive, caused inflammation when injected into skeletal muscle. Secondly, adverse effects of Ad in skeletal muscles have been associated with the de novo expression of Ad genes (311). In this regard, the E4 region of Ad encodes gene products which are known to block host protein synthesis (313) and transcriptional activation of cellular growth control proteins (314). In the liver, toxicity

associated with first generation (E1-deleted) Ad was reduced by the use of doubly E1/E2-deleted Ad (315). Thirdly, powerful promoter/enhancer elements may lead to derangements of myofiber function via competition for host cell transcription factors (316). Other detrimental effects of Ad-mediated gene transfer occur late following delivery and appear to involve antigen-specific, immune reactions. CD8+ CTLs have been implicated in destroying transduced myofibers (311,317), and several reports suggest cells are directed against transgene-encoded proteins not adenoviral antigens (312,318,319). In this regard, it has also been shown that diaphragm myofibers transfected with expression plasmids containing LacZ transgenes undergo immune-mediated destruction in a similar manner to Ad-LacZ-infected cells (320). Thus, Tripathy et al. (318) examined the stability of Ad-mediated gene expression in immunocompetent mice after intramuscular injection of Ad containing either a murine (isogenic) or human (non-isogenic) erythropoietin transgene. Animals receiving the isogenic transgene demonstrated persistent (at least 4 months) and high level erythropoietin expression, whereas a rapid loss of erythropoietin expression was observed in mice receiving the non-isogenic human transgene (318). These results suggest that isogenic transgenes induce less immune cell-mediated negative effects than non-isogenic transgenes and thus may be more beneficial for use in Ad-mediated gene transfer.

The second major drawback of first generation adenoviral vectors is that their insert capacity of only about 8 kb is not large enough to carry the full-length dystrophin cDNA (12 kb). As such, most early first generation Ad vector experiments in mdx mice used the 6.3 kb dystrophin cDNA which is expressed in Becker muscular dystrophy (BMD) patients which have a mild form of the disease. Ad vectors carrying this mini-dystrophin have been

shown to prevent mdx muscle pathology in neonatal mice, which have immature immune systems (15,16), and in immunosupressed mdx adults (18,298). While mini-dystrophins appear to provide some benefit, by definition these BMD-associated truncated gene products are less than fully functional.

The problems associated with first generation adenoviral vectors prompted the development of less toxic, less immunogenic adenoviral vectors with high-insert capacities. Ad vectors devoid of all viral sequences except for the absolutely necessary inverted terminal repeats (ITRs) and DNA packaging sequences have been developed, which have insert capacities up to ~36 kb. Indeed, gene transfer with these so-called large-capacity, fully-gutted or helper-dependent adenoviruses produces less cellular immune response resulting in greater longevity of transgene expression (25-29). Dystrophin gene transfer in neonatal mdx muscle using helper-dependent adenoviruses resulted in improved muscle histology and restoration of the DGC to the sarcolemma (31,311).

Recently, Gilbert et al (24) developed a helper-dependent Ad encoding two tandem full-length human dystrophins under the control of a powerful hybrid CMV enhancer/β-actin promoter. Injection of this helper-dependent Ad in adult mdx muscle resulted in an initially high level of dystrophin expression in treated muscles. However transgene expression was not stable, falling by 70% between 10 and 90 days post-injection. These authors speculated that the instability of dystrophin expression was due the fact that the dystrophin transgene used was non-isogenic. Dellorusso et al (321) reported that helper-dependent AdV gene transfer using full length human and mouse dystrophin transgenes could protect skeletal muscle of old mdx (1 year old) from force-deficits due to eccentric contractions. This study, however, failed to examine the longevity of dystrophin expression afforded by

the helper-dependent Ad. The efficiency and stability of dystrophin expression provided by helper-dependent Ad encoding full-length isogenic dystrophin has not yet been tested in mdx mouse muscles.

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PART I

CHAPTER 2.

Differential effects in diaphragm and limb muscle in a mutant SOD1 transgenic mouse model of amyotrophic lateral sclerosis.

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2.1 ABSTRACT

Muscle weakness is the major cause of patient disability and death in amyotrophic lateral sclerosis (ALS). Indeed, the majority of ALS patients die within 3-5 years of disease onset due to respiratory muscle insufficiency. Oxidative stress is believed to be a major player in ALS as mutations in the ubiquitous antioxidant enzyme superoxide dismutase 1 (SOD1) cause the disease in a subset of patients. Transgenic mice overexpressing mutant SOD1 enzymes display motor neuron disease characterized by muscle paralysis and early death. Skeletal muscles express SOD1 at levels comparable to that of motor neurons. While muscle weakness in ALS is generally attributed to motor neuron dysfunction, independent myogenic and neurogenic pathological processes resulting from the same genetic defect are not unprecedented (21, 22). Little is known about the function of skeletal muscles in transgenic SOD1 mice. In particular, no study has examined respiratory muscle function in this model. In the present study, we compared diaphragm and soleus muscles in SOD1-G37R and non-transgenic (Non-Tg) littermates in terms of muscle contractility parameters and markers of oxidative/nitrosative stress. In addition, we performed a detailed examination of SOD1-G37R muscle for signs of a primary myopathic process including myofiber central nucleation, embryonic myosin heavy chain (eMHC) staining (a sign of muscle regeneration following injury), and serum creatine kinase (CK) levels. The diaphragm of SOD-G37R transgenic mice displayed decreased maximal tetanic force production, slowing of twitch contraction kinetics and signs of increased nitrosative protein modifications relative to Non-Tg mice. The soleus muscle of these mice showed a switch toward a more slow-(type I), fatigue resistant phenotype relative to Non-Tg mice, without

a significant decrease in isometric force production. Neither the diaphragm nor the soleus muscles of SOD-G37R transgenic mice displayed signs of an overt primary myopathic process.

2.2 INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterised by progressive weakness in all skeletal muscle groups (1). In most cases, patient death is due to ventilatory failure caused by the involvement of the diaphragm and other respiratory muscles (2,3). Clinically indistinguishable familial and sporadic forms of the disease exist, with the latter representing 90% of cases. There is no cure and no effective treatment for ALS, and the causes of most occurrences remain unresolved. Current evidence suggests that a complex interaction of genetic and environmental factors contribute to ALS pathogenesis, including oxidative/nitrosative stress, the formation of aberrant protein aggregates, glutamate excitotoxicity, and mitochondrial dysfunction (4).

A major breakthrough came with the discovery that approximately 20% of familial ALS patients have mutations in the gene encoding the ubiquitous antioxidant enzyme, Cu/Zn-superoxide dismutase (SOD1) (5). It is now generally accepted that ALS-linked SOD1 mutations result in an as yet unknown, adverse gain-of-function (6-10). Two leading hypotheses to explain this adverse property are: (1) mutant SOD1 enzymes promote oxidative/nitrosative stress by using abnormal substrates such as peroxynitrite or hydrogen peroxide (11,12); and (2) SOD1 mutant enzymes cause the formation of protein aggregates

which sequester heat-shock proteins and inhibit the ubiquitin-proteasome pathway (13-16). Transgenic mice which have been generated to overexpress various ALS-linked SOD1 mutant enzymes (such as the G93A, G37R, or G85R mutations) develop an ALS-like motor neuron disease characterized by muscle wasting and weakness, and eventually paralysis and death (6-8).

SOD1 is a ubiquitous enzyme, and as such expression of the mutant form of the enzyme may also cause abnormalities in non-neural tissues in ALS patients or transgenic mice. Along these lines, it has recently been shown that expression of mutant SOD1 in motor neurons alone is not enough to cause motor neuron disease (17). One obvious accessory candidate tissue for abnormal neuromuscular function in ALS is skeletal muscle. Muscle weakness is the major cause of patient disability and death in ALS. Respiratory muscle strength, in particular, is an important prognostic factor in ALS patients (2). Muscles of ALS patients have been reported to display decreased force production, slowing of contractile kinetics and increased fatigue (18-20). While these findings are generally attributed to motor neuron dysfunction, independent myogenic and neurogenic pathological processes resulting from the same genetic defect are not unprecedented. Studies in a mouse model of spinal muscular atrophy (SMA), in particular, have suggested the potential for this to occur in motor neuron disease. SMA is caused by mutations in the gene known as survival motor neuron (SMN), which encodes for a protein involved in mRNA processing (21,22). Like SOD1, the protein product of SMN is ubiquitous. Transgenic mice with SMN selectively deleted in neurons, but not in skeletal muscle (23) develop a severe form of motor neuron disease. However, when SMN is deleted in skeletal muscles, but not in motor

neurons, mice display all the signs of a severe muscular dystrophy including myofiber necrosis, central nucleation, inflammation and elevated serum CK levels, while the morphology and number of motor neurons did not change (24). These results suggest the potential for primary skeletal muscle defects in motor neuron disease, especially in cases where the disease is caused by mutations in ubiquitously expressed proteins. Therefore, ALS patients may suffer primary skeletal muscle defects, in addition to denervation effects, which might contribute to disease pathogenesis and progression.

Very few studies have focussed on skeletal muscles of ALS-linked mutant SOD1 transgenic mice (25-27), and despite the importance of respiratory muscle function in ALS, no study has examined contractile function and the role of oxidative/nitrosative stress in the diaphragm of these animal models. In the present study, we compared diaphragm and soleus muscles in SOD1-G37R and non-transgenic (Non-Tg) littermates. Our specific objectives were three-fold: (1) To perform a detailed analysis of contractile parameters of diaphragm and soleus muscles in an SOD1 mutant animal model of ALS; (2) To examine the possibility that mutant-SOD1 overexpressing mice display a primary myopathic process, in addition to motor neuron disease; and (3) To assay for oxidative/nitrosative stress-associated modifications to proteins in the skeletal muscles of these mice.

2.3 METHODS

Transgenic SOD1-G37R Mice

Transgenic SOD1-G37R (line 29) mice and non-transgenic litter mates (4 - 7 mice per group) were generously provided by Dr. Jean-Pierre Julien, of the Centre for Research in Neuroscience, of McGill University. Mutant SOD1 protein levels and activity in this model have been shown to be approximately 5 and 9 times above normal endogenous SOD1 amounts, respectively (7). The age of motor neuron disease onset in these particular animals is 6-8 months. In order to represent early and late stages of disease, two age groups were studied: a six month old group consisting of 5 transgenic and 5 non-transgenic litter mates, and a 10 month old group consisting of 4 transgenic and 7 non-transgenic litter mates. SOD1-G37R mice older than 8 months of age showed the characteristic motor neuron disease features of this model, including muscle wasting, hindlimb paralysis and fine tremors. All experiments were approved by the Institutional Animal Care and Use Committee of McGill University.

Determination of Muscle Contractility Parameters

Costal diaphragm strips or intact whole soleus muscles were dissected from the mice. Muscles (or muscle strips in the case of diaphragms) were quickly mounted at one end in a custom-designed holder between two pairs of platinum plate electrodes. The muscle was submerged vertically in a bath of Ringer solution (119 mM NaCl, 4.7 mM KCl, 2.5 mM

$CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 20 mM $NaHCO_3$) which was continuously oxygenated (95% O_2-5% $C0_2$) and maintained at 25°C. The free tendinous end was attached to the lever arm of a force transducer-servomotor system (model 300B dual mode, Cambridge Technology, Watertown, MA). The force transducer was mounted on a mobile micrometer stage which allowed for fine adjustments of muscle length.

Muscle stimulation was induced through the electrodes placed on either side of the muscle, and the length of the muscle was adjusted to the point at which maximal twitch force was achieved (Lo). A thermoequilibration period was observed before muscle contractility experiments were initiated. Stimuli with a monophasic pulse duration of 2 ms were delivered with a computer controlled electrical stimulator (model S44; Grass Instruments, Quincy, MA) connected in series to a current amplifier (model 6824A, Hewlett-Packard). Muscle force was displayed on a storage oscilloscope (Tektronix, Beaverton, OR) and the data were simultaneously acquired to a computer at a sampling frequency of 1000 Hz for later analysis. Single electrical impulses were delivered to the muscles in order to measure the following twitch contraction parameters: contraction time (time to peak force), half-relaxation time (time for force to decrease to one half its peak value) and maximal twitch force. The force-frequency relationship was assessed by sequentially stimulating the muscle at 10, 30, 60, 90 and 120 Hz and measuring the plateau in force produced at each frequency. In vitro endurance (resistance to fatigue) of the soleus and diaphragm muscle strips was determined by measuring the loss of force production over time in response to repetitive trains of stimulation at 30 Hz for a 2 min period. Finally, the muscle was removed from the bath and its length determined using a microcaliper accurate to 0.1 mm. Muscle

cross sectional area was approximated by dividing muscle mass by its length and density (1.056 g/cm^3), which allowed specific force (force/cross-sectional area, N/cm^2) to be calculated.

Histological & Immunohistochemical Analysis

For each mouse, both soleus muscles and at least two diaphragm strips (including a strip not subjected to muscle contractility experiments) were embedded in mounting medium and immediately snap-frozen in isopentane pre-cooled with liquid nitrogen. Muscle samples were sectioned (10μm) and stained with hematoxylin and eosin (H&E) and examined by light microscopy. In muscles which appeared to display fatty cell infiltration, this was confirmed by oil-red-o staining. Sections for immunostaining were fixed in acetone for 30 seconds and allowed to air dry before treating with monoclonal antibodies specific for either slow type I myosin heavy chain (NOQ7.5.4D, 1:100 dilution) or embryonic myosin heavy chain (2B6, 1:100 dilution) in PBS. Muscle tissue sections were incubated with these primary antibodies overnight at 4°C. The following day the sections were rinsed in PBS and incubated for an hour with biotin-conjugated goat anti-mouse IgG (1:200) secondary antibody at room temperature. For detection, the streptavidin-peroxidase system (Vectastain, Vector Laboratories, Burlington, CA, USA) was used with DAB (3, 3'-diaminobenzadine, Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) substrate to obtain a brown precipitate in immuno-reactive myofibers.

Creatine Kinase Assay

Prior to removal of the diaphragm from the 10 month animals, blood was collected from the jugular vein and serum was obtained. Serum was also collected from an mdx mouse, which displays a dystrophic myopathy characterized by muscle membrane (sarcolemmal) lesions, to be used as a positive control. Total serum creatine kinase activity was measured in the clinical biochemistry laboratories of the Royal Victoria Hospital (Montreal, Canada) on a Beckman Synchron CX-7 automatic analyzer using commercially available reagents (Beckman Instruments Inc, Brea, CA, USA).

Immunoblotting for Markers of Oxidative and Nitrosative Modification

Tibialis anterior (TA) muscle samples were homogenized in protein extraction buffer containing 10mM Hepes pH 7.9, 3 mM $MgCl_2$, 0.2% Non-Idet P-40, 0.04 M KCl, 5% glycerol, 2% 2-mercaptoethanol, 1 μg/ml aprotinin, 0.5 μg/ml leupeptin and 0.5 mM PMSF. Muscle homogenates were centrifuged at 5000 rpm for 30 minutes at 4°C and the protein-containing supernatant was removed. Protein was quantified using the Bradford assay (Bio-rad Laboratories Inc, Hercules, CA, USA).

Protein Carbonyl Formation: Protein carbonyls are formed by direct oxidation reactions of proteins and/or as secondary products of other oxidation reactions such as lipid peroxidations and glycations/glycoxidations (28,29). To examine levels of protein carbonyl

groups in muscles of transgenic SOD1-G37R mice and non-transgenic litter mates, the Oxyblot™ (Intergen Company, Purchase, NY, USA) protein oxidation detection kit was used according to the manufacturers instructions. Briefly, 15 µg of protein was denatured in 5 µl of 12% SDS and derivatized with 10 µl 2,4-dinitrophenylhydrazine (DNPH) solution. This reaction was allowed to proceed for 20 minutes at room temperature and then stopped with neutralization solution. Negative controls were made by omitting DNPH. The derivatized samples were subjected to 8% SDS-PAGE followed by transfer onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 7% nonfat milk for one hour at room temperature and then incubated overnight at 4°C with polyclonal rabbit anti-DNP antibody (1:150), washed, and then incubated with horseradish peroxidase (HRP)-conjugated sheep anti-rabbit secondary antibody (1:300) for 1 hour.

3-Nitrotyrosine Formation: Nitration of protein tyrosine residues can occur via reactive nitrogen species (RNS), such as peroxynitrite ($ONOO^-$), and is widely used as a marker of nitrosative stress. We used immunoblotting to assess the level of 3-nitrotyrosine (3-N-Tyr) in muscles of transgenic SOD1-G37R mice and non-transgenic littermates. Briefly, 15 µg of crude protein was heated, separated by SDS-PAGE and transferred onto PVDF membranes as above. The membranes were blocked in 5% nonfat milk and incubated overnight at 4°C with a primary monoclonal anti-N-Tyr antibody (1:3000) (Caymen Chemicals) and finally with an HRP-conjugated anti-mouse secondary antibody (1:10000) (Transduction Laboratories, Lexington, KY) at 37°C for one hour. For both carbonyl and 3-N-Tyr immunoblotting, antibody-bound proteins were visualized by enhanced

chemiluminescence (ECL Kit, Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England). Coomassie® Brilliant Blue R-250 (Bio-Rad, Hercules, CA) staining of gels was used to ensure equal protein loading across lanes. Quantification of protein band intensities was performed by densitometric analysis using Fluorochem 8000 software (Alpha Innotech, San Leandro, CA).

Statistical Analysis

All data were reported as means ± SE and were analyzed with the statistical analysis program SigmaStat® (SPSS Inc.). Differences between groups were tested using an unpaired 2-tailed T-test. Statistical significance was defined as $p<0.05$.

2.4 RESULTS

Muscle Contractility Parameters

In the 6 month-old group, average contraction time (CT) and half relaxation time (½ RT) of both the diaphragm and the soleus of SOD-G37R mice tended to be greater than that of the non-transgenic littermates, but these trends did not reach statistical significance (Table1). However, by 10 months of age, this slowing of both CT and ½ RT in SOD-G37R mice did achieve statistical significance for the soleus. In addition, the diaphragm of 10 month old SOD-G37R mice showed a significantly increased CT (Table 1). No differences

were observed in twitch forces of the SOD-G37R mice relative to their non-transgenic littermates in either age group.

To examine the tetanic force-generating capacity of SOD-G37R mice, we stimulated diaphragm strips and whole soleus muscles with increasing frequencies from 10 to 120 Hz. No differences were seen between the mutant mice and their non-transgenic littermates at 6 months of age (data not shown). At 10 months of age, however, the diaphragms of mutant mice showed a small but statistically significant decrease in maximal tetanic force (Fig. 1a). A similar trend was observed in the SOD-G37R soleus, but this did not achieve statistical significance.

Diaphragm and soleus muscles were next stimulated repetitively for two minutes, and the force drop over time was used as a indicator of muscle resistance to fatigue. There were no differences between the SOD-G37R mice and their non-transgenic littermates in diaphragm or soleus at 6 months of age (data not shown). However, at 10 months of age, a striking difference was seen between SOD-G37R and non-transgenic solei (Fig. 1b). The soleus of the SOD-G37R mutants displayed significantly greater resistance to fatigue (i.e., less of a decrease in force production over time) than the non-transgenic littermates. In contrast, no such differences were found between the SOD-G37R and non-transgenic diaphragms of these animals.

Slow-(type I) myosin heavy chain immunohistochemistry

The slowing of contractile kinetics and increased resistance to fatigue seen in the soleus of 10 month old SOD-G37R mice indicated a possible shift in muscle fiber type toward more slow-(type I), fatigue resistant fibers. Using immunohistochemistry for slow-(type I) myosin heavy chain expression, we found a dramatic shift from approximately 64% slow-(type I) MHC expressing fibers in soleus sections of non-Tg littermates to 98% in SOD-G37R (Fig. 2) soleus sections. In accordance with the muscle contractility data, no such shift in fiber type profile was observed in the diaphragms of SOD-G37R.

Assessment of a primary myopathic process in SOD-G37R mice

To determine whether the decrease in diaphragm maximal tetanic force in the 10 month old SOD-G37R transgenic mice might be the result of a mutant SOD1-induced primary myopathy, we performed a detailed inventory of several characteristic features of primary myopathic processes including muscle fiber morphology and central nucleation by hematoxylin and eosin (H&E) staining, embryonic myosin heavy chain (eMHC) immunohistochemistry, and serum creatine kinase (CK) levels.

Soleus muscles from SOD1-G37R mice had many small, angular fibers (a sign of denervation atrophy), but otherwise appeared normal (not shown). The diaphragm of SOD-G37R mice, on the other hand, demonstrated a small number of isolated areas of fatty cell infiltration (Fig. 3), which was confirmed by oil-red-o staining (not shown). Such

abnormalities were not seen in the muscles of non-transgenic littermates. Neither the diaphragm nor the soleus of SOD-G37R mice showed typical signs of primary myopathy, such as myofiber necrosis or central nucleation. Immunostaining for eMHC, a marker of myofiber regeneration following muscle damage, was also negative in diaphragm and soleus from SOD-G37R transgenics as well as non-transgenic littermates. Finally, CK activity, an indicator of muscle cell membrane damage, was assayed in the serum from 10 month old SOD-G37R and littermates. Serum from the mdx mouse, which has a primary dystrophic myopathy characterized by sarcolemmal lesions, was also assayed as a positive control. No difference was observed in serum CK levels between the SOD-G37R transgenic mice and non-transgenic mice (data not shown). These results suggest that skeletal muscles from SOD-G37R transgenic mice do not suffer from a superimposed primary myopathic process.

Markers of oxidative/nitrosative stress

Diaphragm and soleus proteins from 10 month old SOD-G37R transgenic mice and littermates were tested for markers of oxidative and nitrosative stress by immunoblotting for protein carbonyls and 3-nitrotyrosine, respectively. No differences in protein carbonyls were seen between the two groups for either the soleus or the diaphragm (data not shown). However, as compared to non-transgenic littermates, diaphragm samples from some SOD-G37R transgenic mice showed increased 3-nitrotyrosine formation for two proteins of approximately 58 and 60 kDa (Fig. 4). Densitometry analysis of these bands revealed a

Table 1. Contraction time (CT) and half-relaxation time (1/2RT)

	CT (ms)		1/2 RT (ms)	
	Diaphragm	Soleus	Diaphragm	Soleus
6 month				
Non-Tg	43.6±1.5	47.7±1.6	47.7±3.2	59.1±5.1
SOD1-G37R	47.5±1.9	49.4±1.6	54.5±6.2	63.2±4.6
10 month				
Non-Tg	42.4±1.4	50.6±1.4	51.7±3.2	66.9±2.3
SOD-G37R	48.4±1.5*	55.7±1.5*	54.5±4.8	89.6±5.2*

Values are means ± SE. *$p<0.05$ for Non-Tg versus SOD1-G37R within an individual muscle group.

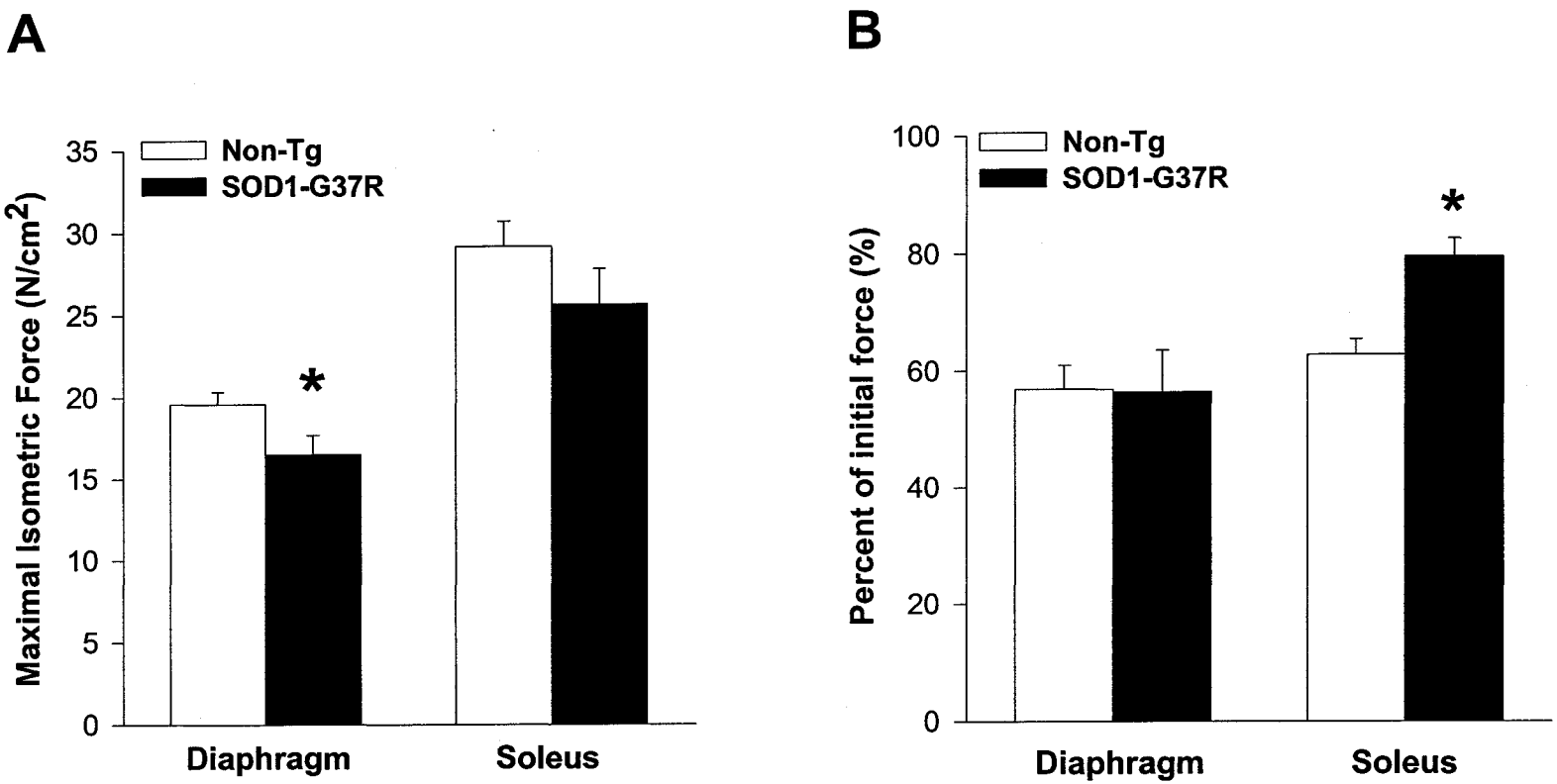

Figure 1. Effect of SOD1-G37R overexpression on muscle contractile properties. Maximal isometric force production (**A**) and resistance to fatigue (**B**) of diaphragm and soleus muscles of 10 month old mutant SOD1 transgenic (SOD1-G37R) versus non-transgenic littermates (Non-Tg). For the diaphragm, sample size (n)=7 for Non-Tg, and 4 for SOD1-G37R. For the soleus, n=12 for Non-Tg, and 6 for SOD1-G37R. All values are group means ± SE. *$p<0.05$ for Non-Tg versus SOD1-G37R within an individual muscle group.

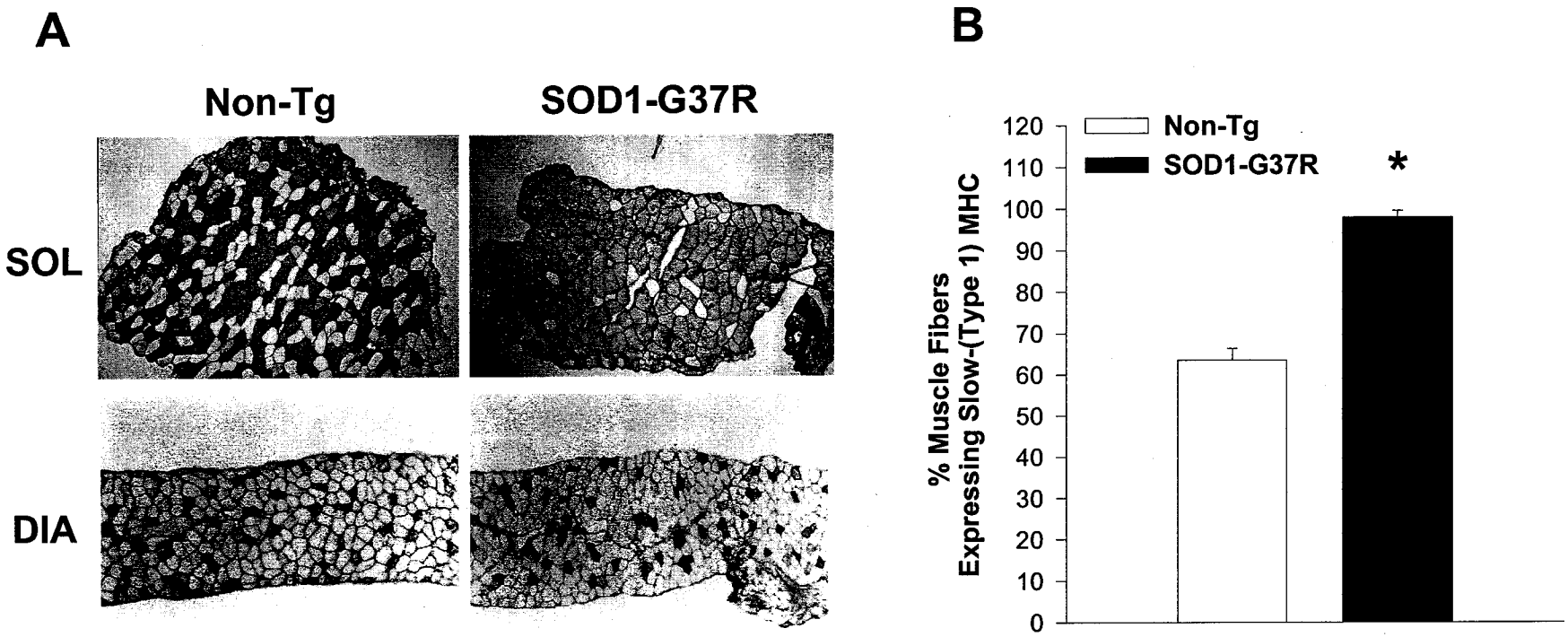

Figure 2. Slow-(type 1) myosin heavy chain (MHC) expression in diaphragm and soleus muscles from 10 month old mutant SOD1 transgenic mice (SOD1-G37R) versus non-transgenic littermates (Non-Tg). (**A**) Representative micrographs of slow-(type 1) MHC immunohistochemistry in muscle cryosections. For the diaphragm, note the increased number of immunopositive (dark-staining) myofibers in SOD1-G37R relative to the Non-Tg muscles. (**B**) Quantification of soleus slow-(type 1) MHC immunohistochemistry. For both mouse strains, sample size (n)=4. All values are group means ± SE. *$p<0.05$ for Non-Tg versus SOD1-G37R.

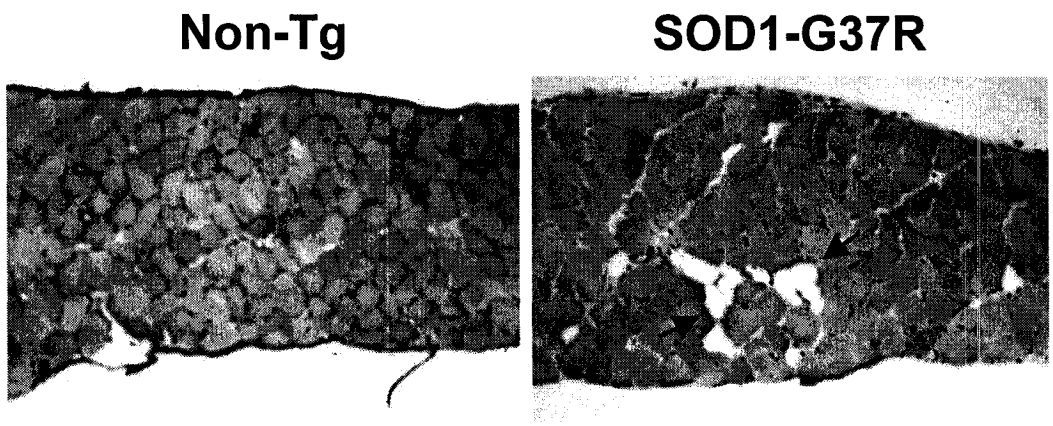

Figure 3. Representative micrographs of hematoxylin and eosin (H&E) staining of diaphragm cryosections from 10 month old mutant SOD1 transgenic mice (SOD1-G37R) versus non-transgenic littermates (Non-Tg). Arrows designate fats cells among myofibers (as determined by oil-red-o staining, not shown).

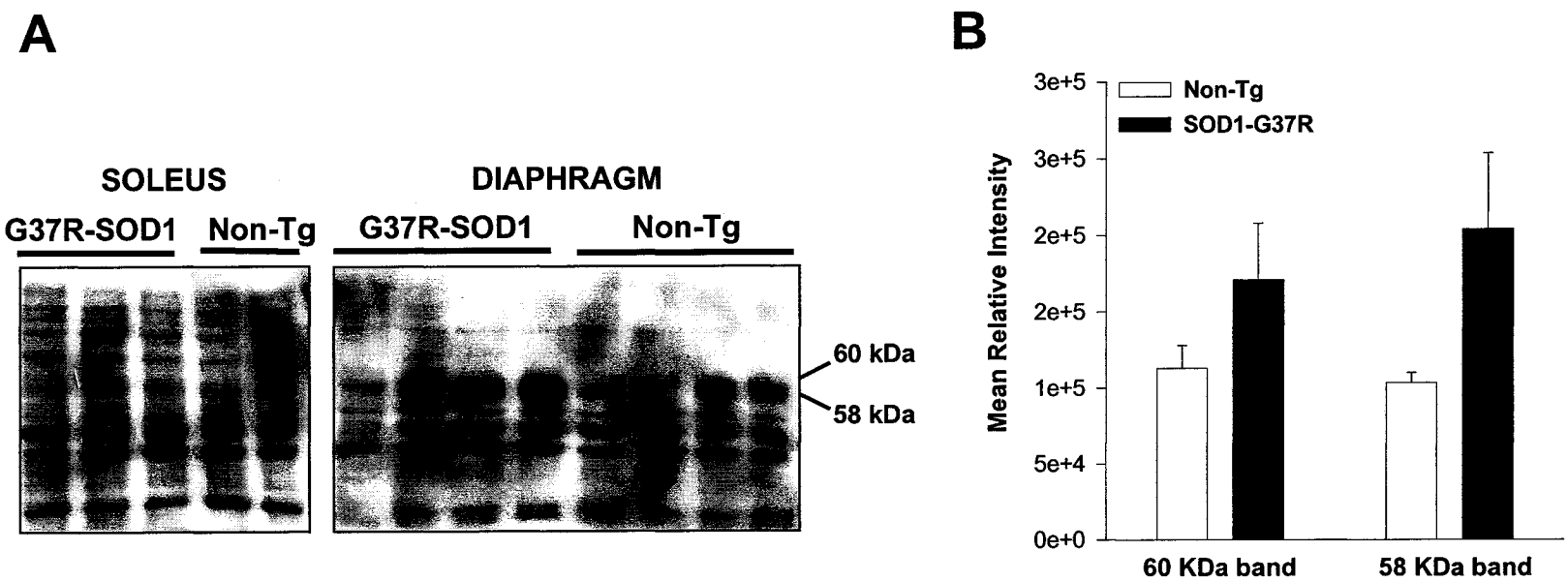

Figure 4. Effect of SOD1-G37R overexpression on 3-nitrotyrosine formation in diaphragm and soleus muscles from 10 month old mutant SOD1 transgenic mice (SOD1-G37R) versus non-transgenic littermates (Non-Tg). (**A**). Anti-3-nitrotyrosine immunoblotting of SDS-PAGE separated proteins. Note that for the diaphragm, two protein bands of approximately 58 and 60 kDa displayed strong immunoreactivity in SOD1-G37R samples. (**B**).Densitometric analysis of proteins bands (~58 and 60 kDa) detected by 3-nitrotyrosine immunoblotting of diaphragm proteins from SOD1-G37R and Non-Tg mice. For both mouse strains, sample size (n)=4. All values are group means ± SE.

trend toward increased mean 3-nitrotyrosine in the SOD-G37R samples (p=0.088 and 0.194 for the 58 and 60 kDa bands, repectively).

2.5 DISCUSSION

Respiratory muscle weakness is the major cause of death in ALS patients. Recently, it has been suggested that primary skeletal muscle defects, in addition to denervation, may contribute to disease pathogenesis (18-20,25-27,30,31,31,32). Mutations in the gene encoding SOD1, the only confirmed cause of ALS, account for 20% of patients with the familial form of the disease and 2% of all cases (5). Because of the striking clinical similarities between the familial and sporadic forms of the disease, mutant SOD1 transgenic mice have become a major experimental tool employed towards understanding the pathophysiology of this invariably fatal condition. However, very few studies have examined skeletal muscle properties of mutant SOD1 transgenic mice (25-27), and none has examined contractile function or oxidative/nitrosative stress in the diaphragm muscle of these animals. To our knowledge, the work presented here is the first detailed investigation of diaphragm muscle properties in a mutant SOD1 mouse model of ALS. Our major findings are: (1) the diaphragm of 10 month old SOD-G37R transgenic mice displayed a decrease in maximal tetanic force, a slowing of twitch contraction time, and fatty cell infiltration; (2) the soleus of these 10 month old transgenic mice showed a switch toward a more slow-(type I), fatigue resistant phenotype characterized by increased contraction and relaxation times, but without a significant decrease in isometric force production; and (3) neither the

diaphragm nor the soleus muscles of SOD-G37R transgenic mice displayed signs of an overt primary myopathic process, but there was a suggestion of increased nitrosative stress in the diaphragm.

Effect of SOD1-G37R on skeletal muscle contractile properties

It is known that ALS progresses independently in different muscle groups (33). Indeed, ALS patients almost invariably present with limb weakness, but eventually die due to respiratory muscle dysfunction (2). Several studies have shown decreased isometric force production in skeletal muscles of ALS patients (18-20), and these force deficits have been attributed to motor neuron dysfunction. Recently, Derave et al (27) studied in vitro muscle contractility parameters in the transgenic SOD1-G93A mouse model of ALS. These authors reported a decline in maximal tetanic force production in the fast-twitch extensor digitorum longus (EDL), which was not found in the slow twitch soleus; and normal twitch force production in both muscles. Similarly here, in SOD1-G37R mice, we found decreased maximal tetanic force production in the fast twitch diaphragm, but no difference in the soleus. Both the diaphragm and soleus displayed normal twitch force production. Both these studies confirm previous reports that muscle weakness occurs earlier in fast-twitch than in slow-twitch skeletal muscles in mutant SOD1-linked ALS mouse models (34,35).

In addition to decreased force production, ALS patients show slowing of skeletal muscle contraction and relaxation times, and this is believed to be due to a selective loss of the larger, fast motor axons with re-innervation by surviving slow axons (18,19). Slowing

of contraction and relaxation times was also found for soleus and EDL muscles in the in vitro muscle contractility studies of SOD1-G93A mice discussed above (27). In the present study, we also found slowing of contractile kinetics in the soleus and diaphragms of SOD1-G37R mice. On the other hand, in chemically-skinned ALS fibers, where contraction is achieved via direct Ca^{2+} activation of the contractile apparatus, Krivickas et al. (26) found increased shortening velocity and no difference in terms of force production. As suggested by Derave et al (27), these combined results point to a contractile defect which is distal to the neuromuscular junction but proximal to cross-bridge interactions, such as deficits of action potential propagation or Ca^{2+} regulation via the sarcoplasmic reticulum.

ALS patients also display increased susceptibility to skeletal muscle fatigue (18,36). In contrast, Derave et al (27) found that the soleus and EDL limb muscles of SOD1-G93A mice actually displayed increased resistance to fatigue. In the present study, we also found the soleus of SOD1-G37R mice to be more resistant to fatigue than that of non-transgenic littermates. No difference in fatigability was found between diaphragms of transgenics and non-transgenic littermates. Because the mouse experiments were done using direct stimulation of muscles without the influence of neural stimulation, these data suggest increased susceptibility to fatigue in ALS patients is most likely due to motor neuron defects, rather than altered muscle properties.

Although slowing of contractile kinetics and resistance to fatigue has been shown previously for transgenic mutant SOD1 skeletal muscle, no mechanism has been elucidated to explain these findings. Here we investigated whether myosin heavy chain expression might be altered in conjunction with these changes, which are at least suggestive of a slow-

twitch muscle phenotype shift. We found a dramatic increase in slow-(type I) myosin heavy chain (MHC) expression in the soleus of SOD1-G37R mice, which was not seen in the diaphragm muscles. Muscle fiber phenotype is determined in large part by innervation. Therefore, this shift in MHC expression may be due to preferential loss of fast-type motor axons, with axonal sprouting and reinnervation by remaining slow-type motor neurons as is suggested to occur in ALS patients and mutant SOD1-G93A transgenic mice (35,37,38). Collectively, the contractility results presented here also suggest that the diaphragm responds differently than limb muscles to transgenic mutant SOD1 expression and/or motor neuron disease. Differential effects in soleus and diaphragm muscle will be discussed in greater detail below.

Effect of SOD1-G37R on markers of oxidative/nitrosative stress in skeletal muscles

In addition to abnormal contractile properties, several lines of evidence suggest that skeletal muscles of ALS patients suffer mitochondrial defects associated with increased oxidative/nitrosative stress. First, mitochondrial respiratory chain dysfunction, including decreased activities of cytochrome c oxidase and NADH:CoQ oxidoreductase, decreased levels of MnSOD (SOD2), and mitochondrial DNA (mtDNA) defects have been found in skeletal muscle biopsy material from ALS patients, but not from patients with other denervating disorders (30,31). Secondly, exercising muscles of ALS patients were shown to display impaired oxidative metabolism, which correlated with increased levels of lipid peroxidation (32,39). Thirdly, saponin-skinned fibers from mutant SOD1-G93A transgenic

mice also displayed aberrant mitochondrial respiratory properties (25). Interestingly, mice which overexpress the wild-type SOD1 do not develop overt motor neuron disease, but do demonstrate increased free radical production in skeletal muscles and, in at least one strain, develop a severe muscular dystrophy-like myopathic phenotype (40,41).

While no differences were seen between transgenic and non-transgenic littermates in terms of protein carbonyl formation for either muscle group, diaphragm samples of the transgenic mice tended to display increased 3-nitrotyrosine immunoreactivity. Increased 3-nitrotyrosine formation has been found in spinal cord material from ALS patients and mutant SOD1-transgenic mice (42,43). Increased nitrosative stress in skeletal muscles of SOD1-G37R mice could result directly from mutant enzyme expression (via adverse gain-of-function reactions), or somehow secondarily to denervation. Several studies have suggested that denervation causes changes in the sarcolemmal expression pattern of the neuronal isoform of nitric oxide synthase (nNOS) (44-49). Nitric oxide ($NO^{\bullet}$) is known to possess antioxidant properties including the ability to directly scavenge hydroxyl radical and to react with lipid peroxyl and alkoxyl radicals to terminate lipid peroxidation reactions (Wink and Mitchell, 1998). In this regard, it has been suggested that denervation and hence down-regulation of $NO^{\bullet}$ production due to decreased nNOS expression could lead to enhanced free-radical damage in muscle fibers (50). However, since nitrotyrosine is formed by the reaction of superoxide with $NO^{\bullet}$, it is unlikely that increased 3-nitrotyrosine formation would be due to decreased nNOS expression in our model. Further studies using larger samples sizes will be needed to determine the importance of the qualitative differences observed

here, and to elucidate possible mechanisms of increased nitrosative stress in mutant SOD-expressing skeletal muscle.

Differential effects in SOD1-G37R soleus and diaphragm muscles

Differential effects in soleus and diaphragm muscles in mutant G37R-SOD1 transgenic mice may simply be due to the different rates of disease progression in different muscle groups. Such differences may result from the level of the spinal cord preferentially affected, an idea which is supported by studies of another earlier ALS mouse model - the wobbler mouse (51). Alternatively, the contrasting muscle-specific pathologies seen here may represent inherent differences in the physiology of different muscle groups. Differences in disease severity between limb muscles and the diaphragm in animal models of neuromuscular disorders is not unprecedented. In the mdx mouse model of DMD, it is well established that the diaphragm is affected to a greater extent than limb muscles (52). The diaphragm differs physiologically from the soleus in its persistent rhythmic activation through both wakefulness and sleep. This enhanced electrophysiological work rate , which could predispose to increased oxidative/nitrosative or mechanical stress, may play a role in accelerating disease progression. This would be consistent with the greater force loss and evidence of nitrosative stress in the SOD1-G37R diaphragm.

2.6 ACKNOWLEDGEMENTS

I would like to again thank Jean-Pierre Julien and Ming-Dang Nguyen of the Centre for Research in Neuroscience of McGill University for providing the animals for these experiments; Johanne Bourdon for her expert technical assistance; Won-Kyung Cho for her important contributions to the muscle physiology experiments presented here; Gawiyou Danialou, Satoru Ebihara, and Ghiabe Guibinga for their technical assistance; and finally Basil J. Petrof for his assistance, advice and supervision.

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CHAPTER 3.

Sarcolemmal weakness in dystrophin-deficient muscle: Potential role of ischemia/reperfusion injury and oxidative/nitrosative

Roy W. R. Dudley, Gawiyou Danialou, Karuthapillai Govindaraju, David E. Eidelman, and Basil J. Petrof

3.1 PROLOGUE

In the preceding chapter we tested the hypothesis that skeletal muscles of the mutant SOD1 mouse model of ALS undergo a primary myopathic process related to oxidative/nitrosative stress. While diaphragm and soleus muscles of these animals did show altered physiological and morphological properties, we did not find any evidence of a myopathic process related to oxidative stress in these muscles. In Duchenne muscular dystrophy (DMD) - a primary myopathy, oxidative stress is believed to play role in the direct destruction of myofibers. Indeed, in vitro evidence suggests that dystrophin-deficient muscle cells are abnormally susceptible to oxidative stress. In the next chapter we will test whether dystrophin-deficient skeletal muscles are abnormally susceptibility to in vivo oxidative/nitrosative stress, in the form of ischemia/reperfusion (I/R) injury. This I/R model is particularly relevant to dystrophin-deficiency in light of recent findings that DMD patients and mdx mice suffer functional muscle ischemia due to the displacement of neuronal nitric oxide synthase (nNOS) from the sarcolemma (1,2).

3.2 ABSTRACT

Duchenne Muscular Dystrophy (DMD) is caused by mutations in the gene encoding the cytoskeletal protein dystrophin resulting in near complete loss of the protein product, but the precise mechanism by which lack of dystrophin leads to muscle destruction remains unresolved. Several lines of evidence have implicated oxidative/nitrosative stress in DMD (3-5). In particular, it has been shown that dystrophin-deficient myotubes are abnormally susceptible to oxidative stress (6,7). In the absence of dystrophin, a secondary displacement of neuronal nitric oxide synthase (nNOS) from the sarcolemma to the cytosol causes recurrent bouts of functional ischemia during muscle activity due to unopposed α-adrenergic vasoconstriction (1,2). It is well established that ischemia/reperfusion (I/R) causes severe muscle damage due to the production of reactive oxygen/nitrogen species. Here we used hindlimb tourniquet application to subject the tibialis anterior (TA) of adult dystrophic (mdx) and normal mice (C57) to I/R-injury in order to examine if dystrophin-deficient muscle is abnormally susceptible to this form of free radical-mediated damage. Immunoblotting for protein carbonyls (a marker of oxidative protein modifications), hydroxynonenal (HNE) protein adducts (an indicator of lipid peroxidation) and 3-nitrotyrosine formation (an marker of peroxynitrite formation) were used to assess susceptibility to I/R-induced oxidative/nitrosative stress. Expression of NOS isoforms and levels of NOx (nitrite/nitrate/nitrosothiols) were measured to examine the role of nitric oxide. Finally, myofiber membrane (sarcolemma) fragility due to I/R-induced oxidative/nitrosative stress was assessed by subjecting muscles to a protocol of in vivo stretch following I/R and examining uptake of the low molecular weight tracer compound Evans Blue dye (EBD).

Dystrophin-deficient muscles demonstrated higher levels of lipid peroxidation, protein tyrosine nitration, and stretch-induced sarcolemmal damage than normal mice following I/R. These changes in mdx muscles were associated with increased NOx production during ischemia, which was not seen in normal C57 muscles. Our results suggest that dystrophin-deficient muscles are abnormally susceptibility to I/R-induced oxidative/nitrosative stress and that I/R predisposes these muscles to membrane fragility. We speculate that nNOS displacement to the cytosol in mdx muscle may play a role in these findings.

3.3 INTRODUCTION

Duchenne muscular dystrophy (DMD) is a fatal X-linked recessive disorder affecting 1 in 3500 male births (8). DMD is caused by genetic mutations, which result in the absence of a large (427 kDa) cytoskeletal protein known as dystrophin, and is characterized by progressive muscle destruction, fibrosis, and fatty cell infiltration leading to muscle weakness (9). Patient death occurs in the late teens to early twenties, usually due to respiratory insufficiency. The mdx mouse, the most widely used animal model of DMD, also has mutation in the dystrophin gene and lacks the protein product (10,11). One of the most prominent features of dystrophic pathology in DMD patients and mdx mice is skeletal muscle membrane (sarcolemmal) damage (12-16).

In normal skeletal muscle, dystrophin exists as part of a multimolecular complex, referred to as the dystrophin-glycoportein complex (DGC), which spans the sarcolemma and thereby permits a physical linkage between the intracellular cytoskeleton and the

extracellular matrix (17,18). In addition to dystrophin, the DGC is comprised of three distinct subgroups of proteins, the dystroglycan, sarcoglycan-sarcospan and syntrophin subcomplexes, as well as a protein with considerable structural homology to dystrophin, known as dystrobrevin (17,19,20). Among several potential signalling molecules associated with the DGC is the multifunctional enzyme neuronal nitric oxide synthase (nNOS) (21). In DMD patients and mdx mice, the primary deficiency of dystrophin destabilizes the entire multimolecular complex, resulting in a secondary loss of other members of the DGC from the sarcolemma. In particular, nNOS is displaced to the cytosol, and its enzymatic activity is significantly reduced (21-25).

Despite enormous progress in understanding the molecular basis of DMD, the precise role of dystrophin and, hence the mechanisms by which its absence causes progressive muscle pathology, remain unclear. One proposed mechanism is that muscle cell damage occurs due to oxygen-derived free radicals (i.e., oxidative stress) (26-29). Indeed, markers of oxidative stress such as protein carbonyls, lipid peroxidation products, and upregulated antioxidant enzymes have been found in DMD and mdx skeletal muscle (61; 185; 18; 4}. In addition, in vitro studies have shown that dystrophin-deficient myotubes are abnormally susceptible to oxidative stress (6,7). It has also been suggested that production of the free radical $NO^{\bullet}$ in the cytosol, due to the mislocalized nNOS, may result in the production of harmful reactive nitrogen species, such as peroxynitrite. While the evidence in regard to this proposed nitrosative stress has not been conclusive, it has been shown in recent years that the loss of nNOS from the sarcolemma results in functional muscle ischemia in DMD patients and mdx mice, due to unopposed sympathetic

vasoconstriction (1,2,30,31). Interestingly, it is well established that ischemia, and subsequent reperfusion, can cause severe skeletal muscle damage by way of the production of free radical species (32-37). Therefore, the first objective of the present study was to examine the in vivo susceptibility of dystrophin-deficient muscle to ischemia/reperfusion (I/R) injury, a model of oxidative/nitrosative stress which has particular relevance to DMD patients.

A second proposed mechanism of DMD pathogenesis suggests that the absence of dystrophin makes the sarcolemma abnormally susceptible to mechanical stress. Several lines of evidence support this hypothesis (14,38-43). In particular, dystrophin-deficient mdx skeletal muscles display increased susceptibility to stretch- and contraction-induced mechanical stress (14,41). Oxidative/nitrosative stress and membrane fragility are not mutually exclusive pathophysiological events. Oxidative/nitrosative stress has been shown to cause membrane fragility through lipid peroxidation and protein-protein cross linking reactions (44-47). Indeed, sarcolemmal damage has been associated with free-radical production in several forms of muscle injury and disease. Recently, Ebihara et al (48) showed that nitrosative stress predisposes cultured muscle cells to abnormally high levels of sarcolemmal injury following mechanical stress. Therefore, the second objective of this study was to examine the susceptibility of dystrophin-deficient muscle to mechanical stress following exposure to I/R-induced oxidative/nitrosative stress.

3.4 METHODS

Materials

A polyclonal antibody directed against hydroxynonenal (HNE)-protein adducts was purchased from Calbiochem (San Diego, CA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was purchased from Cedarlane Laboratories Limited (Hornby, ON, Canada). A monoclonal antibody directed against protein bound 3-nitrotyrosine was purchased from Caymen Chemicals (Ann Arbor, MI, USA). Monoclonal antibodies directed against nNOS, iNOS and ecNOS, and respective positive controls (lysates from rat pituitary, cytokine-stimulated macrophages, and human endothelial cells), as well as HRP-conjugated anti-mouse IgG were purchased from Transduction Laboratories (Lexington, KY). Enhanced chemiluminescence (ECL) kit for detection of immunoblotted proteins was purchased from Amersham Pharmacia Biotech UK Limited (Buckinghamshire, United Kingdom). Coomassie® Brilliant Blue R-250 was purchased from Bio-Rad (Hercules, CA). Evans Blue Dye (EBD), Hepes buffer, sodium nitrate (NaNO3), trichloroacetic acid (TCA), vanadium chloride (VCl_3) and pure human leukocyte MPO were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Frozen Section Medium was purchased from Stephens Scientific (Riverdale, NJ, USA).

Laboratory Animals

Dystrophin-deficient (mdx) and normal dystrophin-expressing C57BL/6 or C57BL/10 mice (collectively referred to as C57) were purchased from the Jackson Laboratory (Bar Harbor, Maine). Animals were studied at 6-8 weeks of age and were exclusively males. The mice were provided with food and water ad libitum, and all experimental protocols were approved by the institutional animal care and ethics committee.

Ischemia/Reperfusion Protocol

Ischemia/reperfusion (I/R) injury is a well established model of in vivo oxidative/nitrosative stress (32-37). In order to induce ischemia in the lower leg tibialis anterior (TA) muscle, a locking plastic pull-tie tourniquet (100 mm length, 2 mm width) was applied just above the knee of the right hindlimb of deeply anesthetized (ketamine:130mg/kg / xylazine:20mg/kg, i.m. injection) mdx and C57 mice for 90 minutes. A thin (3mm thickness) sponge padding was applied between the skin and the tourniquet to prevent skin damage and muscle crush injury. Cessation of blood flow was confirmed by a notable temperature drop to touch and visible discoloration of the lower limb. At the end of the ischemia portion of the protocol the tourniquet was removed and the appearance of the lower limb quickly returned to normal. To examine the effect of ischemia alone, some animals were sacrificed at the end of this first 90 minute period of blood flow cessation. To study the effects of reperfusion following ischemia, animals were sacrificed either one hour or three hours after the removal of the tourniquet, depending on the outcome measure being examined (see below). The time

points of ischemia and reperfusion used here are similar to those reported by other investigators studying short-term I/R injury in mouse hindlimb skeletal muscles (49-52).

Assessment of Susceptibility to I/R-induced Oxidativeand Nitrosative Stress

Immunoblot Analysis

Muscle samples were homogenized in protein extraction buffer containing 10mM Hepes pH 7.9, 3 mM $MgCl_2$, 0.2% Non-Idet P-40, 0.04 M KCl, 5% glycerol, 2% 2-mercaptoethanol, 1 μg/ml aprotinin, 0.5 μg/ml leupeptin and 0.5 mM PMSF. Muscle homogenates were centrifuged (5000 rpm at 4°C) for 30 minutes and the protein-containing supernatant was removed and quantified using the Bradford assay.

Protein Carbonyl Formation: Direct oxidation of certain amino acids, as well as indirect reactions with bi-products of oxidative stress, results in the formation of carbonyl groups (ie. aldehydes and ketones) on proteins (53). Therefore, assays for protein-bound carbonyls are widely used not only to detect protein oxidation, but also as indicators of overall oxidative stress (54). The Oxyblot protein oxidation detection kit (Intergen Company, Purchase, NY) was used for western blotting of protein-bound carbonyl groups according to the manufacturer's instructions. Briefly, 15 µg of total protein was denatured in 5 µl of 12% SDS and derivatized with 10 µl 2,4-dinitrophenylhydrazine (DNPH) solution. This reaction was allowed to proceed for 20 minutes at room temperature and then stopped with an Oxyblot™-

included neutralization solution. Negative controls were made by omitting DNPH. The derivatized samples were subjected to 8% SDS polyacrylamide gel electrophoresis (SDS-PAGE) (BioRad Mini-Protean II gel system) followed by electroblotting onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 7% nonfat dry milk for one hour at room temperature, incubated overnight at 4°C with polyclonal rabbit anti-DNP antibody diluted in blocking buffer (1:150), washed, and then incubated with horseradish peroxidase (HRP)-conjugated sheep anti-rabbit secondary antibody (1:300) for 1 hour. For all immunoblotting experiments, antibody-bound proteins were visualized by enhanced chemiluminescence (ECL) kit, and Coomassie Blue staining was used to ensure equal protein loading across lanes. Quantification of immunoblot protein band intensities was performed by densitometric analysis using Fluorochem 8000 software (Alpha Innotech, San Leandro, CA).

Lipid Peroxidation: Cell membranes are the primary target of lipid peroxidation reactions during periods of oxidative/nitrosative stress. Peroxidation of membrane lipids can lead to protein-protein cross-linking, which decreases membrane fluidity and causes increased membrane leakiness (45). One of the most damaging bi-products of lipid peroxidation reactions is hydroxynonenal (HNE). Once formed it can bind lysine, cysteine and histidine residues of proteins by a process known a Michael addition. In the present study, immunoblotting was performed for HNE-protein adducts. Briefly, 30 µg of crude protein sample in Laemmli buffer (1:1 ratio) was heated for 10 minutes at 97°C and subjected to 10% SDS-PAGE. The proteins were transferred onto PVDF membranes, blocked in 5%

nonfat dry milk and incubated overnight at 4°C with a polyclonal rabbit anti-HNE antibody (1:1000 dilution). After washing, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody (1:2600) at 37°C for one hour.

3-Nitrotyrosine Formation: Nitration of protein tyrosine residues is widely used as a marker of nitrosative stress (55,56). Peroxynitrite ($ONOO^-$), the product of the reaction of superoxide and $NO^{\bullet}$, is considered to be the most potent nitrating species in biological systems (57). Alternatively, neutrophils can induce protein nitrotyrosine formation via myeloperoxidase activity (58,59). In the present study, immunoblotting was performed for protein bound 3-nitrotyrosine. Briefly,15 µg of crude protein was separated by SDS-PAGE and transferred onto PVDF membranes as above. In this case the membranes were blocked in 5% nonfat dry milk and incubated overnight at 4°C with a primary monoclonal anti-3-nitrotyrosine antibody (1:3000 dilution), and finally with HRP-conjugated anti-mouse secondary antibodies (1:10000) at 37°C for one hour.

Nitric Oxide Analysis

NOx levels : NOx (nitrite, nitrate, nitrosothiol) levels were assayed using a Sievers 280 Nitric Oxide Analyser (NOA, Sievers Instruments Inc., Boulder, CO). Two muscles were pooled for each sample and were quickly ground in liquid nitrogen, homogenized in ice cold Hepes buffer and centrifuged (5000 rpm at 4°C) for 30 minutes. The amount of protein in each sample was determined and samples were further diluted to obtain a uniform protein

concentration of 8 µg/µl across all samples. To prevent foaming during analysis, protein was removed by adding an equal volume of 40% trichloroacetic acid, incubating at 4°C for 1 hour and centrifuging at 15000 rpm for 10 minutes. Forty µl of each sample was injected into the purge vessel containing the reducing agent, VCl_3, in HCl at 91°C. By this method any nitrite (NO_2^-), nitrate (NO_3^-), or nitroso (RSNO) compounds present in the muscle homogenate are reduced to nitric oxide ($NO^{\bullet}$). The $NO^{\bullet}$ produced is quickly removed from the reaction chamber by purging with nitrogen gas. It then reacts with ozone to produce a chemiluminescence signal which is read by a detector. These signals were recorded and processed using the Sievers 280 software set up for liquid analysis. Each sample was assayed in triplicate and the concentration of NOx was calculated using a standard curve produced by assaying various concentrations of a sodium nitrate solution.

Immunoblotting for NOS isoforms: Immunoblotting was performed in order to examine the effect of I/R on the expression levels of nNOS, iNOS and ecNOS in the two mouse strains. TA muscle samples were homogenized in 10mM Hepes pH 7.9, 3 mM MgCl2, 0.2% Non-Idet P-40, 0.04 M KCl, 5% glycerol, 1.02 mM DTT, 1 µg/ml aprotinin, 0.5 µg/ml leupeptin and 0.5 mM PMSF. Homogenates were centrifuged (5000 rpm at 4°C) for 30 minutes. Protein-containing supernatants were removed and protein concentrations was quantified as described above. Immunoblotting for nNOS, iNOS, ecNOS was performed on 50ug, 80ug, and 60ug of protein, respectively. Protein samples in Laemmli buffer (1:1 ratio) were heated for 10 minutes at 97°C and subjected to 6% SDS-PAGE. The separated proteins were transferred electrophoretically onto PVDF membranes, blocked for 1 hour at room

temperature in 5% nonfat dry milk, and subsequently incubated overnight at 4°C with monoclonal anti-nNOS (1:700 dilution), anti-iNOS (1:700) and anti-eNOS (1:500) antibodies in blocking buffer. Lysates from rat pituitary, cytokine-stimulated macrophages, and human endothelial cells were included as positive controls for nNOS, iNOS and ecNOS, respectively. After washing, the membranes were incubated with HRP-conjugated anti-mouse IgG antibodies and immuno-labeled protein bands were visualized by enhanced chemiluminescence. Coomassie® Brilliant Blue R-250 was used to stain gels to ensure equal loading of proteins. Densitometric analysis using Fluorochem 8000 software (Alpha Innotech, San Leandro, CA) was performed to quantify immunoblot protein band intensities.

MPO Assay: Neutrophil myeloperoxidase (MPO) activity can result in nitrotyrosine formation via the oxidation of nitrite (NO_2^-) (58,59). Therefore, skeletal muscle (MPO) activity was assayed to examine what role neutrophil sequestration plays in 3-nitrotyrsine formation in the setting I/R injury. The MPO assay method of Koike et al. (60) was used with minor modifications. Briefly, muscle tissue was homogenized in 40mM potassium phosphate buffer, pH 7.4, and centrifuged at 3000 rpm, 4°C, for 10 minutes. The resulting pellet was resuspended in 4ml of 50 mM PPB, pH 6.0, containing 0.5 g/dl centrimonium bromide, and sonicated for 3 minutes. Samples were incubated at 60°C for 2 hours and centrifuged at 13000 rpm for 10 minutes. A 0.1ml volume of supernatant was added to 2.9 ml of 50mM Potassium Phosphate Buffer pH 6.0, containing 0.167 mg/ml o-dianisidine and 5×10^{-4} % H_2O_2 and absorbance was measured at 460 nm for 3 minutes. MPO activity (units/g) =

ΔA460 x 13.5/g, where ΔA460 equals the change in absorbance between 1 and 3 minutes. Pure MPO from human leukocytes was used as a positive control.

Assessment of Sarcolemmal Fragility Following I/R

Sarcolemmal injury is a characteristic feature of dystrophic pathology in DMD patients and mdx mice (12-16). Evans blue dye (EBD), a low molecular weight tracer compound that binds tightly to serum albumin, has been used frequently to evaluate membrane injury in mouse models of muscular dystrophy. EBD-albumin complexes are excluded from normal myofibers, but are readily taken up in fibers with a damaged or leaky sarcolemma (16). Therefore, we used this vital dye to determine the extent of sarcolemmal damage resulting from I/R and/or muscle stretch. We injected EBD (5 μg/μl solution in PBS; 5 μl/g body wt) via the jugular vein of anaesthetized mice either prior to the application of the tourniquet (to examine the effect of ischemia alone), or just prior to removal of the tourniquet (in order to examine the effect of I/R). Again the ischemia period was 1.5 hours. In order to allow sufficient time for the circulating EBD to enter damaged membranes at reproducible levels, in these experiments we sacrificed the animals 3 hours after the release of the tourniquet (i.e., 3 hr reperfusion).

Dystrophin-deficient skeletal muscle of the mdx mouse has been shown to abnormally susceptible to membrane damage due to mechanical stress (14,41,61). In addition, oxidative stress is known to have deleterious effects on membrane stability via lipid peroxidation and protein-protein cross-linking reactions (44-47). Therefore, to examine the potential role of

oxidative stress in the membrane fragility associated with dystrophin-deficiency, we performed a protocol of in vivo muscle stretch following I/R. Briefly, this involved using a computer controlled lever arm to lengthen the TA muscle of stationary anaesthetized mice. The distal tendon of the muscle was connected to the lever arm by a fine 4-0 suture and the muscle was stretched by a distance of 50% of the resting muscle length. As an experimental control for the effect of stretch alone on EBD uptake, we performed the same stretch protocol on TA muscles of mice which had been injected with EBD, but had not been subjected to I/R. At the end of each experiment the TA was quickly removed and mounted in Frozen Section Medium and snap-frozen in isopentane pre-cooled with liquid nitrogen. Transverse sections of frozen TA muscles were cut and fixed in methanol for 5 minutes and visualized by fluorescence microscopy. EBD emits a red fluorescence when activated with green light, allowing the number of EBD positive fibers to be quantified. A digital camera was used to capture images to a computer and the analysis was performed using a commercial software package (Image-Pro Plus, Media Cybernetics, Silver Springs, MD).

Statistical Analysis

All data were reported as means ± SE and were analyzed with the statistical analysis program SigmaStat® (SPSS Inc.). Differences between groups were initially tested by analysis of variance (ANOVA), with post hoc application of the Tukey test where appropriate. Statistical significance was defined as $p<0.05$.

3.5 RESULTS

Susceptibility to I/R-induced Oxidative Stress

Protein Carbonyl Formation: Protein carbonyl formation is a widely used index of oxidative modifications to proteins. By immunoblotting for these protein alterations (Fig. 1), we found that I/R caused increases in protein carbonyl formation in both C57 and mdx skeletal muscles. However, densitometry analysis revealed no statistically significant differences in terms of the amount of increase between the two mouse strains (data not shown). These data suggest that our hindlimb tourniquet model does indeed induce oxidative stress to skeletal muscles of these mice, but that there is no difference between these two strains in terms of susceptibility of total cellular proteins to oxidative stress.

Lipid Peroxidation: Cellular lipids are another potential target of oxidative/nitrosative stress. Hydroxynonenal (HNE) is one of many unsaturated aldehydes formed during lipid peroxidation reactions. Once formed it can bind proteins to form HNE-protein adducts (Halliwell & Gutteridge, 1999). Therefore, to examine the effect of I/R-induced oxidative stress on lipid peroxidation in dystrophin-deficient skeletal muscle, immunoblotting for HNE-protein adducts was performed. HNE-immunoblotting revealed that one protein band, of approximately 70 kDa, was altered by I/R (Fig. 1). This protein band was not detected in either sham- or ischemia-treated C57 muscle, but appeared consistently at a low level in the I/R-treated C57 group. In the dystrophin-deficient mdx muscles, this immunoreactive protein was detected regularly at low levels in the sham- and ischemia-treated groups, and was

increased dramatically after I/R. Densitometry analysis revealed that the mean relative intensity of this band was more than 300% greater in mdx than in C57 muscles following I/R (Fig. 2). These data suggest that dystrophin-deficient skeletal muscle is abnormally susceptible to I/R-induced lipid peroxidation.

Susceptibility to I/R-induced Nitrosative Stress

3-Nitrotyrosine Formation: Peroxynitrite ($ONOO^-$), a highly damaging oxidant formed by the reaction of superoxide and nitric oxide, is believed to be one of the primary mediators of protein nitration in biological systems. As such, nitrotyrosine formation has been widely used as a marker of nitrosative stress. Therefore, we performed immunoblotting for protein-bound 3-nitrotyrosine to examine the effect of I/R-induced nitrosative stress on dystrophin-deficient muscle. These blots revealed several proteins which possessed 3-nitrotyrosine residues, but the intensity of one protein (~58 kDa), in particular, was consistently altered by I/R treatment in mdx muscle (Fig.1). The mean relative intensity of this band was more than 250% greater in mdx than in C57 muscle following I/R (Fig. 3). These data suggest dystrophin-deficient skeletal muscle is abnormally susceptible to IR-induced nitrosative stress. MPO activity from invading neutrophils can also induce nitrotyrosine formation (58,59). To examine the role of neutrophils in the nitrotyrosine formation seen here, MPO activity was assessed. MPO activity was not detected in muscles from either mdx or C57 mice in any of the experimental groups (data not shown) . Human neutrophil MPO, used as a positive control, gave signals in the detectable range from 0.05 U/ml to 2 U/ml. These data suggest that neutrophil

sequestration does not contribute to the increased nitrosative stress found in mdx muscle during I/R.

NOx Levels: To determine the role of NO• production in the response of dystrophin-deficient muscle to I/R, we measured levels of NO• metabolites (NOx) using a Sievers NOA 280i chemiluminescence NO• analyzer (Fig. 4A). At baseline (sham-treatment), NOx levels tended to be lower in mdx than in C57 mice muscle, however, this did not reach statistical significance. In mdx muscles, ischemia alone caused a striking increase in NOx levels. No such difference was seen following I/R in the mdx muscles. In C57 muscles, neither ischemia nor I/R affected NOx levels. These results suggest that the mdx-specific increases in I/R-induced lipid peroxidation and protein nitration follow an increase in NOx production in during ischemia.

NOS Isoform Expression: To examine the potential role of altered NOS expression in the abnormal NOx response found in mdx muscle, immunoblotting for nNOS, eNOS, and iNOS was performed (Fig. 4B). A total of 4 immunoblotting experiments were performed, such that the sample size (n) for each experimental group tested was 4. Densitometric analysis was performed to quantify the level of expression of each isoform (data not shown). By 2-way ANOVA analysis nNOS expression was found to be lower in mdx than in C57 muscles ($p=0.013$). However, nNOS and eNOS expression levels were not altered by ischemia or I/R in either strain of mice. iNOS expression was not detected in any of the experimental groups.

These results suggest that neither altered expression of the constitutive NOS isoforms, ecNOS and nNOS, nor the induction of iNOS contribute to the changes in NOx levels seen in dystrophin-deficient skeletal muscle during ischemia.

Sarcolemmal Fragility Following IR-Induced Oxidative/Nitrosative Stress

After finding that mdx muscles are abnormally suscetible to I/R-induced oxidative/nitrosative stress, we next used Evans blue dye (EBD) (Figures 5 & 6) uptake to examine if I/R might cause increased sarcolemmal injury or increased susceptibility to mechanical stress-induced sarcolemmal injury, two characteristic features of mdx muscle pathology. Sham-treated C57 animals did not display sarcolemmal damage, as EBD positive fibers were not detected in these muscles. Sham-treated mdx muscles, on the other hand, consistently showed low levels of EBD positive fibers. Ischemia alone did not increase sarcolemmal injury in either mouse strain (data not shown). I/R produced significant increases in membrane damage in both mdx and C57 muscle (approximately 163 and 130 EBD positive fibers, respectively), but no significant differences were seen between the two strains (Fig. 6).

To assess whether I/R-induced oxidative/nitrosative stress might make dystrophin-deficient skeletal muscle abnormally vulnerable to mechanical stress-induced sarcolemmal injury, we employed a protocol of repetitive passive muscle stretch (30 x 50% of resting muscle length) following I/R. Stretch alone caused a small, but significant increase in membrane damage in both groups (Fig. 6). The combination of I/R and stretch produced a dramatic increase in EBD uptake in mdx muscle, which was not seen in the C57. These

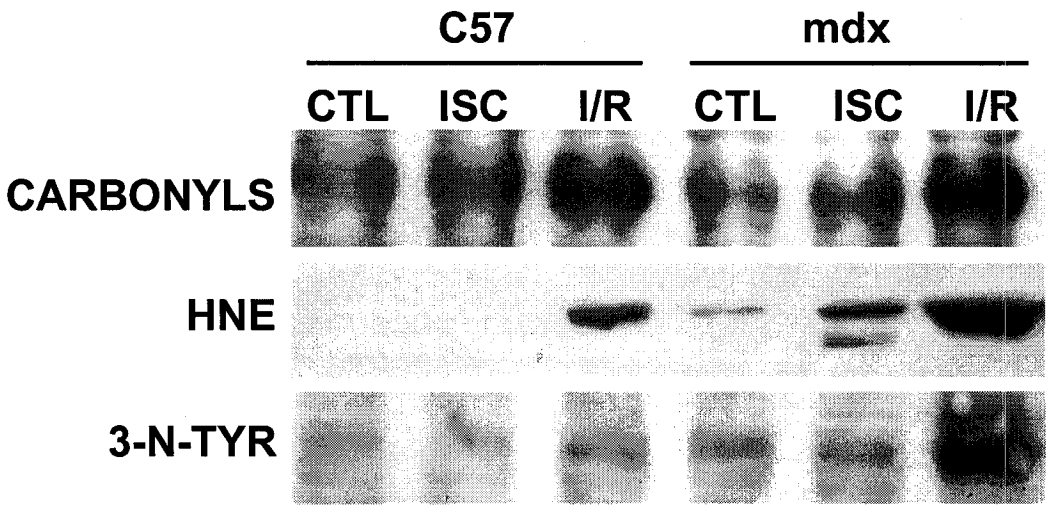

Figure 1. Representative immunoblots of the markers of oxidative and nitrosative stress used in this study. C57 and mdx TA muscles were subjected to sham-treatment (CTL), ischemia-alone (ISC), and ischemia/reperfusion (I/R). Proteins from these muscles were separated by SDS-PAGE, and immunoblotted with antibodies to (from top to bottom) carbonyls, hydroxynonenal (HNE), and 3-nitrotyrosine (3-N-TYR).

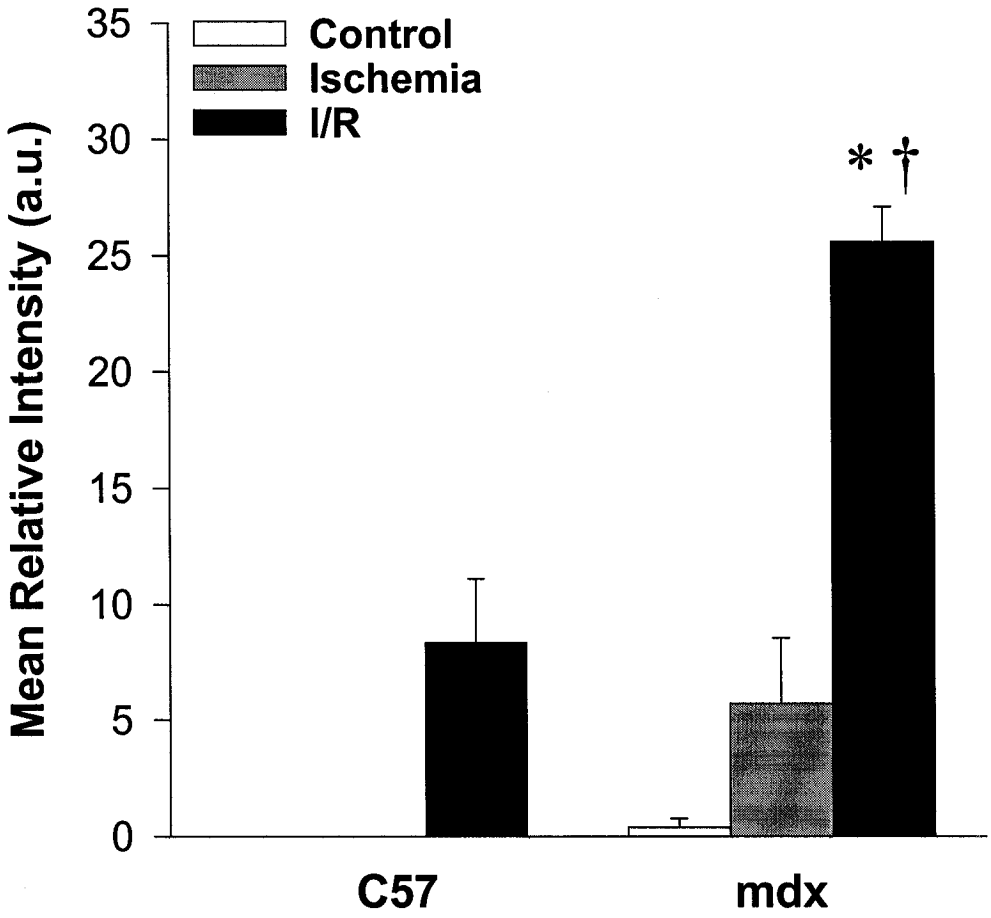

Figure 2. Densitometric analysis of a protein band (~70 kDa) detected by hydroxynonenal (HNE) immunoblotting in C57 versus mdx TA muscles following sham-treatment (Control), ischemia-alone (Ischemia), and ischemia/reperfusion (I/R). Sample size (n)=4 for each treatment group. All values are group means ± SE. *$p<0.05$ for I/R-treated mdx versus sham-treated mdx, and †$p<0.05$ for I/R-treated mdx versus I/R-treated C57.

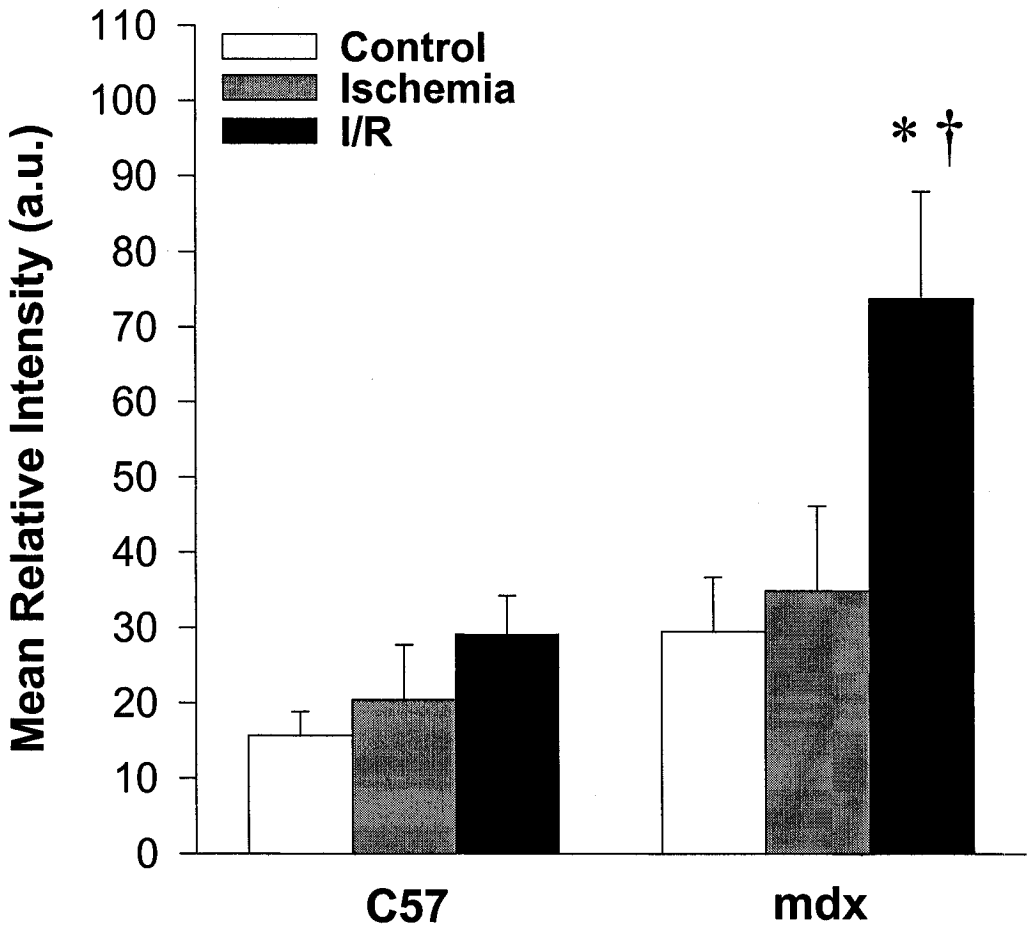

Figure 3. Densitometric analysis of a protein band (~58 kDa) detected by 3-nitrotyrosine immunoblotting in C57 versus mdx TA muscles following sham-treatment (Control), ischemia-alone (Ischemia), and ischemia/reperfusion (I/R). For both the C57 and mdx muscle groups, sample size (n)=4 for Control, 4 for Ischemia, and 5 for I/R. All values are group means ± SE. *$p<0.05$ for I/R-treated mdx versus sham-treated mdx, and †$p<0.05$ for I/R-treated mdx versus I/R-treated C57.

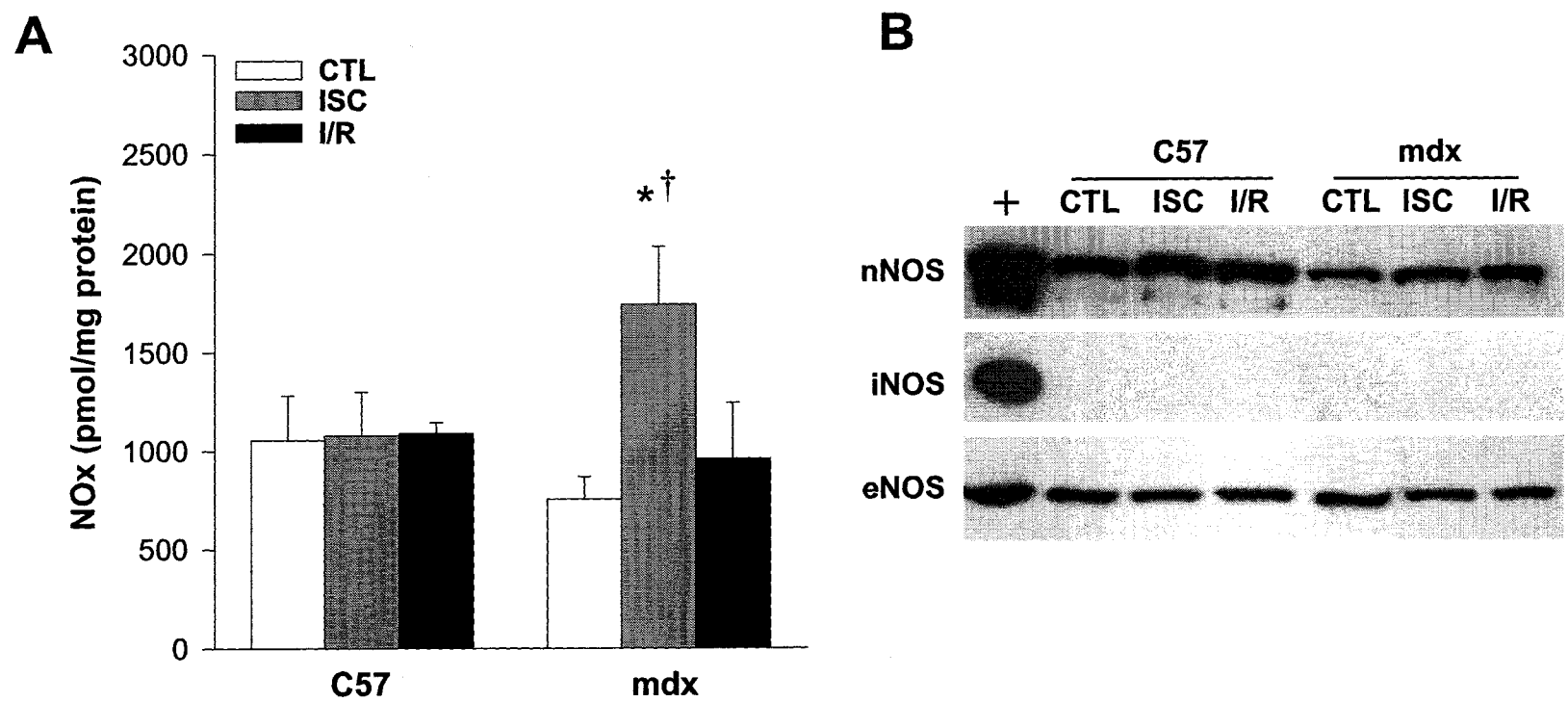

Figure 4. NO production and nitric oxide synthase (NOS) isoform expression in mdx versus C57 TA muscles following sham-treatment (CTL), ischemia-alone (ISC), and ischemia/reperfusion (I/R). (**A**). Levels of NO metabolites (NOx) were measured using a Sievers NOA 280i chemiluminescence NO analyzer. Sample size (n)=6 for each treatment group. All values are group means ± SE. *$p<0.05$ for I/R-treated mdx versus sham-treated mdx, and †$p<0.05$ for I/R-treated mdx versus I/R-treated C57. (**B**). Representative immunoblots of neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) in TA muscle protein samples. Lysates from rat pituitary, cytokine-stimulated macrophages, and human endothelial cells, included as positive controls for nNOS, iNOS and ecNOS, respectively, are shown in the left hand lane (+).

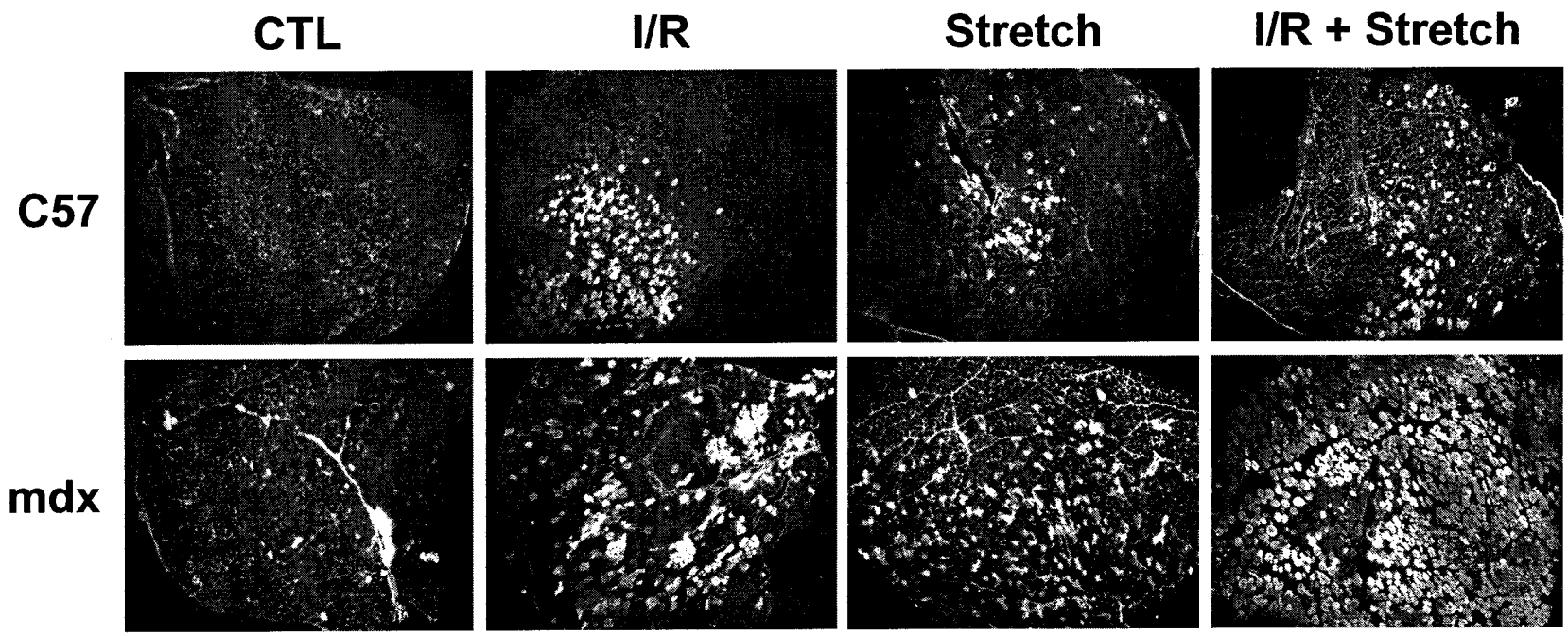

Figure 5. Representative micrographs of TA muscle cross-sections demonstrating Evans Blue Dye (EBD) uptake and fluorescence in C57 and mdx muscles following sham-treatment (CTL), ischemia/reperfusion (I/R), Stretch, and I/R + Stretch. EBD only penetrates those muscle fibers which have a damaged sarcolemma.

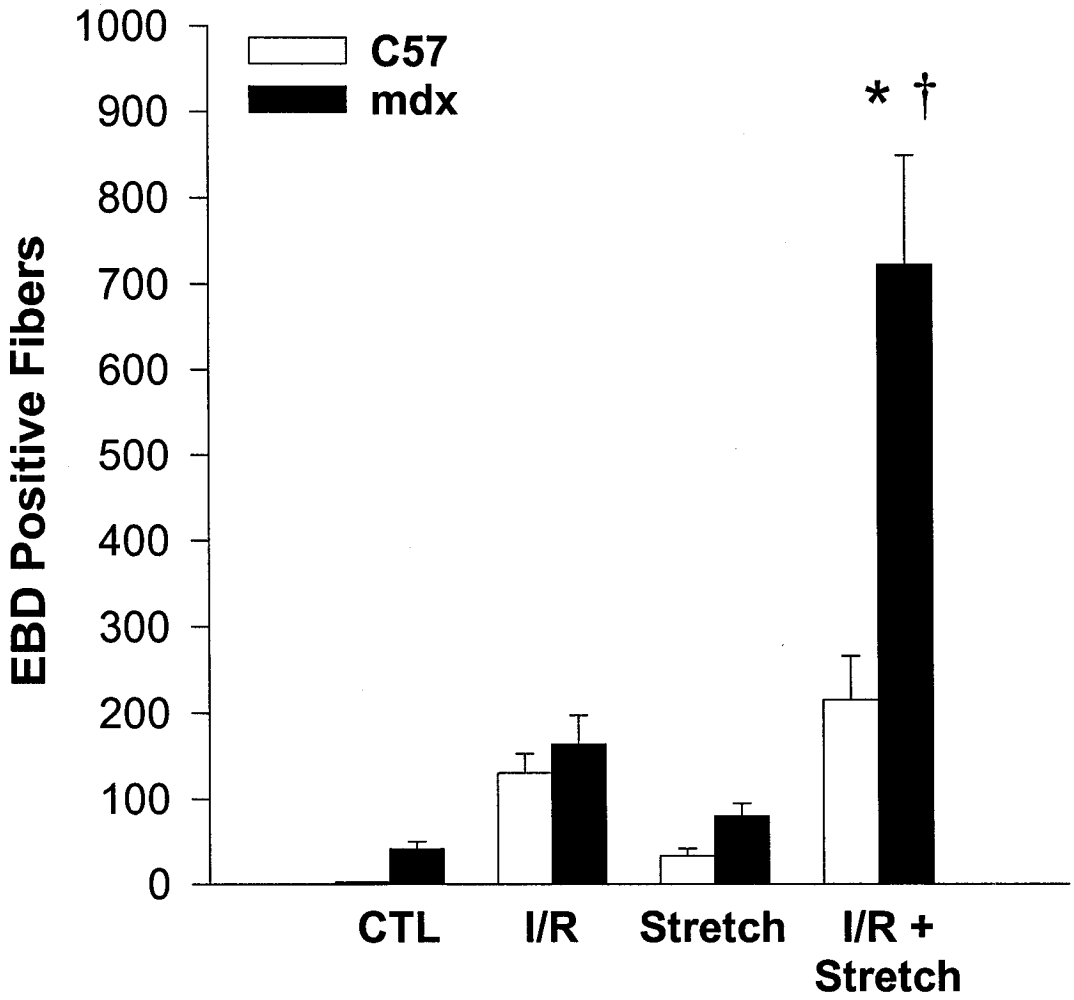

Figure 6. I/R increases the susceptibility of mdx muscle cells to mechanical stress-induced sarcolemmal injury. Sarcolemmal damage, as measured by number of EBD positive fibers, was quantified in C57 versus mdx TA muscle cross-sections following sham-treatment (CTL), ischemia/reperfusion (I/R), Stretch, and a combination of I/R + Stretch. Note that although I/R and Stretch by themselves did not cause a significant increase in sarcolemma damage in mdx versus C57, the combination of I/R + Stretch dramatically increased sarcolemmal injury in dystrophin-deficient muscles. For both C57 and mdx, sample size (n)=6 for each treatment group. All values are group means ± SE. *$p<0.05$ for I/R+Stretch treated mdx versus I/R+Stretch treated C57.

results highlight a potential synergistic interaction between I/R-induced oxidative/nitrosative stress and mechanical stress to induce sarcolemmal damage in dystrophin-deficient muscles.

3.6 DISCUSSION

To our knowledge this is the first study to examine the susceptibility of dystrophin-deficient skeletal muscle to oxidative/nitrosative stress induced by IR-injury, a form of free-radical-mediated injury that is of particular relevance to DMD (1,2). The principle findings of this study are: (1) dystrophin-deficient muscle is abnormally susceptible to I/R-induced oxidative and nitrosative stress; (2) increased susceptibility to I/R-injury in dystrophin-deficient muscle is associated with increased NOx production during ischemia which was not seen in normal mice; and (3) I/R makes the dystrophin-deficient sarcolemma of mdx mice abnormally vulnerable to mechanical stress. The increased protein nitration and NOx production in IR-treated mdx muscle reported here, in the absence of altered constitutive NOS expression or iNOS induction, suggest that aberrant $NO^{\bullet}$ production from mislocalized nNOS may be at least partly responsible for these findings. Importantly, these findings may serve to link several observations concerning dystrophic muscle pathology including abnormalities of vascular control, increased oxidative/nitrosative stress, and sarcolemmal fragility.

Increased susceptibility to I/R-induced oxidative and nitrosative stress.

Several lines of evidence suggest that oxidative stress plays a role in DMD. First, indices of oxidative stress such as protein carbonyls (4) and lipid peroxidation bi-products (3), are abnormally elevated in DMD patients and mdx mice. Secondly, a compensatory activation of antioxidant systems is seen in dystrophic skeletal muscle (3,27). Interpreting the results of these reports is not straightforward because indices such as these may increase secondarily to dystrophic pathology, merely as a consequence of necrotic cell death or inflammatory cell infiltration. However, Disatnik et al (5) showed that antioxidants are upregulated and lipid peroxidation products increased even before the onset of necrosis, suggesting that oxidative stress is involved in disease pathogenesis. Furthermore, in vitro studies using primary cultures of mdx muscle cells have shown that dystrophin-deficient myotubes are abnormally susceptible to oxidant injury, suggesting that increased vulnerability to oxidative stress could be a primary feature of dystrophin deficiency (6,7).

In recent years it has been found that DMD patients and mdx mice suffer functional muscle ischemia due to unopposed sympathetic vasoconstriction during exercise (1,2). It is well-established that ischemia and subsequent reperfusion (I/R) causes skeletal muscle injury due to oxidative/nitrosative stress. If dystrophin-deficient muscles are indeed abnormally susceptible to free-radical damage as has been suggested, I/R may be an important source of oxidative/nitrosative stress which contributes to cell damage and necrosis in these muscles. These reports of functional ischemia in dystrophin-deficient muscles have also renewed interest in an old "vascular hypothesis" of DMD, which was put forward many years ago due to the observation that grouped necrotic fibers in DMD

resemble ischemic muscle lesions, and that experimental ischemia could reproduce these characteristic dystrophic lesions (30,31). Lanari et al. (62) had earlier showed that 10 minutes of ischemia (induced by a blood pressure cuff inflated above systolic pressure) and 1 minute of reperfusion caused increased serum creatine kinase levels in DMD patients. Therefore, we chose to study the susceptibility of dystrophin-deficient muscle to I/R-induced oxidative/nitrosative stress. We found greater increases in lipid peroxidation and nitrotyrosine formation in mdx than in normal muscle following I/R. This study provides the first direct in vivo evidence that dystrophin-deficient muscle is abnormally susceptible to oxidative stress. Undoubtedly, the level of muscle ischemia produced using our model is much greater than would be expected to occur in the mdx mouse during muscle activity. However, our results suggest that, if transient ischemic insults during muscle activity are of sufficient magnitude to induce even low-grade oxidative/nitrosative stress, they may cause damage in dystrophin-deficient muscle, which is abnormally susceptible to such a challenge. Therefore, abnormal susceptibility to oxidative/nitrosative stress may act as a primary pathogenic factor contributing to muscle destruction in DMD.

Role of peroxynitrite, nitric oxide, and nNOS

Immunoblotting for protein 3-nitrotyrosine residues is a widely used marker of nitrosative stress (47). In the present study, significantly greater 3-nitrotyrosine formation was found in mdx than in C57 muscle following I/R. While $ONOO^-$ production from the reaction of $NO^\bullet$ with $O_2^{\bullet -}$ is believed to be the primary noxious agent leading to nitrotyrosine

formation, it is well established that neutrophilic myeloperoxidase (MPO) activity in the presence of nitrite can also induce protein tyrosine nitration. However, we did not detect MPO activity in either mouse strain under any of the experimental conditions tested. Therefore, this suggests that the increased nitrotyrosine formation in mdx muscles following I/R was due to the production of $ONOO^-$ rather than myeloperoxidase. We found that the increased nitrotyrosine formation in mdx muscle followed a significant increase in NOx production during ischemia, which was not seen in the normal muscle. This suggests that levels of $NO^\bullet$ increased during ischemia in mdx muscle, and this increased $NO^\bullet$ could have reacted with $O_2^{\bullet-}$ to produce $ONOO^-$. Possible source of this increased $NO^\bullet$ production will be discussed in detail below.

$ONOO^-$ is also a potent initiator of lipid peroxidation reactions (57) and here, immunoblotting for HNE-protein adducts revealed that I/R-treatment also caused significantly greater lipid peroxidation in mdx than in C57 skeletal muscle. Protein-bound HNE was also significantly increased in mdx during ischemia, corresponding to the time point at which we also observed the mdx-specific increase in NOx production. HNE is formed when lipid peroxides are cleaved in the presence of transition-metals, such as iron. This would suggest that the release of stored transition-metals may also be involved in the free radical-mediated damage seen here. This is in agreement with the study of Bornman et al. (63) which reported that iron deprivation decreased muscle pathology in mdx skeletal muscle.

The increased NOx formation seen here in mdx mice during ischemia occurred in the absence of iNOS expression. This would suggest that the constitutive forms of NOS play a role in increased NOx production during ischemia. While nNOS and eNOS are constitutively

expressed in myofibers, nNOS is most abundant and is believed to be the main regulator of $NO^{\bullet}$ homeostasis in muscle (64). This has been demonstrated using eNOS knock-out mice, which are indistinguishable from control mice in terms of skeletal muscle $NO^{\bullet}$ production and contractile function. Using normal mice, Thiemermann et al (65) showed that specific inhibition of nNOS reduced skeletal muscle damage due to I/R. Together these results point to nNOS as the most likely candidate for aberrant $NO^{\bullet}$ production during I/R.

Some controversy exists as to the level of nNOS expression in dystrophin-deficient muscle. In normal skeletal muscle nNOS is most abundant at the sarcolemma, but it is also present in the cytosol (21,66). While it is generally accepted that the sarcolemmal portion of nNOS is displaced to the cytosol in dystrophin-deficieny muscle, there is less agreement as to the overall nNOS levels in these muscles. Reports range from modest reductions (~20%) to near complete loss of nNOS. By western blotting of crude protein homogenates, Brenman et al. (21) found that overall nNOS expression was modestly decreased in mdx muscle and decreased by ~75% in muscles from DMD patients. By examining individual components of muscle fibers by cellular fractionation, immunohistochemistry, western blotting and enzyme activity assays, these authors found that nNOS was absent from the sarcolemma, but actually increased in the cytosol of mdx skeletal muscle fibers. Grozdanovic et al. (67,68) used nNOS immunohistochemistry in DMD muscle biopsy samples and found that nNOS was greatly reduced both at the sarcolemma and in the cytosol. Chang et al. (23) performed cellular fractionation and immunoblotting experiments and found that nNOS was absent from the sarcolemma, and reduced in the cytosol of mdx muscle, but completely absent from both the fractions in muscles from DMD patients. The discrepancies between

these studies, particularly the different findings regarding cytosolic localization of nNOS in DMD patient muscle, may have resulted from variations of methodology, or the muscles analysed, or the age of patients from which these biopsy samples were derived. While some disagreement remains as to the level of nNOS reduction, it is now widely accepted that the enzyme is displaced from the sarcolemma of dystrophin-deficient muscle, and overall expression and activity is reduced. Here, using western blotting of crude protein homogenates we found a small decrease in total cellular nNOS in mdx muscle relative to C57. Similar levels of average decrease were seen for nNOS protein expression and NOx levels. Therefore, our data support the contention that modest deficits in total nNOS protein occur in mdx muscle, and that the major abnormality is one of mislocalization.

Potential deleterious and beneficial roles of NO• in muscle

Prior to the knowledge that the loss of nNOS from the sarcolemma caused unopposed vasoconstriction during muscle activity, aberrant nNOS localization was hypothesized to contribute to dystrophic pathology by increasing cytosolic nitrosative stress (21). Our data would support such a deleterious role for nNOS in dystrophin-deficient skeletal muscle, at least during I/R. However, several reports have refuted this earlier hypothesis. Two independent groups crossed mdx mice with nNOS knock-out mice and found no improvements in the mdx dystrophic pathology, despite the complete absence of nNOS (69,70). More recently, Zhuang et al (71) experimentally altered nNOS expression in

normal and mdx myotubes and found that the susceptibility of these cultures to various forms of oxidative stress was independent of nNOS expression.

It can be argued, however, that none of the above experimental systems accurately reproduced the in vivo situation of mislocalized nNOS expression which accompanies dystrophin-deficiency. The study of Zhuang et al (71) was performed in vitro and therefore may not adequately reproduce the kind of oxidative/nitrosative stress that skeletal muscle is subjected to in vivo. The studies which showed that double nNOS/dystrophin-deficient mice do not display reduced muscle pathology most strongly oppose the idea that aberrant localization of nNOS contributes to dystrophic myopathy. However, skeletal muscles in these animals were not stressed in any fashion. It is well established that dystrophic pathology is worsened by muscle activity. For instance, mdx muscle damage is greatly increased by controlled protocols of in vitro or in vivo muscle contractions or by exercise (14,72,73). To accurately test if the genetic removal of nNOS can afford some protection against dystrophic pathology, skeletal muscles from these animals would have to be challenged with some form of muscle activity. In addition, not only has $NO^{\bullet}$ been implicated in vascular control, but this it has also been shown to be involved in muscle repair (74,75), contractile function (76), glucose metabolism (77), calcium release from the SR (78,79), mechanical-mediated signal transduction (80), and inhibition of leukocyte adhesion (81). Although the overall level of nNOS may be reduced in the mdx, the portion which remains may play dual beneficial and deleterious roles in muscle homeostasis. As such, it is difficult to envisage that total removal of nNOS would actually improve pathology, when these beneficial functions would also be lost. Along these lines, Wehling et al (82) showed that

overexpression of nNOS ameliorates dystrophic pathology in transgenic mdx mice, and these authors suggested the protective affect might be due to anti-inflammatory properties of nitric oxide. However, the possibility of prevention of functional ischemia in the mdx mice overexpressing nNOS, as well as other potential beneficial effects of $NO^{\bullet}$ as outlined above cannot be excluded.

The increased NOx levels found following ischemia were not associated with any change in NOS expression. The question remains then, as to the source of these increased $NO^{\bullet}$ metabolites. Constitutive NOS isoforms, including nNOS, are activated by calcium (Ca^{2+}). Therefore, NOS activity may have increased due to increased intracellular free Ca^{2+} levels. Inappropriate free Ca^{2+} levels in myofibers, a characteristic feature of dystrophic-pathology, is also known to occur in normal muscle during ischemia (83-85). Therefore, increased intracellular calcium induced by I/R could have activated the displaced nNOS, leading to excess $NO^{\bullet}$ and peroxynitirite production in the cytosol.

Little is known about the regulation of nNOS at the normal DGC, or how this regulation might be altered in dystrophin-deficient muscle. However, it has been reported that binding of the enzyme to membrane associated caveolins inhibits its activity (86). Mutations in the gene encoding caveolin-3, an additional protein which binds nNOS at the sarcolemma of skeletal muscle, have been found in patients with autosomal dominant limb-girdle muscular dystrophy (LGMD1C) (87). Intriguingly, transgenic mice expressing one of these mutant caveolin-3 proteins displayed a severe myopathy, which was associated with loss of caveolin-3 and increased nNOS activity at the sarcolemma (88). Therefore, nNOS mislocalization in dystrophin-deficient muscle may not only initiate recurrent bouts of

functional ischemia, but displaced nNOS in the cytosol might also be under less rigorous control mechanisms, thereby allowing for excess $NO^{\bullet}$ production and increased nitrosative stress during these ischemic periods. In this regard, it would be interesting to see how double nNOS/dystrophin-deficient mice (discussed above) would respond to IR-induced oxidative/nitrosative stress. Indeed, it is well established that nNOS knock-out mice are less susceptible to cerebral I/R injury (89,90). Similarly, one could envisage nNOS/dystrophin-deficient mice, with similar baseline pathology as regular mdx mice, being less susceptible to skeletal muscle IR-injury. Further studies are needed to test these hypotheses.

I/R-induced hyperfragility of the sarcolemma

Membrane fragility is a characteristic feature of dystrophin-deficiency. In particular, the mdx skeletal muscles have been shown to display increased susceptibility to sarcolemmal injury due to mechanical stress. In addition, Ebihara et al. (48) showed that oxidative/nitrosative stress and mechanical stress work synergistically to produce membrane injury in normal rat myotubes. In the present study we found that I/R and mechanical stress interact synergistically to induced sarcolemmal injury in mdx skeletal muscles. Specifically, we found that in mdx muscles, stretch following I/R caused significantly greater sarcolemmal injury than either treatment alone. No such statistically significant increase was found in C57 muscles subjected to I/R and stretch. Our results suggest that mdx muscles are abnormally susceptible to I/R-induced oxidative/nitrosative stress, and that I/R predisposes these muscles to increased sarcolemmal damage when subjected to mechanical stretch. In

addition, we found that I/R caused greater lipid peroxidation in mdx than in normal skeletal muscles. Lipid peroxidation, and HNE formation in particular (91), within biological membranes decreases membrane fluidity and increases membrane permeability to molecules, such as calcium ions, which normally only enter the cell through specific channels (47). Therefore, it is possibility that I/R caused greater lipid peroxidation of the sarcolemma in mdx muscles, which predisposed the membrane to rupture during stretch. Together, our findings suggest that increased susceptibility to oxidative/nitrosative stress, may play a role in the characteristic sarcolemmal hyperfragility seen in dystrophin-deficient muscle.

In conclusion, we have provided the first direct evidence that dystrophin-deficient muscle is abnormally susceptible oxidative/nitrosative stress induced by IR injury, a form of insult that is now believed to occur in DMD and mdx skeletal muscle on a frequent basis during muscle activity due to the absence of nNOS at the sarcolemma. In addition, we have shown that the abnormal increases in protein nitration and lipid peroxidation in mdx muscle are associated with increased production of nitric oxide derivatives during ischemia, which may result from the aberrant mislocalization of nNOS to the cytosol in skeletal muscle of these mice. Finally, we have demonstrated that I/R predisposes the dystrophin-deficient sarcolemma to heightened injury when these muscles are subjected to mechanical stress. These findings may serve to link several previous hypotheses concerning the means by which the absence of dystrophin leads to muscle destruction. This suggests the possibility of a new conceptual framework for pathogenesis in DMD.

3.7 ACKNOWLEDGEMENTS

I would like to again thank Won-Kyung Cho for her important contributions to the development of the animal model presented here; Duska Gvosdic for her expert technical assistance; Gawiyou Danialou for his assistance with the I/R experiments and microscopy, Maziar Divanghani and Weisheng Bao for their help with the MPO assay, Karuthapillai Govindaraju for his assistance with the NOx analysis; and finally Basil J. Petrof for his assistance, advice and supervision.

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CHAPTER 4.

Response of the glutathione antioxidant system to ischemia/reperfusion injury in dystrophin-deficient (mdx) skeletal muscle.

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4.1 PROLOGUE

In the preceding chapter, we reported that dystrophin-deficient muscle is abnormally susceptible to ischemia/reperfusion-induced oxidative/nitrosative stress. Furthermore, this enhanced susceptibility appears to play a role in the mechanical fragility of the sarcolemma in these animals. It remains unresolved how the absence of dystrophin makes skeletal muscles more vulnerable to oxidative stress, as the cytoskeletal protein is not known to possess any antioxidant functions itself. It may simply be that in dystrophin-deficient muscle, the structural reinforcement is lost such that a membrane subjected to oxidative/nitrosative damage can be more easily torn apart during muscle contraction. Alternatively, antioxidant systems may be disturbed in the setting of dystrophin-deficiency. In the next chapter we investigate the response of the glutathione antioxidant system to I/R-induced oxidative/nitrosative stress in mdx muscle.

4.2 ABSTRACT

Several lines of evidence have implicated oxidative/nitrosative stress in Duchenne muscular dystrophy (DMD). In particular, reports have suggested that dystrophin-deficient cells are abnormally susceptibility to oxidative damage (1). In this regard, we have recently shown that dystrophin-deficent mdx mouse muscles display increased susceptibility to ischemia/reperfusion (I/R)-induced oxidative/nitrosative stress. It remains to be determined how the absence of dystrophin might confer this deleterious property to skeletal muscles. One possibility is a disturbance of antioxidant systems, however, dystrophin itself is not known to possess antioxidant function, and some antioxidants such as SOD1 have actually been shown to be upregulated in dystrophin-deficient muscle. One of the most important antioxidant systems in skeletal muscle involves glutathione (GSH) and its associated enzymes, glutathione peroxidase (GPx) and NADPH-dependent glutathione reductase (GR). It is well established, that the citric acid cycle enzymes isocitrate-dehydrogenase (ICDH) and aconitase are highly susceptible to inactivation due to oxidative/nitrosative stress, and ICDH, in particular, plays a role in glutathione recycling as it the major supplier of NADPH to GR in skeletal muscle. Despite the importance of this system, a thorough analysis of GSH and its associated enzymes, and specifically how these respond to oxidative/nitrosative stress in dystrophic muscle, has not been reported. Therefore, in the present study we measured total GSH, the oxidized-to-reduced glutathione (GSSG/GSH) ratio, GPx, GR, ICDH, and aconitase activity in mdx versus normal C57 muscles at baseline (sham-treatment) and following I/R-treatment. We found that total GSH is decreased and the GSSG/GSH ratio is increased in mdx relative to C57

muscle. GPx and GR activities are increased, while ICDH and aconitase activites are decreased in mdx muscles. In C57 muscle, I/R caused a drop in total GSH, increased the GSSG/GSH ratio, increased GPx activity, and decreased activity of both ICDH and aconitase. In contrast, the only outcome measure to change in mdx muscles following I/R treatment was GR activity, which decreased relative to sham-treated mdx. These results suggest that the natural history of dystrophin-deficient mdx muscles involves oxidative/nitrosative stress which alters the glutathione status in this tissue.

4.3 INTRODUCTION

Duchenne muscular dystrophy (DMD) is caused by mutations in the dystrophin gene which result in the absence of this sarcolemma-associated protein. The exact mechanism by which the loss of dystrophin induces skeletal muscle destruction remains unresolved, but several lines of evidence suggest that oxidative/nitrosative stress plays a role in the pathogenesis of this disease. Skeletal muscles of DMD patients and dystrophin-deficient mdx mice display abnormal increases in several indices of oxidative stress including protein carbonyls (2), lipid peroxidation bi-products (3), and compensatory antioxidant systems (1,4). With regard to the latter, Disatnik et al (1) showed that antioxidants are increased even before the onset of necrosis, suggesting that oxidative stress may be a primary pathogenic event, rather than simply a secondary consequence of muscle damage. In addition, in vitro studies using primary cultures of mdx muscle cells have

shown that dystrophin-deficient myotubes are abnormally susceptible to oxidant injury via reactive oxygen species (ROS) (5,6). Together, these data suggest that an increased exposure and vulnerability to oxidative stress may be a primary feature of dystrophin deficiency.

It is recognized that dystrophin-deficient muscles are abnormally susceptible to mechanical stress-induced injury, such as sarcolemmal rupture, during muscle contractions (7). In recent years, it has also been shown that skeletal muscles of DMD patients and mdx mice suffer recurrent bouts of functional ischemia due to the mislocalization of neuronal NOS (nNOS) from the sarcolemma, secondarily to the absence of dystrophin (8-11). Ischemia/reperfusion (I/R) is a well known cause of oxidative and nitrosative stress (12-17). We recently tested the hypothesis that dystrophin-deficient muscles might be abnormally susceptible to I/R injury. Using a hindlimb tourniquet model of skeletal muscle I/R, we found that mdx muscles are indeed abnormally susceptible to I/R-induced protein nitration and lipid peroxidation. Importantly, we also found that these dystrophin-deficient muscles were abnormally vulnerable to mechanical stress-induced sarcolemmal damage following I/R-induced oxidative/nitrosative damage (see preceding Chapter 3).

While recurrent bouts of I/R may help to explain an increased exposure of dystrophin-deficient skeletal muscles to oxidative stress, the mechanisms responsible for an increased vulnerability to cellular damage (Chapter 3) and death (5,6) caused by oxidative/nitrosative stress in dystrophic muscle remain to be determined. One possibility is a disturbance in antioxidant systems. Dystrophin, itself, is not known to possess

antioxidant activity. It is, however, associated with the neuronal nitric oxide synthase (nNOS) at the sarcolemma in normal muscle cells. This enzyme catalyses the formation of NO•, which has been ascribed antioxidant, as well as pro-oxidant roles in different scenarios. The proposed antioxidant activities of NO• include direct scavenging of •OH, and the ability to terminate lipid peroxidation reactions (18). As for its pro-oxidant role, NO• itself is not a potent oxidant, but it reacts with other molecules to form more damaging reactive nitrogen species (RNS). Two such reaction products of NO•-derived species, peroxynitrite ($ONOO^-$) and nitroglutathione (GSNO), have both been shown to inactivate the antioxidant enzyme glutathione reductase (GR) (19-21).

Glutathione (GSH) is a critically important antioxidant in skeletal muscle, such that its depletion results in severe muscle degeneration (22). This low molecular weight tri-peptide scavenges ROS/RNS directly, and is used as a cofactor by the antioxidant enzyme glutathione peroxidase (GPx). In both cases, the antioxidant activity of GSH results in its conversion to oxidized glutathione (GSSG). Therefore, the ratio of GSSG/GSH is a widely used indicator of oxidative/nitrosative stress. Under normal circumstances, reduced GSH levels are maintained via recycling of GSSG by the NADPH-dependent enzyme, GR. In muscle cells, the main source of NADPH for GR is the TCA cycle enzyme NADP(+)-specific isocitrate dehydrogenase (NADP-ICDH) (23,24). For this reason NADP-ICDH is also considered to have an important role to play in the glutathione antioxidant system (25,26). NADP-ICDH like the citric acid cycle (CAC) enzyme, aconitase, which supplies isocitrate for the ICDH reaction, is known to be highly susceptible to inactivation by ROS/RNS (27-31).

The purpose of our study was to examine the status of the glutathione antioxidant system of mdx muscle and to see how this is altered by I/R, a form of oxidative/nitrosative stress of particular relevance to dystrophin deficiency. Our specific objectives were: (1) to measure the baseline levels of GSH and GSSG, as well as activities of the GSH-associated enzymes, GPx and GR; (2) to examine activities of the TCA cycle enzymes known to be highly susceptible to oxidative/nitrosative stress, NADP-ICDH and aconitase; and (3) to determine how these glutathione-associated systems are affected by I/R-induced oxidative/nitrosative stress.

4.4 METHODS

Laboratory Animals

Dystrophin-deficient (mdx) and normal dystrophin-expressing C57BL/6 or C57BL/10 mice (collectively referred to as C57) were purchased from the Jackson Laboratory (Bar Harbor, Maine). Animals were studied at either 2 weeks, or 6-8 weeks of age, and were exclusively males. The mice were provided with food and water ad libitum, and all experimental protocols were approved by the institutional animal care and ethics committee.

Ischemia/Reperfusion Protocol

Ischemia/reperfusion (I/R) injury is a well established model of in vivo oxidative/nitrosative stress (12-17). In order to induce ischemia in the lower leg tibialis anterior (TA) muscle, a locking plastic pull-tie tourniquet (100 mm length, 2 mm width) was applied just above the knee of the right hindlimb of deeply anesthetized (ketamine:130mg/kg / xylazine:20mg/kg, i.m. injection) mdx and C57 mice for 90 minutes. A thin (3mm thickness) sponge padding was applied between the skin and the tourniquet to prevent skin damage. Cessation of blood flow was confirmed by a notable temperature drop to touch and visible discoloration of the lower limb. At the end of the ischemia portion of the protocol the tourniquet was removed and the appearance of the lower limb quickly returned to normal. Animals were sacrificed one hour after the removal of the tourniquet. The time points of I/R used here are similar to those reported by other investigators studying short-term I/R injury in mouse hindlimb skeletal muscles (32-35).

Glutathione Analysis

Frozen muscles were homogenized in 20 µl of 5% (w/v) 5-sulfosalicylic acid (SSA)/mg tissue and centrifuged at 11,500 rpm for 5 min at 4°C . The supernatant was diluted 1:5.5 in ddH2O. Separate aliquots of 400 μl were taken for total glutathione (GSH) and oxidized glutathione (GSSG) determination.

Total GSH was determined by Tietze's glutathione reductase recycling method adapted for the Cobas Mira spectrophotometer (Roche Diagnostics). The Cobas Mira was set up to pipette 250 µl of NADPH (0.3mmol/l), 30 µl of DTNB (6.0 mmol/l), and 95 µl of sample, standard, or 5% SSA into cuvettes. The reaction mixture was allowed to incubate at 37°C for 4 minutes. Next 15 µl of glutathione reductase enzyme (1.0 U/100 µl) was added and the reaction was monitored at 405nm every 24 sec for a period of 12 min. Using these conditions, the glutathione reductase method was linear for GSH concentrations ranging from 0 to 6 µM. Finally, the concentration of GSH in each sample was determined from a calibration curve produced using known glutathione standards. Reproducibility of GSH determination using this method is very high with an intra-assay coefficient of variation of less than 2%.

The 400 µl portion of the sample supernatant for the GSSG assay was derivatized in 50 μl of the following solution: 45.5% triethanolamine, 0.4% SSA and 9% 2-vinylpyridine in ddH2O. The mixture was vortexed vigorously for ten minutes at room temperature. To quantify the amount of GSSG, known standards of 2.5 µM GSH, 2.5 µM GSH + 0.1 µM GSSG, and 2.5 µM GSH + 0.5 GSSG were derivatized and assayed. The samples were analyzed as for total GSH above. A regression curve was used to obtain the value of the GSSG recovered in each assay, and from this a correction factor was obtained to calculate the GSSG for each unknown.

Enzyme Activity Assays

GPx and GR Activities: For the GR activity assay, muscles were homogenized in PBS (pH 7.4) containing 0.1 mM EDTA. For the GPx activity assay, muscles were homogenized in the same PBS buffer except that 1 mM dithiothreitol (DTT) was added. In both cases, approximately 200 mg of muscle was homogenized per mL of buffer. Homogenates were spun at 8500 g, for 10 minutes at 4°C, and the supernatant was collected. GR and GPx activities were measured using Randox kits GR 2368 and RS 505, respectively (Randox Laboratories Ltd, San Francisco, CA, USA). Briefly for the GR activity assay, 10 µL of sample was added to 250 µL GSSG substrate (2.2 mM in potassium phosphate/EDTA buffer) and incubated for 5min at 37 °C. Then, 30 µL NADPH (0.17 mM in ddH2O) was added and the decrease in absorbance at 340 nm was followed for 5 min at 37°C. Background interference is assessed by assaying each sample using buffer instead of substrate. For the GPx activity assay, 5 µL of sample was added to 220 μL reagent buffer (4 mM GSH, 0.5 U/L GR, 0.28 mM NADPH in phosphate/EDTA buffer) and mixed at 37 °C. Then, 10 µL cumene hydroperoxide (0.18 mM in ddH2O) was added and the decrease in absorbance at 340 nm was followed for 2 min at 37 C. Background interference was assessed by assaying each sample using ddH2O instead of cumene hydroperoxide.

ICDH and Aconitase Activities: $NADP^+$-ICDH and aconitase maximal activities were assessed in 50 mg of powdered skeletal muscle tissue after being homogenized on ice

in 1 ml of buffer containing 0.1 mM Tris-HCl and 15 mM tricarballylic acid (pH 7.8), and centrifuged for 10 min at 6,000 g at 4°C. Enzyme activities were assayed in supernatants, by monitoring the kinetics of optical density of NADPH (340 nm) measured with a spectrophotometer (Cobas Fara, Roche). $NADP^+$-ICDH activity was measured with a commercial kit (Sigma Diagnostics) (36). As for measurements of aconitase activity, the procedure of Nulton-Persson and Szweda was slightly modified (37). Supernatants were added to an incubation mixture containing 5 mM citrate, 0.5 mM $MgCl_2$, 1 mM $NADP^+$, and 1 U/ml ICDH, (pH 7.4). Protein contents were determined with a Bio-Rad kit with BSA serving as a standard. Activities were expressed in units/mg of protein, where 1 unit is defined as the amount of enzyme necessary to catalyze the conversion of 1 mmol substrate/min at 37°C.

Statistical Analyses

All data were reported as means ± SE and were analyzed with the statistical analysis program SigmaStat® (SPSS Inc.). Differences between groups were initially tested by analysis of variance (ANOVA), with post hoc application of the Tukey test where appropriate. Statistical significance was defined as $p<0.05$.

4.5 RESULTS

Glutathione status

Glutathione analysis in mdx and C57 muscle revealed that, at baseline, dystrophin-deficient muscle had significantly less total glutathione (Fig. 1A) and a significantly greater ratio of oxidized to reduced glutathione (GSSG/GSH) (Fig.1B) than normal muscle. Whereas I/R caused a drop in total glutathione in C57 muscle (Fig. 1A), I/R produced no significant change in total GSH in mdx muscle. No significant changes in GSSG/GSH ratio were observed in either mouse strain when subjected to I/R, although the latter tended to increase in the C57 group.

GPx and GR activity

Glutathione peroxidase (GPx) uses GSH as a cofactor for the removal of peroxides, and glutathione reductase (GR) is responsible for recycling GSH. To examine the role that these enzymes play in the abnormal glutathione status of mdx mouse muscle, we assessed the activity levels of these enzymes at baseline and following I/R. At baseline, mdx muscle had significantly greater GPx and GR activity than C57 muscle. I/R caused a small, but significant, increase in GPx activity in C57 muscle, but no change in GR activity. In mdx muscle, I/R also tended to increase GPx activity, but this did not achieve statistical significance. However, GR activity was significantly decreased following I/R in mdx muscle.

ICDH and Aconitase activity

The enzymes ICDH and aconitase are both known to be particularly susceptible to inactivation via oxidative stress. ICDH is regarded as having an important antioxidant function as it provides the reducing equivalent, NADPH, for the recycling of GSSG to GSH by GR. Aconitase supplies isocitrate for the ICDH reaction, in addition to its crucial participation to the TCA cycle. At baseline, both ICDH and aconitase activities were significantly decreased in mdx muscle relative to C57. In C57 muscle, I/R caused activities of both enzymes to decrease. In contrast, the activities of these enzymes did not change with I/R in the mdx group.

Glutahione status in pre-necrotic mdx muscle

Abnormal glutathione status in adult mdx muscles may reflect a primary factor in dystrophic pathogenesis, or may reflect secondary effects of free-radical exposure associated with necrotic cell death and inflammation. Therefore, we also examined the glutathione status of pre-necrotic muscles from 14 day old mdx mice. At baseline, no difference was seen in total glutathione content between 14 day old mdx and C57 muscles (Fig. 4A). However, a greatly increased GSSG/GSH ratio was seen in mdx relative to C57 muscles at this age (Fig. 4B). Therefore, these results suggest that abnormal glutathione status, and hence oxidative stress is an early feature of dystrophic pathology which cannot be solely attributed to myofiber necrosis or inflammation in mdx muscles.

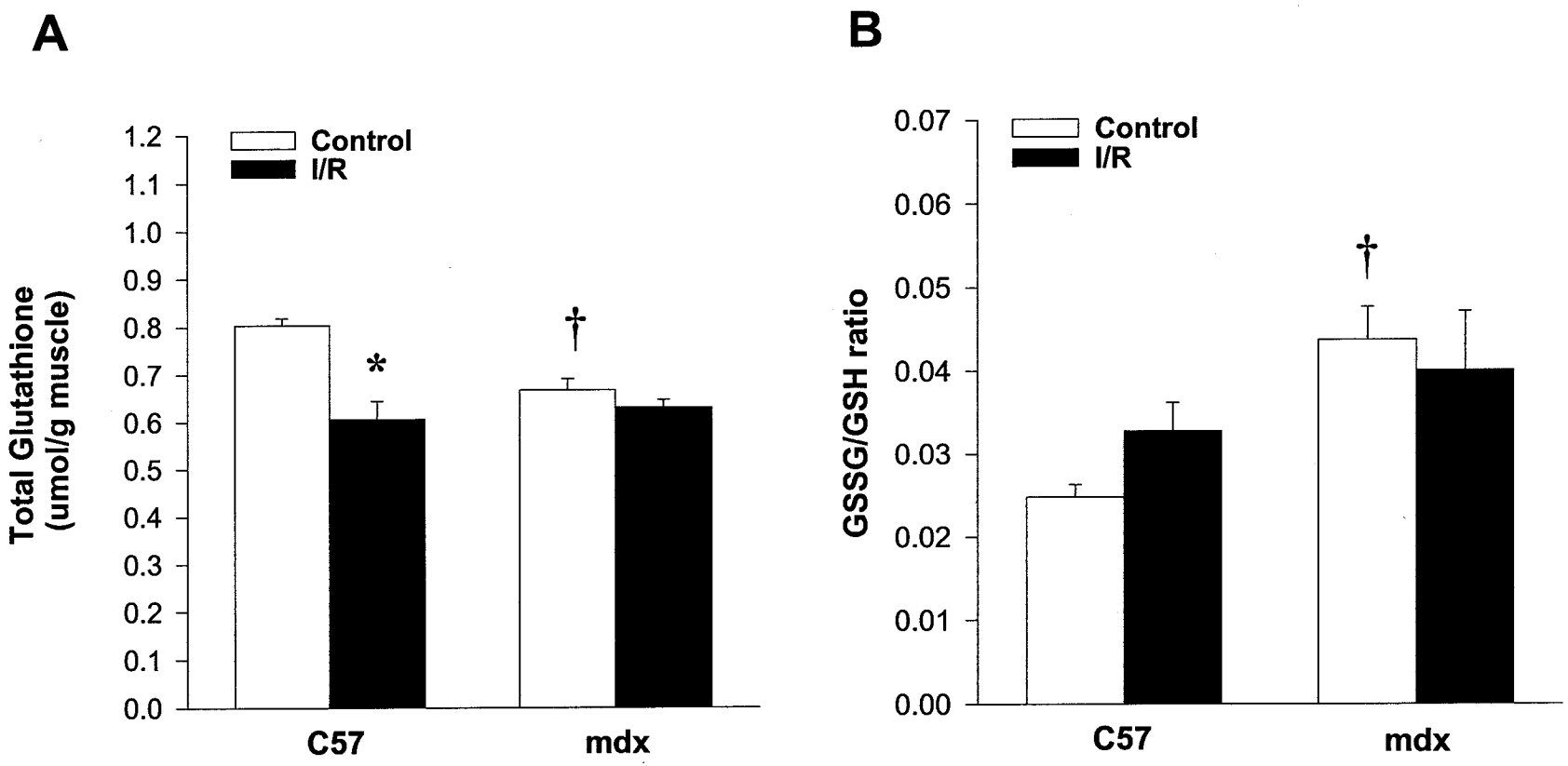

Figure 1. Total glutathione (**A**) and GSSG/GSH ratio (**B**) in adult mdx versus C57 TA muscles following sham-(Control) and ischemia/reperfusion (I/R)-treatment. For C57, sample size (n)=20 for Control, and 9 for I/R; for mdx, n=16 for Control, and 9 for I/R. . All values are group means ± SE. *$p<0.05$ for sham versus I/R-treatment within an individual mouse strain (mdx or C57), and †$p<0.05$ for mdx versus C57 for a particular treatment condition (Control or I/R).

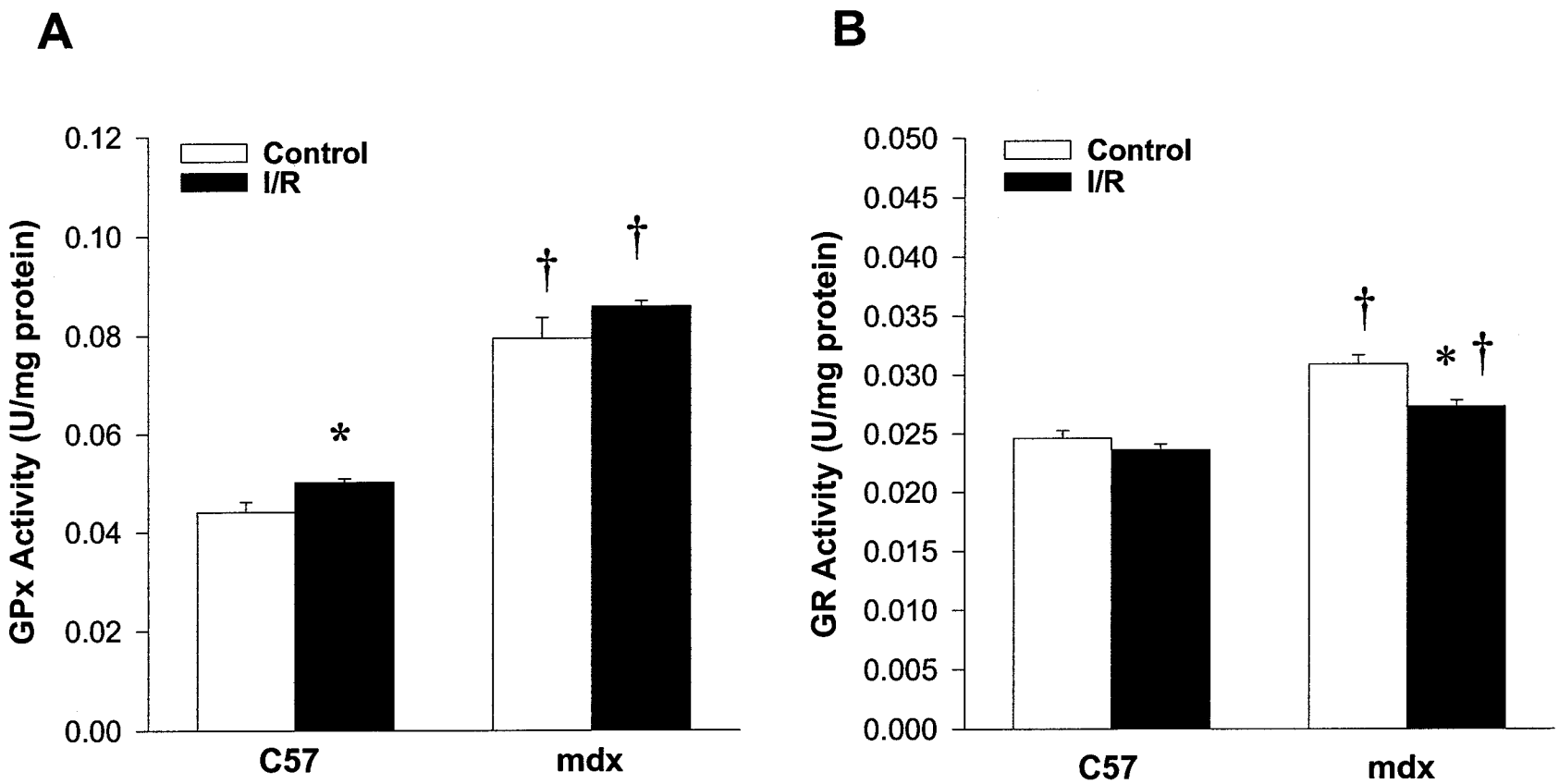

Figure 2. Enzymatic activities of GPx (**A**) and GR (**B**) in mdx versus C57 TA muscles following sham- (Control) and ischemia/reperfsuion (I/R)-treatment. Sample size (n)=5 for each treatment group. All values are group means ± SE. *$p<0.05$ for sham versus I/R-treatment within an individual mouse strain (mdx or C57), and †$p<0.05$ for mdx versus C57 for a particular treatment condition (Control or I/R).

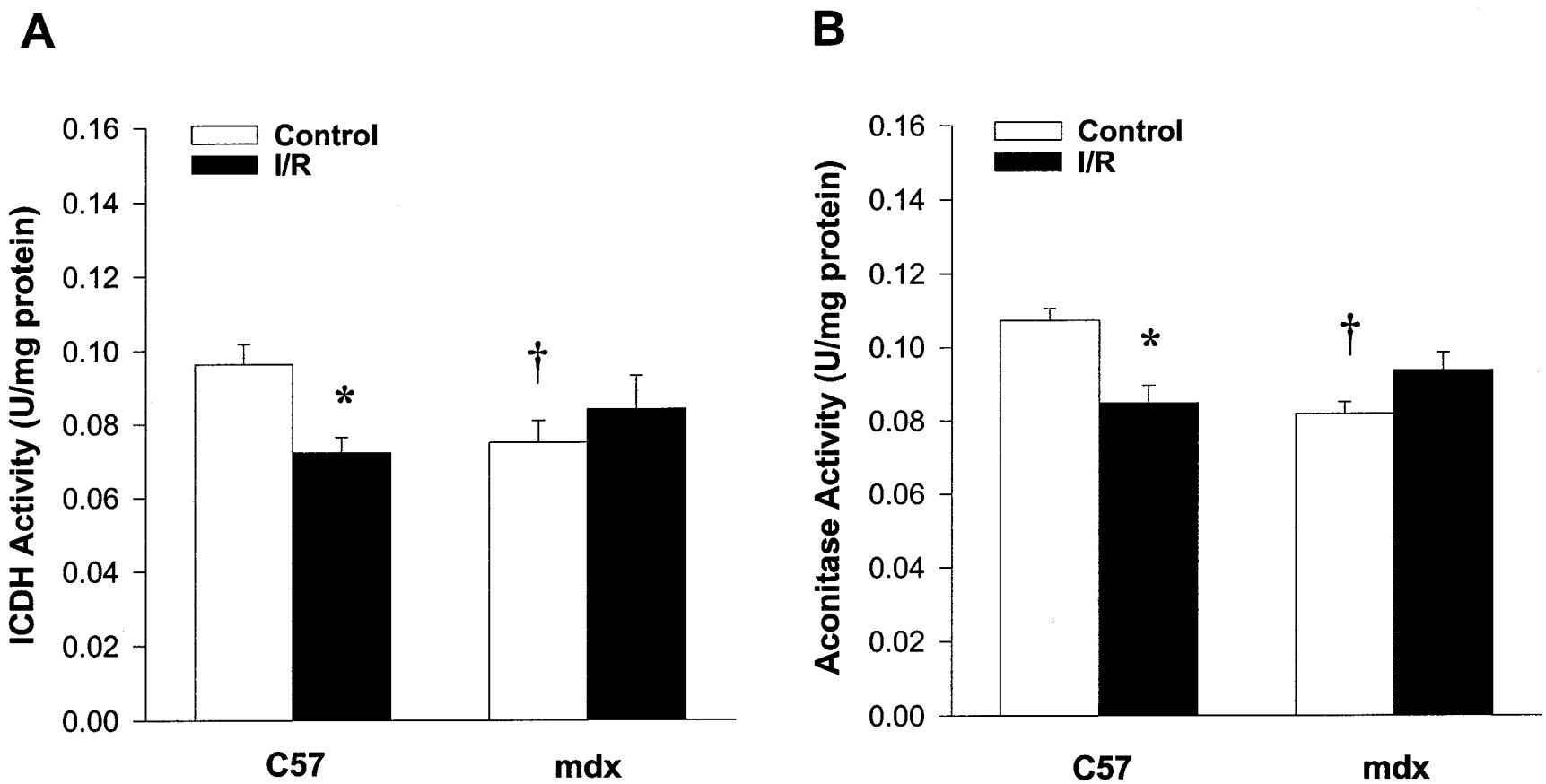

Figure 3. Enzymatic activities of ICDH (**A**) and Aconitase (**B**) in mdx versus C57 TA muscles following sham-(Control) and ischemia/reperfsuion (I/R)-treatment. Sample size (n)=9 for each treatment group. All values are group means ± SE. *$p<0.05$ for sham versus I/R-treatment within an individual mouse strain (mdx or C57), and †$p<0.05$ for mdx versus C57 for a particular treatment condition (Control or I/R).

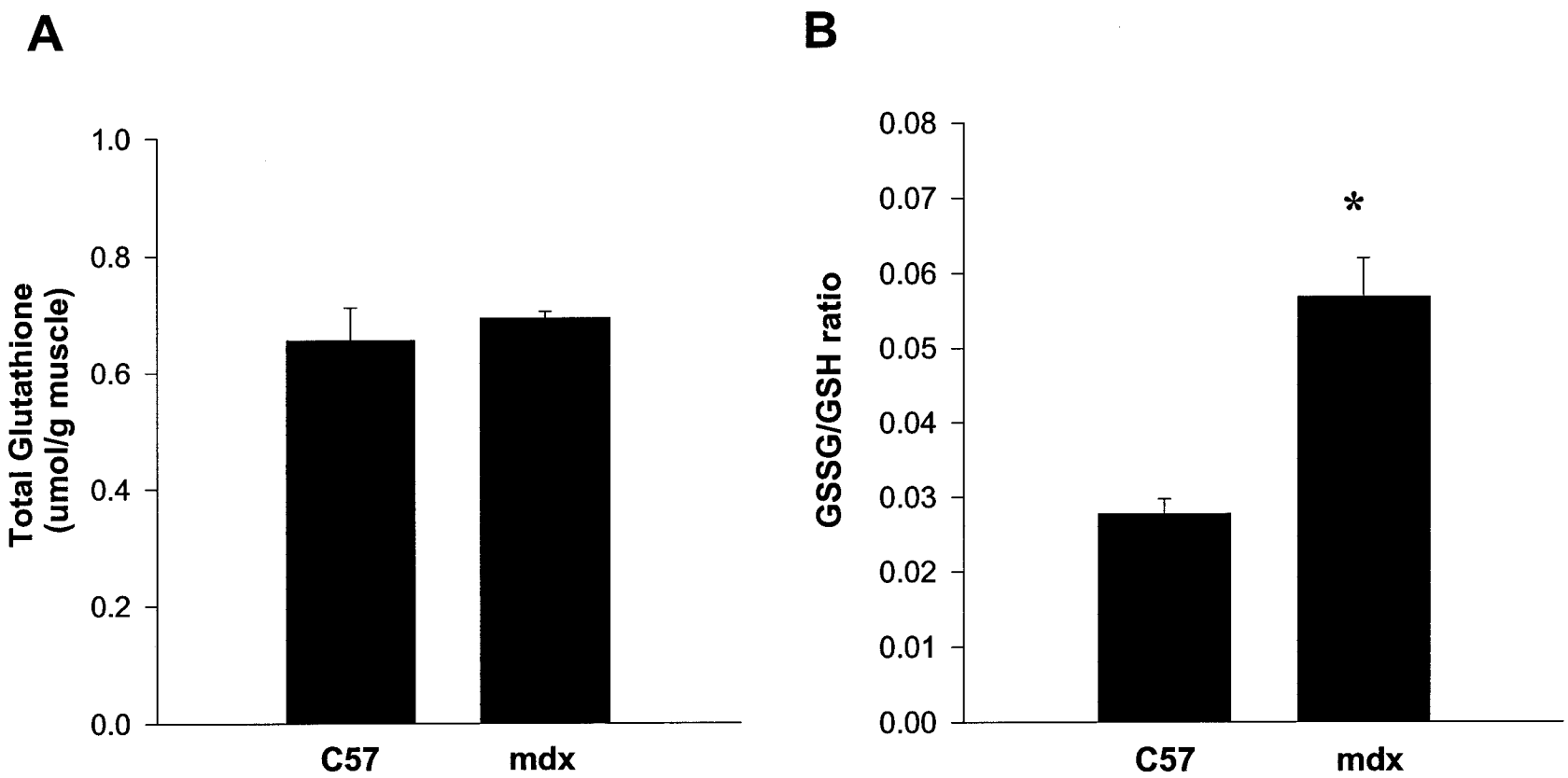

Figure 4. Total glutathione (**A**) and GSSG/GSH ratio (**B**) in 14-day-old (pre-necrotic) mdx versus C57 TA muscles. For C57, sample size (n)=9; for mdx, n=7. All values are group means ± SE. *$p<0.05$ for mdx versus C57.

4.6 DISCUSSION

Glutathione is one of the most abundant and important antioxidants in skeletal muscle (38). Indeed, the most prominent feature of systemic glutathione depletion in animal models is skeletal muscle cell necrosis and inflammation (22). Few studies have examined the GSH antioxidant system in the setting of dystrophin deficiency. Degl'Innocenti et al (39) found no difference in the levels of GSH, GSSG, and total glutathione in cultures of skin fibroblasts from DMD patients and normal subjects under baseline conditions. However, when challenged with sub-lethal doses of H_2O_2, DMD cultures showed significant reductions, relative to normal cells, of both reduced and total GSH. To our knowledge, GSH status has never been tested in mdx mouse muscles. The most consistent finding in regard to the GSH-associated enzymes, GPx and GR, in dystrophin-deficient muscle has been an upregulation of GPx expression and enzyme activity in mdx and DMD skeletal muscle relative to normal controls (1,4,40,41). Given the context of recurrent bouts of functional ischemia in dystrophin-deficient muscle, it is noteworthy that GPx is one of the antioxidant enzymes shown to be upregulated during ischemic preconditioning in animal models of cardiac muscle I/R (42). Studies which have examined GR activity in dystrophin-deficient muscle have produced somewhat contradictory results (3,39,43). To our knowledge, the fates of ICDH and aconitase in dystrophin-deficient skeletal muscle have not yet been examined in prior studies.

In the present study, we performed a broad evaluation of the glutathione antioxidant system in dystrophin-deficient muscle. The principal findings of this study are as follows: (1) the GSSG/GSH ratio is higher in mdx muscle (including 14 day old pre-

necrotic mdx muscle) than in age-matched normal C57 muscle; (2) GPx and GR activities are increased, whereas ICDH and aconitase activities are decreased in mdx muscle relative to C57 muscle; and (3) upon being subjected to the oxidant stress associated with I/R, glutathione antioxidant system in mdx muscle does not respond in the same manner as in normal C57 muscle. Hence, mdx muscle demonstrated increased resistant to oxidative/nitrosative stress-mediated changes, such as decreases in total glutathione, ICDH and aconitase activity, that occurred in C57 muscle following I/R. The one exception was, GR activity, which was unaffected by I/R in C57 muscles, but reduced by I/R in mdx muscles.

Baseline abnormalities in dystrophin-deficient muscle

We found that at baseline (i.e., prior to I/R challenge) the GSSG/GSH ratio was increased in both adult mdx and young (pre-necrotic) mdx muscles. These findings support previous reports of increased oxidative stress in dystrophin-deficient muscle(1). In particular, our finding of an increased GSSG/GSH ratio in pre-necrotic mdx muscles supports increased oxidative stress as an early phenomenon which is not related to necrosis in these muscles.

The increased GSSG/GSH ratio in mdx adults relative to controls was accompanied by a loss of total glutathione, which was not seen in 14-day-old mdx mice. Accumulation of GSSG due to oxidative stress is known to have two major consequences (44). The first is an altered redox status in the cell, which can activate

oxidant-responsive transcription elements (45,46). The second, is the secretion of GSSG from the cell, which is believed to occur to keep the intracellular redox status constant (47). Therefore, in the case of the mdx muscle some GSSG may have been actively secreted from the cell, thus leaving total glutathione levels lower than normal. In addition to this controlled secretion, membrane damage in adult mdx muscle may allow for increased efflux of GSSG from myofibers, which would be driven by the extreme intracellular (mM) to extracellular (μM) glutathione concentration gradient (44). This could explain why a similar decrease in total glutathione did not occur in pre-necrotic mdx muscle.

It is well established that GSH can react with either $NO^{\bullet}$ or $ONOO^-$ to form S-nitrosoglutathione (GSNO) (48,49). Neuronal nitric oxide synthase (nNOS) expression/activity is believed to be modestly reduced in mdx muscle, but some $NO^{\bullet}$ is still produced (50,51). Tietze's method of glutathione measurement, which was used here, does not detect GSNO (52). Therefore, another potential mechanism to explain the loss of total GSH is through the formation of GSNO. It is of interest to note that in the present study, we also found ICDH activity to be decreased at baseline in mdx muscle, since one of the most potent inhibitors of ICDH activity is GSNO (discussed in more detail below).

The major rate limiting enzyme for the de novo synthesis of GSH is γ-glutamylcysteine synthase (GCS). The level of expression and activity of this enzyme in mdx muscle is not known. Therefore, we cannot exclude the possibility that the decreased total GSH found here is due to a decrease in GCS activity/expression in mdx

muscle. However, several studies have shown that in the setting of increased oxidative/nitrosative stress (which appears to occur in mdx muscle) GCS activity/expression is actually increased, not decreased. Therefore, it is unlikely that GCS inhibition played a role in decreasing total GSH in mdx muscle.

To further determine the effect of dystrophin deficiency on glutathione status, we examined the activity levels of two major enzymes which regulate GSH levels in skeletal muscle, GPx and GR. We found increased GPx and GR at baseline in mdx mice. The increased GPx activity found here is in agreement with other reports (1,4,40,41). While some reports described increased GR activity in mdx muscle, contradictory statements have also been published (3,39,43). Our data suggest that activities of both these enzymes are increased in mdx muscles.

Increased GPx activity should cause an increase in GSSG at the expense of GSH, and increased GR activity should reverse this effect. In the method of GR activity measurement used here, NADPH is added to the reaction buffer, such that the measured activity is based on inherent properties of the enzyme itself and not the availability of its cofactors. In vivo, GR activity is dependent on the production of NADPH from ICDH. We found that activities of both ICDH and aconitase were decreased in mdx muscles at baseline. Therefore, although GR activity was found to be increased, its function may actually be compromised to some extent in living mdx skeletal muscle because of a deficiency of ICDH-produced NADPH.

Aconitase is vulnerable to oxidative inhibition due to its easily oxidizable active-site iron-sulfur cluster (27,28,53). ICDH does not contain iron-sulfur clusters, but in vitro studies of this enzyme have shown that it is susceptible to inactivation via protein carbonyl formation, conformational changes, peptide cleavage, and thiol nitrosylation (26,29). While several reactive oxygen species, including superoxide, singlet oxygen, and hydroxyl radical have been shown to inactivate this enzyme, one of the most potent inhibitors of ICDH in vitro is GSNO (19,20). As discussed above, the finding of decreased total GSH in mdx muscle might suggest that reduced GSH reacts with $NO^{\bullet}$ or ONOO to form GSNO, which could inactivate ICDH, thereby decreasing NADPH production for GR and the recycling of GSH. Further studies are needed to examine the role of $NO^{\bullet}$, and GSNO, in the antioxidant abnormalities in mdx muscle seen here. In addition to these proposed deficiencies related to antioxidant enzymes, decreased ICDH and aconitase activities may contribute to dystrophic pathology via metabolic disturbances.

Changes in normal C57 and dystrophic mdx muscle following I/R

In the present study, we found that I/R in normal muscle produced an altered redox status very similar to that seen in mdx skeletal muscle at baseline (i.e., not subjected to I/R). Just as in mdx muscles at baseline, C57 muscles subjected to I/R displayed decreased total glutathione, increased GPx activity, and decreased ICDH and aconitase activities. On the other hand, while total GSH, GSSG/GSH ratio, GPx, ICDH, and

aconitase activities were abnormal at baseline, these properties did not change further in mdx muscle following I/R. This might suggest that the non-challenged (baseline) mdx muscles had already experienced I/R or or some other form of oxidative/nitrosative stress and had thus already adapted to this situation. This is in agreement with the study of Ragusa et al (3) which examined the effect of hyperoxia on antioxidant and TBAR levels in mdx and normal muscle. Greater CAT and MnSOD upregulation and TBAR levels were seen in normal mice than in mdx following exposure to 80-85% oxygen for 3 days.

Skeletal muscle of DMD patients and mdx mice are known to suffer recurrent bouts of functional ischemia. It is well known that brief periods of ischemic stress in cardiac (54-56) and skeletal muscle (57,58) can induce cellular adaptations that protect against subsequent I/R insults. This so-called ischemic preconditioning appears to be mediated by signal transduction pathways involving adenosine receptors, mitochondrial ATP-sensitive potassium channels (mK_{ATP}), protein kinase C (PKC), and free radicals (59-62). The downstream end-effectors of ischemic preconditioning pathways are believed to include antioxidant enzymes including superoxide dismutase (SOD), catalase, and GPx (42,55,61,63-65). Our data support the notion that compensatory increases in GPx and GR occur in dystrophin-deficient muscle in an attempt to protect against oxidative/nitrosative damage. This upregulation of GPx and GR may be the result of recurrent bouts of functional ischemia during exercise with subsequent reperfusion at rest, in a mechanism similar to that proposed to explain the phenomenon of ischemic pre-conditioning in cardiac muscles.

Interestingly, the one outcome measure which was adversely affected by I/R in mdx but not C57 muscles was GR activity. GR is known to be inactivated by several mediators of oxidative/nitrosative stress including GSNO, ONOO, and HNE. The former of these compounds, GSNO, is believed to inhibit GR by nitrosylating a cysteine at position 63 in the active site of the enzyme (19,20). As discussed earlier, our findings of decreased GSH and ICDH inhibition in mdx muscle at baseline suggest that GSNO may be produced in mdx muscle during periods of ischemia. Peroxynitrite, $ONOO^-$, has also been shown to inactivate GR, by nitration of two tyrosine residues at positions 106 and 114 at the GSSG binding site (21). In our previous study of I/R in mdx mice (Chapter 3), we found that dystrophin-deficient muscle was abnormally susceptible to I/R-induced tyrosine nitration as well as HNE formation. GSNO and $ONOO^-$ are derivatives of $NO^{\bullet}$ (NOx). Our previous I/R study (Chapter 3) found increased NOx in mdx muscle following ischemia alone. Increased NOx produced during ischemia might cause lipid peroxidation and/or combine with other molecules such as $O_2^{\bullet -}$ (to form $ONOO^-$) or GSH (to form GSNO) during reperfusion. An end result of these reactions could then be the inactivation GR.

Implications for disease pathogenesis

In the present study, we found that mdx muscles (including those at the pre-necrotic stage) display an abnormal glutathione antioxidant phenotype, characterized by an increased an GSSG/GSH ratio and upregulation of glutathione-associated enzymes.

This supports previous reports of increased oxidative stress in dystrophin-deficient muscles (1,4,40,41). We also found that by subjecting normal C57 mouse muscles to I/R-induced oxidative/nitrosative stress we reproduced many aspects of the altered antioxidant status of dystrophin-deficient muscles. This suggests that the abnormal glutathione status of mdx muscles may be the result of an ischemic phenomenon in these muscles. Decades ago, Mendell et al. (8) found that ischemia in rat muscles could reproduce the characteristic signs of dystrophic muscle pathology including group myofiber necrosis, which suggested a vascular cause for DMD. In recent years, it has become well established that ischemia occurs in dystrophin-deficient muscles during exercise due to the loss of nNOS at the sarcolemma (10,11). The endogenous oxidative/nitrosative stress and altered glutathione phenotype of mdx muscles may therefore be the result of this functional ischemia.

Not only does oxidative stress appear to occur in dystrophin-deficient muscles (1,3,4,66), but evidence also suggests that these muscles are abnormally susceptible to oxidative and nitrosative stress (Chapter 3) (5,6). The mechanisms responsible for these observations have yet not been elucidated. One possibility is that a deficiency of antioxidant systems occurs in these muscles. In this study, we found that the GPx and GR activites were increased in mdx muscles, which is in agreement with several reports of upregulated antioxidant enzymes, such as SOD1 and catalase, in dystrophin-deficient muscles (1,4). Therefore, it is unlikely that increased oxidative stress or increased vulnerability thereto is due to antioxidant deficiencies. In contrast, it would appear that antioxidants are upregulated in these muscles due to chronic oxidative/nitrosative stress,

perhaps due to functional ischemia. However, the fact that ICDH and aconitase activities were decreased and the GSSG/GSH ratio increased in mdx muscles at baseline, suggests that this antioxidant upregulation is not fully protective. This might imply that the upregulated antioxidant systems are eventually overwhelmed by the oxidation/nitrosative stress which occurs in these muscles, perhaps due to its persistent nature.

We also found here that, when subjected to I/R, mdx muscles were protected against further inhibition of aconitase ICDH and further increases in the GSSG/GSH ratio. This suggests that a form of preconditioning may occur in mdx muscles, such that the upregulation of antioxidant enzymes provides protection against acute exposures to oxidative/nitrosative stress, such as that imposed in our I/R model. However, we previously showed that I/R caused greater 3-nitrotyrosine formation, HNE formation, and sarcolemmal fragility in mdx muscle than in C57 muscle (Chapter 3). Therefore, it appears that while the preconditioning in mdx muscles is sufficient to defend against some aspects of acute I/R challenge, it is unable to protect against protein nitration, lipid peroxidation, and membrane damage.

In conclusion, it appears that mdx muscles adapt to some form of endogenous oxidative/nitrosative stress by upregulating antioxidant enzymes. This compensatory mechanism appears to provide some protection during acute insults (experimental I/R injury), but may be overwhelmed in the long run by constant, repeated exposures. Finally, the similarities between the glutathione status of C57 muscles subjected to I/R

and baseline mdx muscles, suggest functional ischemia in mdx muscles as a potential source of this endogenous oxidative/nitrosative stress.

3.7 ACKNOWLEDGEMENTS

I would like to again thank Maya Khairallah for her important contributions to this study, in particular for the GPx and GR analyses which she performed; Christine des Rosiers for her assistance and critical analysis of this work; Shawn Mohammed for his contributions to the glutathione analysis; Larry Lands for his advice and technical support; Duska Gvosdic for her expert technical assistance; and finally Basil J. Petrof for his assistance, advice and supervision.

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PART III

Thus far we have shown that dystrophin-deficient mdx muscles are abnormally susceptible to ischemia/reperfusion (I/R)-induced oxidative/nitrosative stress and sarcolemmal fragility (Chapter 3). In addition, we have found that the baseline oxidative/nitrosative stress in mdx muscles is associated with an upregulation of antioxidant enzymes, which protects against some of the negative effects of acute I/R injury (Chapter 4). However, the findings of Chapter 3 imply that these compensatory antioxidant mechanisms are not sufficient to protect against I/R-induced sarcolemmal fragility. While these results suggest that antioxidant supplementation therapies might be beneficial in DMD patients, trials of such treatments have been not been successful. Many cellular antioxidants depend on partner biological compounds to replenish them once they have been oxidized. This might explain the disappointing results of antioxidant interventions tried thus far. Since the loss dystrophin is fundamentally responsible for all cellular disturbances in dystrophin-deficient muscle (including oxidative/nitrosative stress), dystrophin gene replacement is another very promising therapeutic option. In the final chapters of this thesis we investigate the efficacy of a fully-gutted (helper-dependent) adenovirus to deliver a full-length murine dystrophin gene in mdx muscles.

CHAPTER 5.

Prolonged dystrophin expression and functional correction of mdx mouse muscle following gene transfer with a helper-dependent (gutted) adenovirus encoding murine dystrophin.

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5.1 ABSTRACT

Dystrophin gene transfer using helper-dependent adenoviruses (HDAd), which are deleted of all viral genes, is a promising option to treat muscles in Duchenne muscular dystrophy. We investigated the benefits of this approach by injecting the tibialis anterior (TA) muscle of neonatal and juvenile (4- to 6-week-old) dystrophin-deficient (mdx) mice with a fully deleted HDAd (HDCBDysM). This vector encoded two full-length murine dystrophin cDNAs regulated by the powerful cytomegalovirus enhancer/β-actin promoter. At 10 days post-injection of neonatal muscles, 712 fibers (42% of the total number of TA fibers) were dystrophin positive (dys+), a value that did not decrease for 6 months (the study duration). In treated juveniles, maximal transduction occurred at 30 days post-injection (414 dys+ fibers, 24% of the total number of TA fibers), but decreased by 51% after 6 months. All studied aspects of the pathology were improved in neonatally-treated muscles: the percentage of dys+ fibers with centrally localized myonuclei remained low, localization of the dystrophin associated protein complex was restored at the plasma membrane, muscle hypertrophy was reduced, maximal force generating-capacity and resistance to contraction-induced injuries were increased. The same pathological aspects were improved in the treated juveniles, except for reduction of muscle hypertrophy and maximal force generating capacity. We demonstrated a strong humoral response against murine dystrophin in both animal groups, but mild inflammatory response occurred only in the treated juveniles. HDCBDysM is thus one of the most promising and efficient vectors for treating DMD by gene therapy.

5.2 INTRODUCTION

Duchenne muscular dystrophy (DMD) is a fatal disease that affects one male birth in 3500 (1,2). It is caused by mutations of the dystrophin gene that encodes an elongated 427 kDa protein associated with the plasma membrane of skeletal and cardiac muscles. The hallmark of DMD is a progressive wasting and weakness of skeletal muscles leading to death around the age of 20. A promising approach to treat DMD is to restore dystrophin expression in the diseased muscle by injecting the muscle with naked DNA or viral vectors carrying the dystrophin cDNA. An ideal viral vector for treating DMD in this manner, in addition to being non-toxic, should be able to produce sufficient and sustained dystrophin expression in a major proportion of the body musculature.

Efficient and long-term transgene expression has been achieved in muscle after gene transfer using recombinant adeno-associated virus (rAAV) (3). One of the major problems with rAAV is its small insert capacity of about 5-kb, which is too small to carry the full-length dystrophin cDNAs (12-kb). Improvement of the histology and of the force-generating capacity of muscle was demonstrated after treatment of dystrophin-deficient (mdx) mouse muscle with rAAV carrying very small truncated version of dystrophin (microdystrophin) (4-7). However, it is unclear to what extent will the expression of these microdystrophin cDNAs mitigate the dystrophic phenotype in large animals and humans.

E1-deleted adenovirus (Ad), also known as first generation Ad (FGAd), can transduce muscle fibers in vivo. Indeed, several research groups have demonstrated the potential usefulness of FGAd-mediated dystrophin gene transfer (8-11), or that of its

functional homologue utrophin (12-14), as a means to mitigate the dystrophic phenotype of the mdx mouse muscle. However, FGAd encode most of their viral genes. Low grade synthesis from these genes after in vivo gene transfer, triggers a cellular immune response that subsequently eliminates and/or silences transgene expression (15,16). In addition, the insert capacity of FGAd is only about 8-kb, which is sufficient for the Becker minidystrophin cDNA (6.5-kb), but not for the full-length dystrophin cDNA (13.6-kb). To reduce the immune response caused by FGAd, all the viral sequences except for the inverted terminal repeats (ITR) and the packaging signal of Ad were removed (17-20). Gene transfer using these completely deleted Ad vectors, also referred to as "gutted Ad", large capacity Ad or helper-dependent Ad (HDAd), is associated with a reduction in the cellular immune response, which has led to an improvement in the duration of transgene expression (21-25). Another advantage of HDAd is the proportional gain in their insert capacity, which, in the case of a fully deleted HDAd, is increased to approximately 36-kb.

Despite their potential advantages as vectors for treating DMD by gene therapy, relatively few preclinical studies using HDAd carrying dystrophin exist. Dystrophin gene transfer with HDAd in neonatal mdx mice can improve the muscle histology and can restore the dystrophin associated protein complex (DPC) at the cell surface (26,27). Treatment of old mdx muscle with HDAd carrying dystrophin can increase their resistance to contraction-induced injury but not their force production (28). We previously observed weak dystrophin expression level after treatment of mdx mouse and dystrophic golden retriever dog muscles with HDAd carrying dystrophin regulated by the cytomegalovirus (CMV) promoter (29). Because of this low expression level, we

constructed an HDAd encoding two tandem human dystrophin cDNAs regulated by the powerful hybrid CMV enhancer/β-actin (CB) promoter. However, despite excellent dystrophin expression level, the use of this vector still only resulted in transient dystrophin expression in mdx mouse muscle, most likely because dystrophin was non-isogenic (30). Transient dystrophin expression was also observed after treatment of mdx mouse using HDAd encoding human dystrophin cDNA or murine dystrophin plus β-galactosidase (26,31). Reduction of dystrophin expression was also observed even when transgenic mdx mice expressing β-galactosidase were injected with the latter vector (27). However in this study, the mice were not completely tolerant to β-galactosidase. Gene transfer of an isogenic dystrophin transgene using an HDAd into immunocompetent dystrophin-deficient muscle has thus not been completely investigated.

In the present study, to gain a better insight of the potential usefulness of HDAd for DMD treatment, we tested in mdx mouse muscle a fully deleted HDAd (HDCBDysM) encoding two, tandem murine dystrophin full-length cDNAs, each regulated by the powerful (CB) promoter. Massive necrosis of the mdx mouse limb muscle fibers do not occur before 3 to 4 weeks of age (32). We thus treated the tibialis anterior (TA) muscle of neonatal and juvenile (4- to 6-week-old) mdx mice with HDCBDysM to verify if the pathology would be mitigated to the same extent when the muscle is treated before or after the onset of the necrotic process. In addition, treatment of neonatal and juvenile mdx muscle allowed us to compare the duration of dystrophin expression in animals with immature (neonatal) and fully developed immune system (juvenile).

Following injection of the TA muscle of neonatal mdx mice with HDCBDysM, we demonstrated unabated dystrophin expression to a level (more than 40% of the TA was transduced) and duration (6 months) not achieved before using an HDAd. In the treated juvenile mdx TA, maximal transduction occurred at 30 days post-injection (24% of the TA), but decreased by 51% after 6 months. An appreciable but mild inflammatory response, whose exact triggering agent is not known, was detected only in the juvenile-injected muscle. Marked improvement of muscle histology and physiology was achieved in both animal groups. HDCBDysM is thus a promising vector for treating DMD by gene therapy. Further studies are required to fully characterize the factor(s) triggering the inflammatory response, which might prevent long-term application of this vector in fully immunocompetent subjects.

5.3 MATERIAL AND METHODS

Construction, purification and characterization of HDCBDysM

To construct a HDAd encoding the full-length cDNA of murine dystrophin regulated by the strong hybrid CMV enhancer/β-actin (CB) promoter, we first made plasmid pCBmDys5'ITR. The muscle creatine kinase promoter of pMDA which also contains the full-length murine dystrophin cDNA (57) was removed by digestion with XhoI and replaced with the CB promoter obtained by digesting pCAGGS (58) with XhoI and SalI. The resulting plasmid was further modified by inserting into the SpeI/NotI sites at the 3' end of dystrophin, the β-globin poly (A) signal followed by the packaging signal and the 5' inverted terminal repeat (ITR) of Ad type V derived from pAdCMV-dys (10). Plasmid pCBmDys5'ITR thus contains (in a 5' to 3' orientation) a unique NotI site, the 5'ITR and the packaging sequence of Ad, the rabbit β-globin poly (A) signal, the full-length murine dystrophin cDNA, the CB promoter and a unique XhoI site. HDCBDysM was generated by digesting pCBmDys5'ITR with NotI and the ends were dephosphorylated. After phenol/chloroform extraction and ethanol precipitation, the DNA was digested with XhoI, and the NotI/XhoI fragment containing the dystrophin expression cassette was purified by electrophoresis. The purified NotI/XhoI fragment was self ligated, extracted by phenol/chloroform, and precipitated by ethanol. The ligation product was used to transfect 293Cre4 cells (20) with LipofectAMINETM (Life Technologies, Burlington, Ont.) according to the manufacturer's recommendation. HDCBDysM was then amplified using 293Cre-loxP system and purified by two consecutive continuous CsCl gradient centrifugations as described previously (29). After ultracentrifugation, the

CsCl was removed by chromatography on Sephadex G25 columns (Amersham Pharmacia Biotech Inc, Piscataway, NJ) and HDCBDysM was eluted with 50 mM HEPES pH 7.5, 2 mM MgCl2, 150 mM NaCl, 5% sucrose (freezing buffer). The titer (virus particles/ml) was determined by measuring the optical density at 260 nm (59) and the level of helper virus contamination, which was determined by measuring the cytopathic effect after serial dilution on 293A cells (60) ranged between 0.02% to 0.17% in different vector batches. When not used the same day, the vector was kept at –80°C. We confirmed the structure of the viral DNA by restriction analysis of purified DNA (61) followed by Southern blot as described previously (30). The probe consisted of a purified 16-kb NotI/XhoI DNA fragment of pCBmDys5'ITR containing the 5'ITR and the complete dystrophin expression cassette, or a purified 1.6-kb SalI/XhoI DNA fragment of pCCAGS containing the CB promoter.

Animal injection

We performed all animal experiments according to McGill University guidelines for animal care. We injected the left and right tibialis anterior muscles (TA) of neonatal (2- to 4-day-old) or juvenile (4- to 6-week-old) mdx mice (C57BL/10ScSn-mdx/J; The Jackson Laboratory, Bar Harbor, ME) once with 10 or 30 µl respectively of Ad vectors at a titer of 2 x 1012 virus particles/ml as described previously (10). Before the injection, the juvenile mdx mice were anesthetized by intraperitoneal injection of 2.5% Avertin. At 10, 30, 60,

90 or 180 days post-injection, the mice were euthanized by an overdose of pentobarbital, the TA was removed and frozen in liquid nitrogen-cooled isopentane.

Staining and western blot of muscle tissues

Transverse cryostat sections were stained for dystrophin, using a rabbit polyclonal antibody raised against the C-terminus of human dystrophin that recognizes murine and human dystrophin (10). We visualized the staining using either horseradish peroxidase- or Cy-3-conjugated streptavidin (Jackson Immuno Research Lab. Inc, West Grove, PA). For each injected muscle, we determined the total number of transduced fibers by counting the number of dys+ fibers on a single cryostat section, which spans the entire TA cross-section. To calculate the percentage of transduced fibers, the number of dys+ fibers was divided by 1700, which corresponds to the mean number of fibers in the TA muscle of 1- to 9-week-old mdx mice. We calculated the percentage of muscle fibers with centrally located nuclei by counterstaining with hematoxilin, cryostat sections that were previously stained for dystrophin. Some sections were also stained using antibody against β-dystroglycan (NCL-43DAG; Novocastra, Newcastle upon Tyne, U.K.), α-sarcoglycan (NCL-50DAG; Novocastra) and utrophin (NCL-DRP2; Novocastra) as described previously (55). Dystrophin expression was analyzed by western blot using 10 µg of muscle protein (30). The membrane was processed using the ECF western blotting kit (Amersham Pharmacia Biotech. Buckinghamshire, England) according to the manufacturer's recommendations. The signal was visualized and quantified using a

PhosphorImager system (STORM, Molecular Dynamics Inc, Sunnyvale, CA). The blots were also stained with a monoclonal antibody against vinculin (V2638, Sigma-Aldrich, St-Louis, MI) as a loading control.

Cellular and humoral response

We measured the cellular immune response caused by HDCBDysM by staining consecutive cryostat sections for dystrophin as described above, and for macrophages, CD8+, or CD4+ T-lymphocytes using rat monoclonal antibodies Mac1, anti-CD8a (Ly 2, Cedarlane, Hornby, Ont.) and anti-CD4 (L3T4, BD Biosciences Pharmingen, Mississauga, Ont.) respectively. Mac1 was prepared from supernatant of rat hybridoma (M1/70.15.11.5HL) obtained from the American Type Culture Collection (Rockville, MD). The signal was visualized using a rat anti-mouse biotinylated antibody (Jackson Immuno Research Lab. Inc, West Grove, PA) followed by horseradish peroxidase-conjugated streptavidin (Jackson Immuno Research Lab. Inc, West Grove, PA). For each muscle analyzed, we computed the number of positive inflammatory cells in the transduced areas (regions with more than 50% dys+ fibers), and we divided this value by the total number of fibers (dys+ and dys-) of the area. The TA of age-match mdx mice previously injected with freezing buffer, and the TA of juvenile mdx injected with a FGAd encoding human dystrophin (AdCMV-dys) (10) at a titer of 1.4 X 10^{12} virus particle/ml and analyzed 10 days later, were used as control. We measured the humoral response against murine dystrophin by transfecting dishes of 293A cells with pCBmdys5'ITR using

Cytofectene (Bio-Rad Laboratories, Hercules CA) according to the manufacture's recommendations. The next day, the cells were lysed and 50 µg of protein were separated on a 5% gel and processed for western blot by enhanced chemiluminescence as described previously (29). The sera of uninjected mdx mice or of mdx mice injected with HDCBDysM were used a primary antibody. The detection of a dystrophin band on the blot indicated the presence of antibodies against dystrophin in the tested serum.

In vivo measurement of force generation

To measure the force of neonatally-injected muscle, the right TA of 2- to 4-day-old mdx mice were injected as described above with HDCBDysM. The left TA, which was used as control, was injected with 10 µl of freezing buffer. To measure the force of juvenile-injected muscle, 4-to 6-week-old mdx mice were immunosupressed by daily subcutaneous injections of FK506 (generous gift of Fujisawa Inc, Japan) for seven days. On the second and fifth day of FK506 treatment, the right TA was injected with 30 µl of HDCBDysM as described above and the left TA with 30 µl of freezing buffer. At 60 days post-injection, mice were anesthetized with ketamine (130 mg/kg) and xylazine (20 mg/kg) to achieve a loss of deep pain reflexes and immobilized in the supine position. Two 27.5 gauge needles were used to secure the knee and ankle to a surgical platform. The distal tendon of the TA muscle was isolated and tied with 4-0 nylon suture to the lever arm of a force transducer/length servomotor system (model 305B dual mode; Cambridge Technology, Watertown, MA, USA). The latter was mounted on a mobile

micrometer stage to allow fine incremental adjustments of muscle length. Exposed portions of the TA were kept moist with a 37°C isotonic saline drip, and the TA was then stimulated directly via an electrode placed on the belly of the muscle. Supramaximal stimuli with a monophasic pulse duration of 2 ms were delivered using a computer-controlled electrical stimulator (model S44; Grass Instruments, quincy, MA, USA). Muscle force and length signals were displayed on a storage oscilloscope (Tektronix, Beaverton, OR, USA) and simultaneously acquired to a computer (Labdat/Anadat software; RHT-InfoData, Montreal, Qc, Canada) via an analog-to-digital converter at a sampling rate of 1000 Hz.

After adjusting the TA to optimal muscle length (L0; the length at which maximal twitch force is achieved), two twitch stimulations were recorded and the mean value was considered as maximal isometric twitch force. Maximal isometric tetanic force was then measured by stimulating the muscle at 120 Hz for 300 ms, allowing a clear plateau in force to be attained. Following a 2 min recovery period, the ability of the TA to withstand a series of high-stress eccentric (lengthening) contractions was determined. Each contraction involved supramaximal stimulation at 120 Hz for a total of 300 ms; the muscle was held at L0 during the initial 100 ms (isometric component), and then lengthened through a distance of 25% of L0 during the last 200 ms (eccentric component). Peak muscle length was maintained for an additional 100 ms after cessation of the stimulation, followed by a return to L0 during the next 100 ms. A total of five such contractions were imposed on the TA, each being separated by a 2 min recovery period. Lastly, a 120 Hz stimulation was performed at L0 to determine the final

level of isometric force production following the eccentric contraction protocol. The isometric force deficit induced by each eccentric contraction was normalized to take into account the magnitude of mechanical stress placed upon the muscle. This was done by dividing the percent force drop by the level peak stress (N/cm^2) attained during the preceding eccentric contraction. All measurements were performed in HDCBDysM-treated and untreated (contralateral limb) TA muscles of mdx mice, as well as in untreated TA muscles of normal wild type C57Bl/10 mice (The Jackson Laboratory, Bar Harbor, ME) or normal wild type C57Bl/6 mice (Charles River Lab., St-Constant, QC, Canada) of the same age. We did not observe any differences between the force generating capacity of C57Bl/10 and C57Bl/6.

Statistical analysis

Unless stated otherwise the data are expressed as the mean ± SEM. The data were analyzed using an unpaired 2-tailed T-test, or with an analysis of variance (ANOVA) followed by the Fisher's LSD procedure to compare the means. Comparisons of the response to eccentric contractions were analyzed by 2-way ANOVA, with both treatment status and the number of eccentric contractions being incorporated into the ANOVA model. Statistical significance was set at $P < 0.05$.

5.4 RESULTS

Characterization of HDCBDysM

The structure of the fully deleted HDAd (HDCBDysM) encoding two identical murine dystrophin expression cassettes appears in Figure 1A. Dystrophin expression by this vector was controlled by the strong hybrid CMV enhancer/β-actin (CB) promoter. We constructed this vector because efficient transgene expression using a similar HDAd encoding human dystrophin was demonstrated in muscle (30). HDCBDysM was produced using the 293Cre-loxP system (20) and purified by CsCl gradient centrifugation. To verify that the structure of HDCBDysM corresponded to the one depicted in Figure 1A, we isolated DNA from purified viral particles and analyzed it by Southern blot analysis. Following digestion with BamH1 and hybridization with a probe against the whole vector, or with probe against the CB promoter, the observed bands pattern matched exactly the predicted structure of HDCBDysM (Fig. 1B).

Dystrophin expression in neonatally-injected muscle

To study the level and duration of dystrophin expression bestowed by HDCBDysM in vivo, we injected the TA of neonatal mdx mice with HDCBDysM at a titer of 2.0×10^{12} viral particles/ml and analyzed dystrophin expression by immunocytochemisty and by western blot at various time points. At 10 days post-injection, the earliest time point investigated, we observed an excellent transduction level, with an average of 712 fibers per muscle positive for dystrophin, a value corresponding to 42% of the fibers of the TA

(Fig. 2A,B). Dystrophin expression was not homogeneous throughout the transduced area, as many fibers demonstrated an intense cytoplasmic immunostaining, a clear sign of dystrophin overexpression, whereas other fibers did not (see also Fig. 5A). Efficient dystrophin expression was further confirmed by western blot analysis, where the average quantity of dystrophin produced at 10 days post-injection in the tested muscles was 2.3 times the amount in wild type mouse muscle (Fig. 2C). The number of dystrophin positive (dys+) fibers and the amount of dystrophin produced by the treated muscle, did not decrease at 30, 60, 90 and 180 days post-injection, indicating that the abundant early dystrophin expression was stable (Fig. 2B).

Treatment of neonatal mdx mice with HDCBDysM mitigates the dystrophic phenotype

The mdx muscles are characterized by cycles of muscle fiber necrosis followed by regeneration. The regenerated fibers can be easily identified because they contain conspicuous internal myonuclei (33). To evaluate if expression of dystrophin by HDCBDysM could protect muscle fibers from necrosis, the percentage of fibers having centrally located nuclei (centronucleation index) was analyzed at various time points. For this study, we compared the centronucleation index of the dys+ fibers to the centronucleation index of the dystrophin negative (dys-) fibers of the same muscles (Fig. 3A). At 10 days post-injection, the centronucleation index was low (less than 7.5%) in both dys+ and dys- fibers and no significant difference existed between these two

groups. At this early age, muscle fibers have not gone yet through cycles of necrosis and regeneration and have their nuclei still located at the periphery (32,33). At later time points, muscle necrosis occurred in the dys- fibers, which is reflected by their elevated centronucleation index (higher than 45%). In contrast, this index remained low in the dys+ fibers throughout the course of this study, indicating that treatment with HDCBDysM protects muscle from necrosis.

The absence of dystrophin in muscle causes a dramatic secondary reduction of the DPC at the plasma membrane (34,35). To evaluate if dystrophin gene transfer would restore the normal distribution of the DPC at the cell surface, we stained cryostat sections of muscles that were treated 90 days earlier with HDCBDysM, forβ-dystroglycan and α-sarcoglycan, two major components of the DPC. In the control mdx muscle, the signal intensity of β-dystroglycan and α-sarcoglycan at the cell surface was markedly reduced compared to wild type mouse muscle. In contrast, the signal intensity of these two components in the dys+ fibers of the treated muscle, was comparable to the signal found in wild type muscle, thus indicating restoration of the DPC at the cell surface (data not shown).

The lack of dystrophin in mdx muscle causes a reduction in force-generating capacity of the muscle, and increases its sensitivity to mechanical stress-induced injury following the application of lengthening (eccentric) contractions (36). To verify if treatment with HDCBDysM could improve the force-generating capacity of mdx muscle, we injected the right TA of neonatal mdx mice with HDCBDysM as described above, and the left TA, which was used a control, with only buffer. Isometric force parameters were

then measured in both legs at 60 days post-injection and compared to age-matched wild type control mice (Fig. 4A). Quantification of the transduction level of this experiment appears in Figure 2B. Maximal twitch force was significantly higher in HDCBDysM-treated mdx muscles relative to untreated TA muscles. In addition, the twitch force of the HDCBDysM-treated TA muscles was restored to normal wild type levels. Maximal tetanic force was also greater in the HDCBDysM-treated mdx muscles compared to the contralateral untreated, but in contrast to the twitch force data, remained lower than wild type control mice.

The increased susceptibility of dystrophin-deficient myofibers to damage by eccentric contractions is manifested by a significant reduction in maximal isometric force production after each eccentric contraction. In addition, the level of damage and dysfunction induced by eccentric contractions is directly correlated with the magnitude of peak mechanical stress imposed on the muscle (36). Therefore, the effect of eccentric contractions on maximal isometric force production by the TA was determined, and this was normalized to account for any differences in peak mechanical stress placed on the muscles. As shown in Figure 4C and 4D, the isometric force deficit after eccentric contractions was significantly lower in HDCBDysM-treated mdx muscles than in the contralateral buffer-injected TA. However, the force deficit after eccentric contractions remained higher in HDCBDysM-treated mdx muscles compared to wild type control mice. Inflammation caused by intramuscular injection of FGAd encoding an immunogenic transgene such as β-galactosidase resulted in the upregulation of extrasynaptic utrophin, a functional dystrophin homologue (37). To verify that treatment

with HDCBDysM did not upregulate utrophin in our study, sections of muscles treated 60 days earlier with HDCBDysM were immunostained for utrophin. No difference of the extrasynaptic utrophin staining intensity was observed between the muscle injected with HDCBDysM and the muscle injected with the buffer (data not shown). Thus, treatment with HDCBDysM does not upregulate extrasynaptic utrophin expression.

The limb muscles of mdx mice are hypertrophic and weigh significantly more than normal muscle (32). To determine if treatment with HDCBDysM would reduce the hypertrophy, the weight of the TA treated 60 days earlier with HDCBDysM was compared to contralateral buffer-injected TA. The average muscle weight of the TA injected with HDCBDysM was 47.5 ± 1.7 mg (n = 17) which was significantly less ($P < 0.001$) than the average of 63.4 ± 2.2 mg observed for the contralateral control TA (n = 17). This result indicated that treatment with HDCBDysM at an early age reduces muscle hypertrophy.

Prolonged dystrophin expression after treatment of juvenile mdx mice with HDCBDysM

Neonatal mice can become partly tolerant to the Ad vector and transgene because they do not have a completely matured immune system. For this reason, and to verify the extent to which treatment of HDCBDysM could mitigate the pathology after the onset of muscle necrosis, we repeated all of the above experiments using juvenile (4- to 6-week-old) mdx mice, which are fully immunocompetent. We injected the TA of juvenile

mdx mice with HDCBDysM at a titer of 2.0 X 10^{12} viral particles/ml and analyzed dystrophin expression by immunocytochemistry and by western blot analysis at various time points. At 10 days post-injection, the earliest time point investigated, a good transduction level was observed because an average of 276 dys+ fibers were counted in the treated TA, a value corresponding to 16% of the total fibers of the TA (Fig. 5A,B). The average number of dys+ fibers was 415 (24% of the TA) at 30 days post-injection and remained high at 60 days post-injection. However, a slight but significant reduction in the number of dys+ fibers occurred at 90 and 180 days post-injection. Despite this, an average of 204 muscle fibers were still dys+ at 180 days post-injection. In previous experiments, we never observed such a prolonged transgene expression following gene transfer in juvenile mdx muscle using FGAd encoding dystrophin or utrophin, or using a fully deleted HDAd encoding human dystrophin (12,13,30,38,39). The presence of full-length dystrophin in the treated muscle was also demonstrated by western blot analysis at all the time points investigated (Fig. 5C). The highest amount of dystrophin was observed in the muscle analyzed at 30 and 60 days post-injection. At these two time points, the average quantity of dystrophin produced in the four muscles analyzed corresponded to 33% of the amount of wild type murine muscle.

HDCBDysM treatment of juvenile mdx muscle mitigates the dystrophic phenotype

We computed the centronucleation index of the dys+ fibers of juvenile-injected muscles at various time points and compared this value to the index of dys- fibers of the

same muscle. At every time point investigated, the centronucleation index was lower in the dys+ fibers, thus demonstrating a protective effect against necrosis (Fig. 3B). The centronucleation index was much higher in the dys+ fibers of juvenile-treated muscles compared to the neonatally-treated ones, because at the time of injection, a significant proportion of muscle fibers already had centrally located nuclei (Fig. 3A). The centronucleation index of the dys+ fibers did not decrease significantly at later time points, indicating the internally located nuclei do not readily move back to the periphery. We also stained for β-dystroglycan and α-sarcoglycan, cryostat sections of juvenile-injected mdx muscles that were treated 60 days earlier with HDCBDysM. The signal intensity of these two components was increased at the cell surface of the dys+ fibers of the treated muscle, thus indicating restoration of the DPC at the plasma membrane (data not shown).

We also tested if HDCBDysM would ameliorate the physiological indices of the juvenile-treated muscle. For this experiment, in an attempt to increase the transduction level, the right TA of juvenile mdx mice was injected twice (78 hrs between each injection) with HDCBDysM as described above and the left TA was injected with buffer. To avoid potential adverse effects on transduction efficiency caused by greater non-antigenic-specific inflammation (innate immune response) triggered by the second exposure to HDCBDysM capsid proteins (40), mice were transiently immunosuppressed with FK506 (38) for seven days, starting one day before treatment with HDCBDysM. Isometric force parameters were then measured in both legs at 60 days post-injection and compared to age-match wild type control mice. In contrast to the observation made

with neonatally-injected muscle, no improvement of the maximal twitch force and maximal tetanic force was observed (Fig. 6A). The lack of improvement was not due to poor transduction level, because the average number of dys+ in the 6 muscles used for the physiology was 345 ± 102. This value was not statistically different from the one obtained after a single injection of HDCBDysM (Fig. 5B). The effect of eccentric contractions on maximal isometric force production by the TA was also determined. As shown in Figure 6B, the isometric force deficit after eccentric contractions was significantly lower in HDCBDysM-treated mdx muscles than in the contralateral buffer-injected TA, but remained, nonetheless, higher than wild type control mice.

The weight of the TA treated 60 days earlier with two doses of HDCBDysM was compared to the contralateral TA. In contrast to the observation made with the neonatally-injected muscles, the average muscle mass of the juvenile TA treated with HDCBDysM (59 ± 5 mg, n = 6) was not significantly different from the control TA (59 ± 3 mg, n = 6).

Immune response after treatment with HDCBDysM

To test if injection of HDCBDysM would trigger a cellular inflammatory response, the transduced areas of muscle injected once 60 days earlier with HDCBDysM, were stained for markers for macrophages, CD8+ and CD4+ T-lymphocytes. No significant increase of immune cells compared to mdx control muscles occurred in the neonatally injected animals (Fig. 7). In contrast, we observed slightly more CD4+ and CD8+ T-lymphocytes in the transduced areas of the juvenile-injected animals, but the number of macrophages was not higher. Thus, treatment with HDCBDysM causes a slightly stronger inflammatory response in juvenile-injected compared to neonatally-injected muscles in agreement with a previous study (27). However, we considered this inflammatory response to be mild, because significantly more immune cells were observed after treatment with a FGAd encoding dystrophin (Fig. 7A). This mild inflammatory response was clearly insufficient to eliminate all the dys+ fibers, because an average of 204 fibers per TA were still expressing dystrophin at 180 days after injection of HDCBDysM (Fig. 5B). Because mdx muscles do not express full-length dystrophin, we then determined if treatment with HDCBDysM would generate a humoral response against dystrophin. Sera were collected from the treated animals and analyzed for the presence of antibody against murine dystrophin by western blot analysis using lysate of cells transfected with the murine dystrophin cDNA as a source of antigen. All the sera analyzed (7 juvenile-injected and 6 neonatally-injected animals) demonstrated the presence of antibody against the murine dystrophin (data not shown).

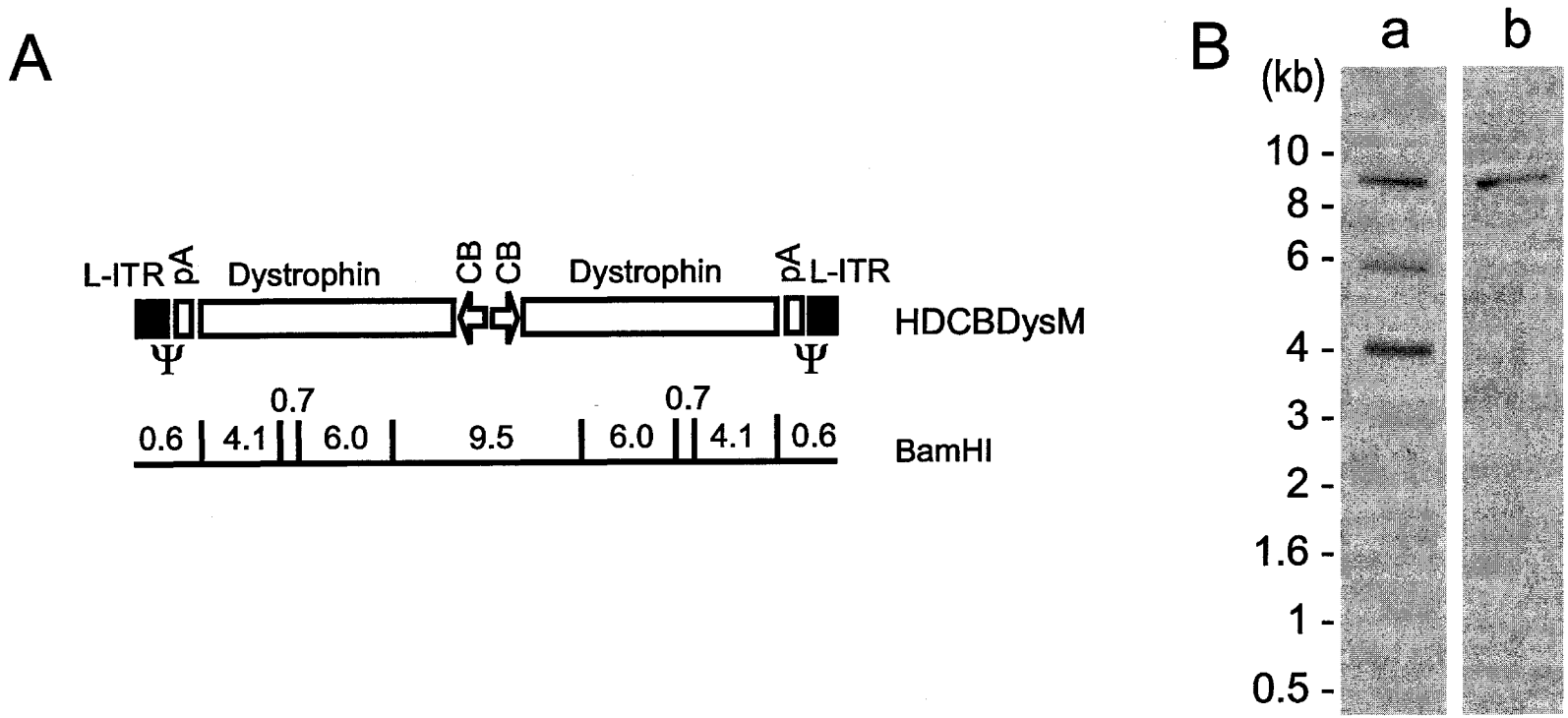

Figure 1. Characteristics of the vector used in this study. (**A**) Structure of HDCBDysM. The positions of the CB promoters, full-length murine dystrophin cDNAs, polyadenylation signals (pA), packaging signals (Ψ), and left inverted terminals repeats of Ad (L-ITR) are indicated. The lines under HDCBDysM show the position of cleavage sites for BamHI. The numbers correspond to the size of the fragments in kilobases (kb) after digestion with BamHI. (**B**) Southern blot analysis of HDCBDysM DNA after digestion with BamHI. In lane a, the blot was hybridized to a probe consisting of the L-ITR, the dystrophin cDNA and CB promoter. In lane b, the blot was hybridized to a probe consisting of the CB promoter. The positions of DNA size markers (kb) is indicated at the left of the gel. A 0.6-0.7 kb band is observed in (lane a) after longer exposure.

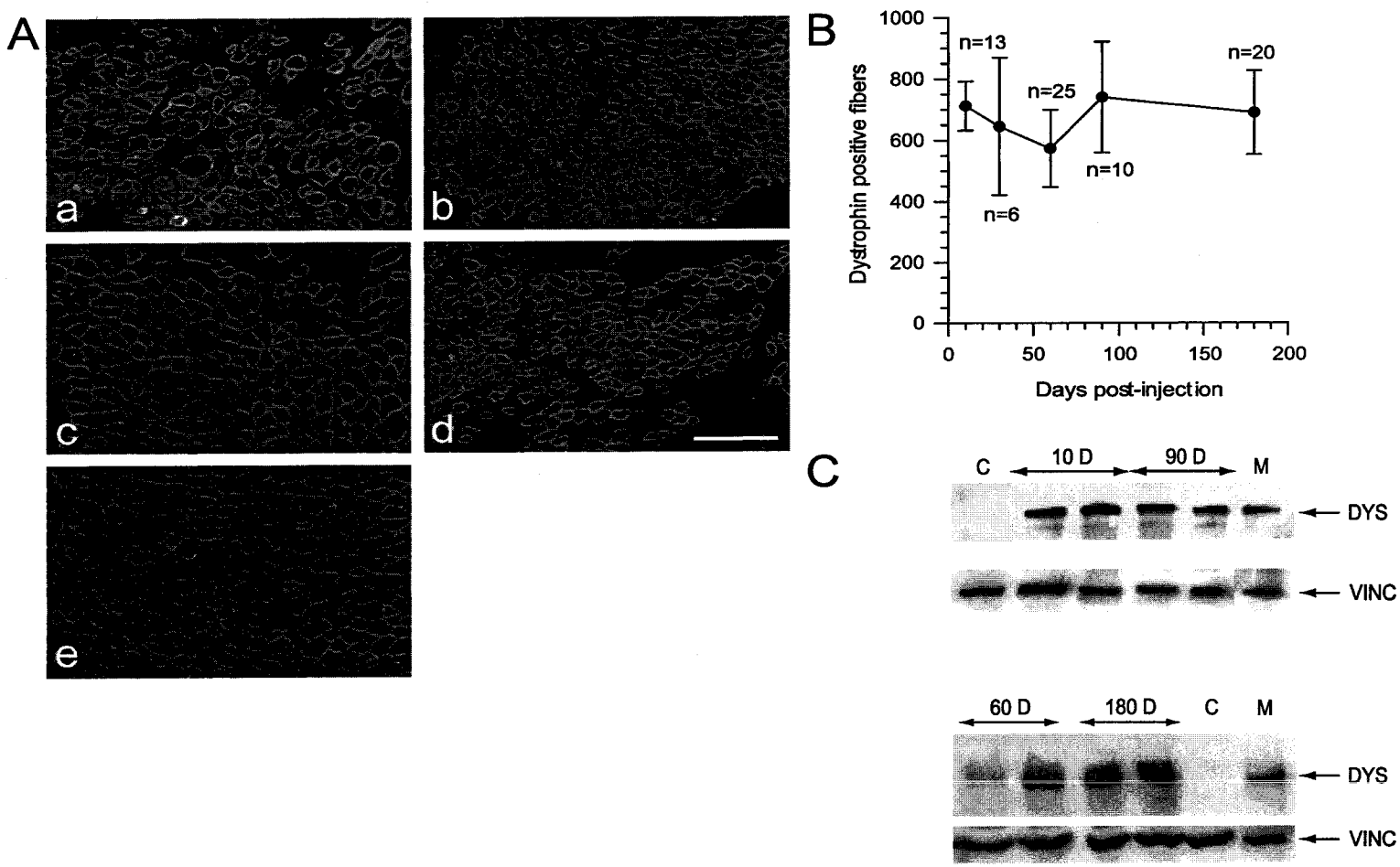
A
a
b
c
d
e
B
Dystrophin positive fibers
1000
800
600
400
200
0
n=13
n=6
n=25
n=10
n=20
0
50
100
150
200
Days post-injection
C
C
10 D
90 D
M
DYS
VINC
60 D
180 D
C
M
DYS
VINC

Figure 2. Dystrophin expression after treatment of neonatal mdx muscle with HDCBDysM. The TA of neonatal mdx mice was injected with HDCBDysM at a titer of 2.0 X 10^{12} virus/ml and analyzed for dystrophin expression at the indicated time points. (**A**) Cryostat sections stained for dystrophin by immunohistochemistry at 10 (a), 30 (b), 60 (c), 90 (d) and 180 days post-injection. Scale bar = 250 µm, except for (a) where it is 125 µm. (**B**) Quantification of the number of dystrophin positive fibers. The data are the mean number of dystrophin positive fibers per TA ± SEM. n, number of muscles analyzed. (C) Western blot analysis of dystrophin expression. 10 µg of two different muscle extracts for each time point post-injection in days (D) were analyzed using an antibody against dystrophin (DYS) or vinculin (VINC), which serves as loading control. 10 µg of normal mouse muscle (lane M) and non-injected mdx muscle (lane C) were also analyzed simultaneously.

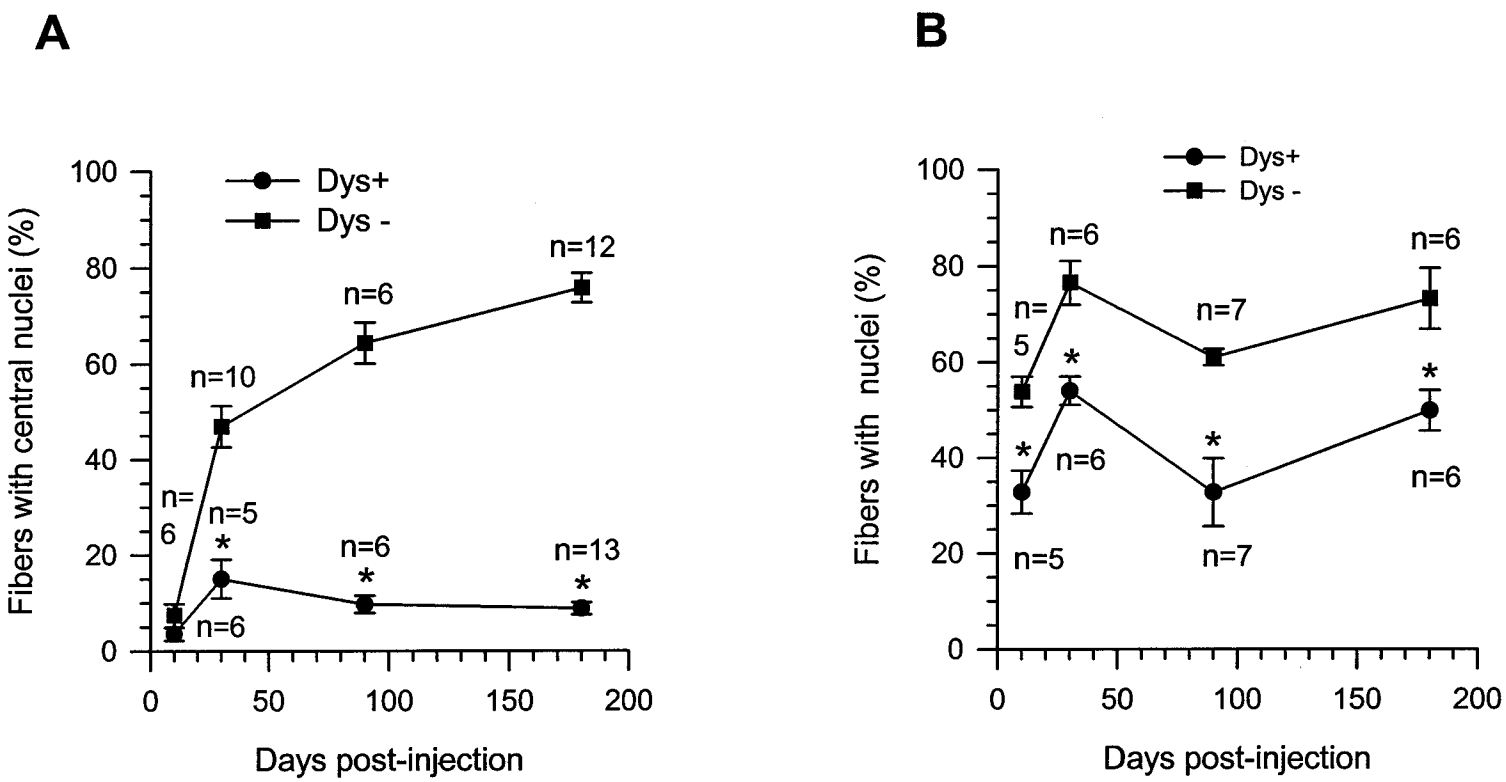

Figure 3. Treatment with HDCBDysM protects mdx muscle from necrosis. The TA of neonatal (**A**) or juvenile (**B**) mdx mice were injected with HDCBDysM at a titer of 2.0 X 10^{12} virus/ml. At the indicated time points post-injection, the percentage of fibers with centrally located nuclei was determined in the dystrophin positive (Dys +) and dystrophin negative (Dys-) fibers of the injected muscles. The data are the mean number of dystrophin positive fibers per TA ± SEM. n, number of muscles analyzed; *, means significantly less than dystrophin negative fibers.

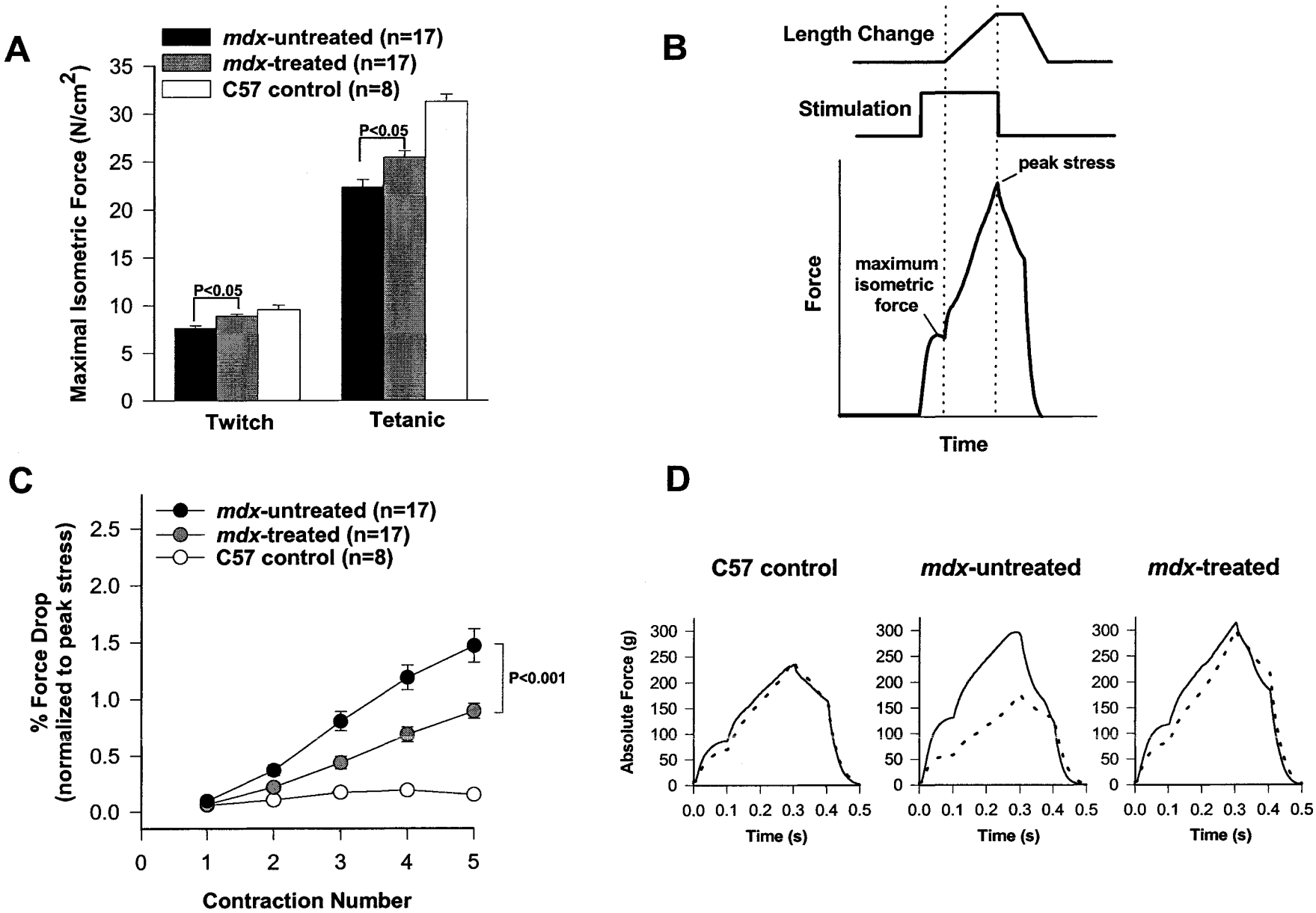
A
mdx-untreated (n=17)
mdx-treated (n=17)
C57 control (n=8)
Maximal Isometric Force (N/cm2)
35
30
25
20
15
10
5
0
P<0.05
P<0.05
Twitch
Tetanic
B
Length Change
Stimulation
peak stress
maximum isometric force
Force
Time
C
mdx-untreated (n=17)
mdx-treated (n=17)
C57 control (n=8)
% Force Drop (normalized to peak stress)
2.5
2.0
1.5
1.0
0.5
0.0
P<0.001
0
1
2
3
4
5
Contraction Number
D
C57 control
mdx-untreated
mdx-treated
Absolute Force (g)
300
250
200
150
100
50
0
0.0 0.1 0.2 0.3 0.4 0.5
Time (s)

Figure 4. Treatment of neonatal mdx muscle with HDCBDysM improves muscle physiology. The right TA of neonatal mdx mice was injected with HDCBDysM (mdx-treated) and the contralateral TA with buffer (mdx-untreated). The force generating capacity of the muscle was analyzed at 60 days post-injection. (**A**) Maximal isometric force production. (**B**) Schematic representation of physiological signals obtained during an eccentric contraction showing simultaneous tracings corresponding to muscle force, length change and stimulation. (**C**) Group mean data for the isometric force deficit induced by eccentric contractions are shown. (**D**) Representative signals from eccentric contractions imposed on C57 control, mdx-untreated, and mdx-treated muscle. The solid line indicates the first eccentric contraction in a series of 5 such contractions, while the dashed line represents the tracing from the fifth eccentric contraction. In the C57 control and treated mdx muscles, note the relative maintenance of isometric force at the fifth eccentric contraction in comparison to the untreated mdx. Values are group means ± standard error. n, number of muscles analyzed.

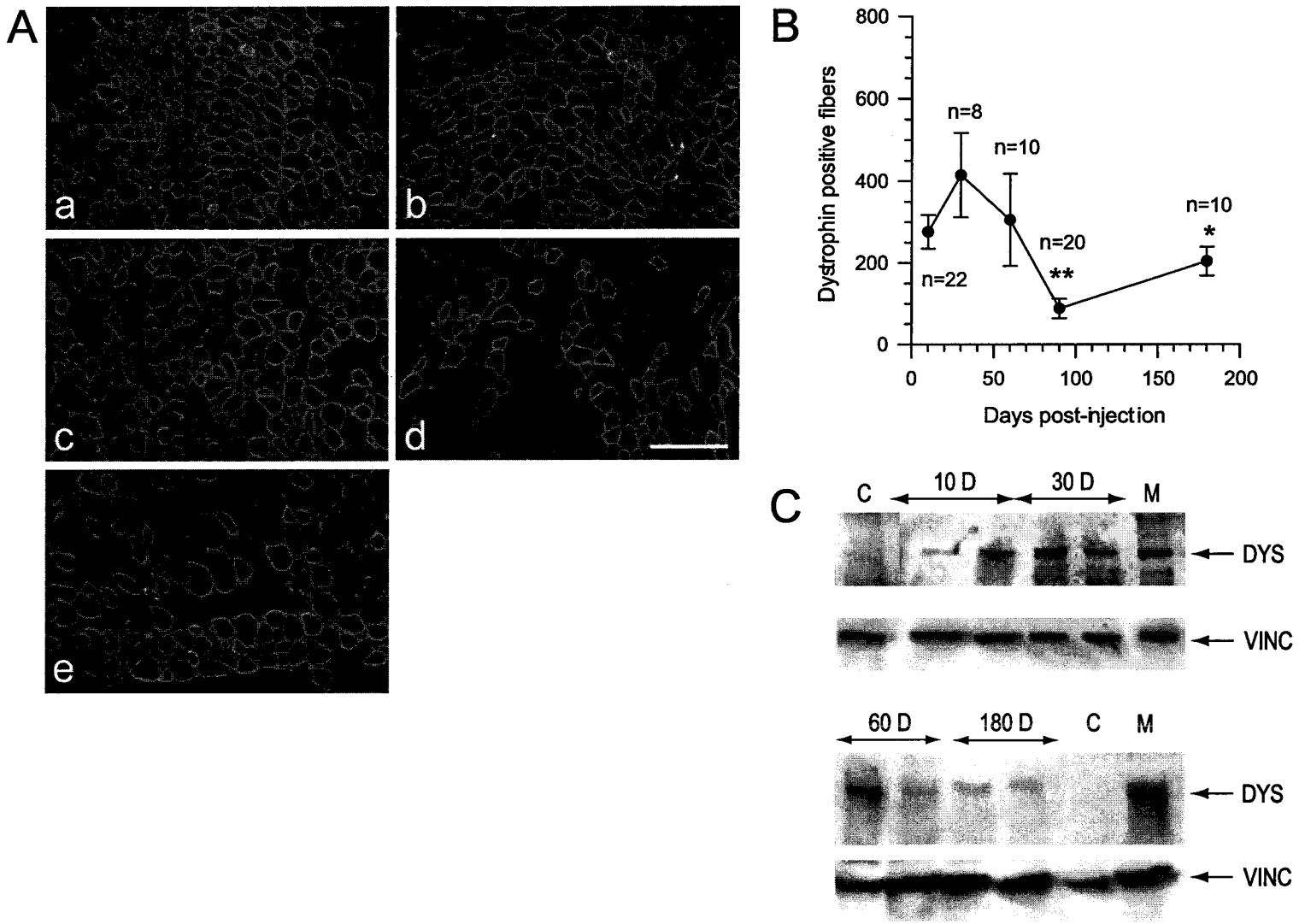
A
a
b
c
d
e
B
Dystrophin positive fibers
800
600
400
200
0
n=8
n=10
n=20
n=10
n=22
**
*
0
50
100
150
200
Days post-injection
C
C
10 D
30 D
M
DYS
VINC
60 D
180 D
C
M
DYS
VINC

Figure 5. Dystrophin expression after treatment of juvenile mdx muscle with HDCBDysM. The TA of juvenile mdx mice was injected with HDCBDysM at a titer of 2.0 X 10^{12} virus/ml and analyzed for dystrophin expression at the indicated time points. (**A**) Cryostat sections stained for dystrophin by immunohistochemistry at 10 (a), 30 (b), 60 (c), 90 (d) and 180 days post-injection. Scale bar = 250 µm. (**B**) Quantification of the number of dystrophin positive fibers. The data are the mean number of dystrophin positive fibers per TA ± SEM. n, number of muscles analyzed; ** mean significantly smaller than 10, 30 and 60 days; * mean significantly smaller than 30 days. (**C**) Western blot analysis of dystrophin expression. 10 µg of two different muscle extracts for each time point post-injection in days (D) were analyzed using an antibody against dystrophin (DYS) or vinculin (VINC), which serves as loading control. 10 µg of normal mouse muscle (lane M) and non-injected mdx muscle (lane C) were also analyzed simultaneously.

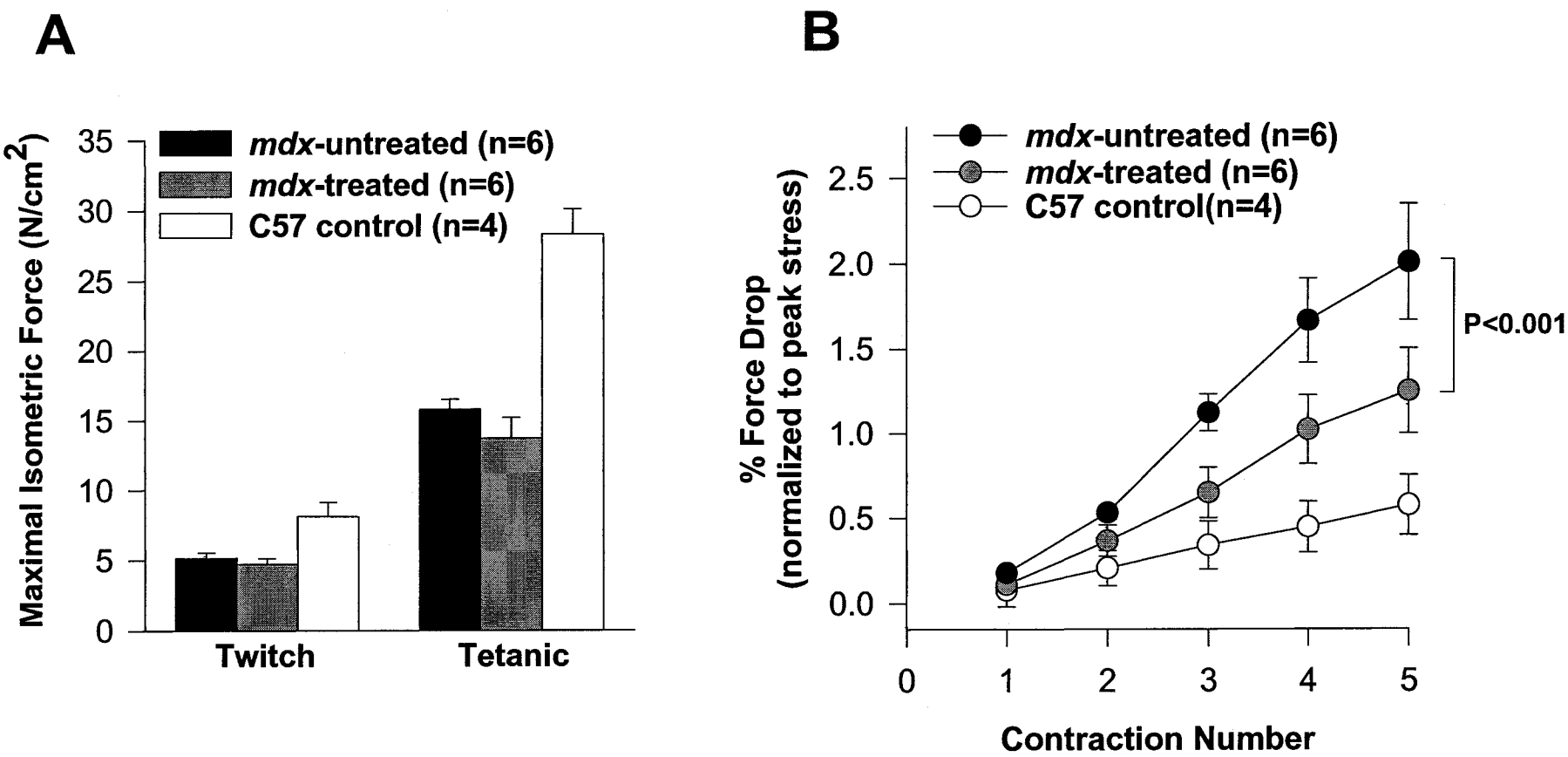

Figure 6. HDCBDysM treatment of juvenile mdx muscle improves resistance to contraction-induced injury. The right TA of juvenile mdx mice was injected with HDCBDysM (mdx-treated) and the contralateral TA with buffer (mdx-untreated). The force-generating capacity of the muscle was analyzed at 60 days post-injection. (**A**) Maximal isometric force production. (**B**) Group mean data for the isometric force deficit induced by eccentric contractions are shown. C57 control, TA of normal mice of the same age. Values are group means ± standard error; n, number of muscles analyzed.

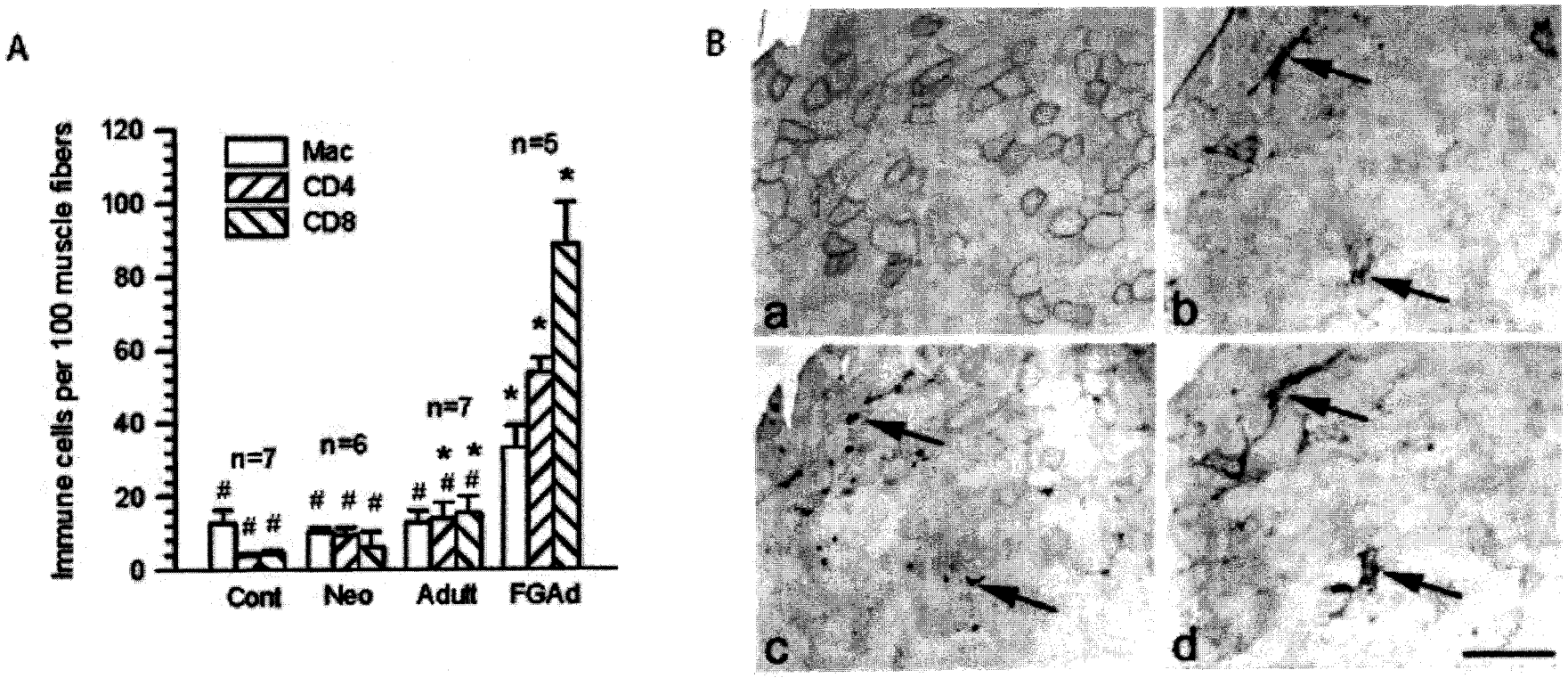

Figure 7. Treatment of juvenile muscle with HDCBDysM generates a mild inflammatory response. (**A**)The TA of juvenile (Adult) and neonatal (Neo) mdx mice were injected with HDCBDysM at a titer of 2.0 X 10^{12} virus/ml. 60 days later, cryostat sections of injected muscles were stained using antibody against macrophages (Mac), CD4 or CD8 T-lymphocytes. Buffer-injected juvenile mdx TA (Cont) at 60 days-post-injection, and juvenile mdx TA injected with FGAd encoding the human minidystrophin cDNA (FGAd) (8) at 10 days post-injection, were also analyzed. The data are the means ± SEM; n, number of muscle analyzed. *, means significantly higher than buffer-injected mdx; # , means significantly smaller than FGAd-injected mdx. (**B**) Example of mild inflammatory response in juvenile-treated muscle. Consecutive cryostat sections of mdx TA injected 60 days earlier with HDCBDysM, were stained for dystrophin (a), macrophages (b), CD4 (c) and CD8 T-lymphocytes (d). Arrows: localization of inflammatory cells in the muscles. Scale bar = 210 µm.

5.5 DISCUSSION

The present work represents the most comprehensive published study of full-length isogenic dystrophin gene transfer into mdx muscle using an HDAd in both neonatal and older animals. To obtain high-level dystrophin expression in vivo, we employed a unique and efficient Ad vector (HDCBDysM) encoding double tandem dystrophin expression cassettes controlled by a very strong hybrid promoter. No extraneous "stuffer" sequences were present in this vector: it contained only the expression cassette flanked by the Ad ITR, and the Ad packaging signal. We used neonatal and juvenile mdx mice to verify that the pathology would be mitigated to the same extent when the muscle is treated before or after the onset of necrosis, and to compare dystrophin expression in animals with immature and fully developed immune system. We believe juvenile mdx mice are a better model to investigate the immune response on dystrophin expression, compared to old mdx mice (one-year-old) used in a recent study (28), because of higher prevalence of immune cells in juvenile mdx muscles (2). Our major findings are unabated dystrophin expression, and correction of all pathological aspects investigated in neonatal animal, despite induction of a strong humoral response again the murine dystrophin. In contrast, in juvenile-treated muscle, a slight but significant reduction of dystrophin expression occurred at 90 and 180 days post injection, and muscle hypertrophy as well as maximal isometric force-generating capacity were not ameliorated. Our data thus suggests that treatment of dystrophin-deficient muscle by gene therapy before the onset of necrosis would mitigate the pathology more efficiently,

and that cellular immune response might prevent long-term (more than six months) application of our vector in fully immunocompetent individuals.

A successful treatment of DMD using a dystrophin gene transfer approach will most likely require that a large proportion of the body muscle mass is transduced and expresses significant and sustained amount of dystrophin. In an attempt to achieve this goal, we have previously constructed a fully deleted HDAd encoding two human dystrophin cDNAs controlled by the strong CB promoter (30). Although efficient, dystrophin expression was transient in mdx muscle, most likely because the dystrophin used was of human origin. In the present study, using the identical vector, but this time carrying the murine (isogenic) dystrophin cDNA, we demonstrated efficient and prolonged dystrophin expression after gene transfer in mdx mouse muscle. The fact that expression of murine dystrophin transgene was more stable compared to human dystrophin transgene is in agreement with various studies (41-43) demonstrating that isogenic proteins (murine dystrophin) are expressed for longer periods of time in muscle.

In juvenile mdx muscles that were treated with HDCBDysM, we observed a mild cellular infiltration by T-lymphocytes. The presence of low level of immune cell infiltrates was also observed by other researchers after gene transfer of fully deleted HDAd carrying the murine dystrophin cDNA in adult mdx muscle (27,28). It is possible the reduction of transgene expression we observed at 90 and 180 days post-injection was due to destruction of some of the transduced fibers by the immune cell infiltrate (41,44) and/or resulted from down regulation of the transgene expression due to the release of cytokines by the immune cells (45,46). The exact nature of the immunological insult

responsible for triggering this low level of inflammation in the muscle is not known. The muscles of mdx mice do not normally synthesized the full-length dystrophin and thus, the recombinant murine dystrophin could appear as a neoantigen and trigger the immune response. This is supported by the fact that dystrophin antigen produced by transplanted myoblasts in dystrophin deficient mice can induce a cytotoxic T-lymphocyte response specific for dystrophin (47). We and others (27) have demonstrated the presence of a humoral response against murine dystrophin in the mdx mice treated with HDAd encoding dystrophin cDNA, indicating it could act as a neoantigen. In contrast, no humoral response was observed after intramuscular injection of plasmid DNA encoding murine dystrophin in mdx mice (48). The reason for this discrepancy is not clear. The Ad viral capsids could act as an immunological adjuvant. Alternatively, the immune response against murine dystrophin could be due to the higher transduction level conferred by the HDAd. However, it is unlikely the humoral response against dystrophin was responsible for the loss of transgene expression, because no such loss occurred in neonatally-injected animals, despite the fact that a comparable humoral response against dystrophin was generated. Normal and dystrophin deficient muscles contain low level of utrophin, a functional analogue of dystrophin (49). Forced utrophin expression in transgenic animals or after gene transfer using FGAd (12,13,50,51) can mitigate the pathology associated with the lack of dystrophin. Utrophin gene transfer is thus an interesting alternative for treating DMD, because this protein is already present in dystrophin-deficient muscle and its overexpression should be well tolerated by the immune system of the host. Another approach to reduce the immune response against the transgene would be to control its expression using a muscle specific promoter

(44,52). However, a clear advantage of this strategy to treat DMD using HDAd technology has yet to be demonstrated (27). Another factor that could trigger the inflammatory response is the low-grade synthesis of viral protein from the residual level of helper virus contaminating our preparation of HDAd. Long-term transgene expression has been achieved in healthy adult muscle after gene transfer with FGAd and with HDAd encoding isogenic transgene and containing comparable level of helper Ad (23,24,41,42). The mdx muscle, because of its chronic inflammatory state (due mostly to phagocytes) (44,53,54), is presumably highly sensitive to the presence of non-self proteins, and for this reason, even low level of helper Ad contamination could be sufficient to trigger a damaging immune response.

The excellent and prolonged transduction level obtained after treatment of neonatal and juvenile mdx mice with HDCBDysM allowed us to compare the extent to which this treatment mitigates the pathology when the muscle is treated before (neonates) or after the onset of the necrotic process (juveniles). After treatment of neonates with HDCBDysM, the centronucleation index remained low in the dys+ fibers, indicating the transduced fibers were protected from necrosis for the complete duration of the study. In addition, the localization of the DPC was restored at the plasma membrane and we observed a significant increase in the resistance to contraction-induced injury as well as an improvement of isometric force generating capacity of the muscle. Comparable reduction of the centronucleation index and restoration of the DPC at the cell surface were documented following gene transfer of neonatal mdx mice using HDAd encoding dystrophin (26,27) or with FGAd carrying dystrophin or utrophin (8,10,12,55). Our current

study is the first to analyze the muscle physiology after treatment of neonates with an HDAd. In this respect, our data are comparable to those obtained with FGAd carrying dystrophin or utrophin in neonates (9,12,13). We further demonstrated that treatment with HDCBDysM is able to significantly reduce muscle hypertrophy, suggesting that homeostasis of muscle fiber volume is abnormal in the absence of dystrophin. Although no previous dystrophin gene transfer study exists about improvement of muscle hypertrophy in neonates, transgenic mouse experiments have shown that forced dystrophin or utrophin transgene expression in mdx muscle can reduce muscle weight (7,56).

At the time of injection, about 50% of the fibers of juvenile mdx TA have gone through at least one cycle of muscle necrosis followed by regeneration, and consequently possess centrally located myonuclei (32). The fact that we were able to demonstrate a stable 35% reduction of the centronucleation index after treatment of juvenile mdx muscle with HDCBDysM, indicated that muscle fibers having peripheral nuclei at the time of transduction, were subsequently protected from necrosis. Improvement of the centronucleation index of juvenile and adult mdx muscle was also documented after treatment with FGAd carrying utrophin or dystrophin transgenes (11,13), or after treatment with rAAV carrying microdystrophin (4,7). In one study (4), in agreement with our data, the percentage of dys+ fibers with centrally located nuclei did not decrease at later time points, indicating the internally located nuclei do not readily move to the periphery. In contrast, a significant reduction of the percentage of dys+ fibers with centrally located nuclei was observed at five months post-injection in another

study (7). The reason for this apparent discrepancy is not clear. Because we observed a mild inflammatory response in the juvenile mdx muscles treated with HDCBDysM, we cannot rule out that the presence of cellular infiltrate was responsible for maintaining the myonuclei in a central position. In juvenile muscles treated with HDCBDysM, we observed restoration of the amount of the DPC at the cell surface and a significant increase of the resistance to contraction-induced injury, which however, still remained lower than normal muscle. By contrast, we did not observe an increase of the normalized specific isometric force. Similar observations concerning these two physiological parameters were recently made following dystrophin gene transfer in adult mdx muscle using HDAd (28). In contrast, improvement of the specific muscle isometric force was demonstrated after gene transfer of microdystrophin in adult mdx muscle using AAV (5). In the same study, an improvement of the resistance to contraction-induced injury was also demonstrated, which, however, was not statistically different from that of normal muscle. It is not clear why these parameters of functional muscle recovery were better after gene transfer with AAV, despite the fact the transgene was a microdystrophin. However, it is worth mentioning that the transduction efficiency was slightly higher with AAV, and that treatment with the latter vector did not increase the number of cytotoxic T-lymphocytes in the transduced muscle areas (4,5), when compared to HDAd (28). Finally, in agreement with our data, treatment with AAV (5) and HDAd (28) of adult mdx muscle did not reduce muscle hypertrophy.

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5.7 ACKNOWLEDGMENTS

We thank Jun-Ichi Miyazaki (Osaka University Medical School, Osaka, Japan) for the generous gift of plasmid pCAGGS, as well as Merk & Co., Inc (West Point, PA) for the 293Cre4 cells and AdLC8cluc. We acknowledge the expert technical assistance of Carol Allen, Mylène Bourget, Stephen Prescott and Klara Rostworowski. JN is a National Scholar of the Fonds de la recherché en santé du Québec and a Killam Scholar. This work was supported by the Canadian Institutes for Health Research, the Muscular Dystrophy Association of USA and Canada, and the Association Francaise Contre les Myopathies.

CHAPTER 6.

Sustained improvement of muscle function one year after full-length dystrophin gene transfer into mdx mice by a gutted helper-dependent adenoviral vector.

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6.1 PROLOGUE

In the preceding chapter, we reported high level dystrophin expression and functional correction of dystrophin-deficient (mdx) mouse skeletal muscle 60 days after gene transfer with a helper-dependent adenovirus (HDAd) encoding two full-length murine dystrophin cDNAs (referred to as HBCBDysM). Next, we tested the long-term efficacy of HDCBDysM by examining muscle contractility parameters and the stability of dystrophin expression one year after injection into neonatal mdx muscles.

6.2 ABSTRACT

Dystrophin gene transfer using helper-dependent adenoviral vectors (HDAd) deleted of all viral genes is a promising option to treat muscles in Duchenne muscular dystrophy (DMD). Previously, we reported high-level dystrophin expression and functional correction of dystrophin-deficient (mdx) mouse muscle 60 days following gene transfer with a HDAd encoding two full-length murine dystrophin cDNAs (referred to as HDCBDysM). In the present study, we tested the long-term efficacy of HDCBDysM by examining muscle contractility parameters and the stability of dystrophin expression one year following injection into neonatal mdx muscles. At this point, HDCBDysM-treated muscles averaged 52% dystrophin-positive fibers. Treated muscles also displayed significantly greater isometric force production as well as greater resistance to the force deficits and damage caused by eccentric contractions. The level of protection against eccentric contraction-induced force deficits correlated with the percentage of dystrophin-positive fibers. Furthermore, HDCBDysM treatment restored the dystrophin-glycoprotein complex (DGC) to the sarcolemma and improved other aspects of mdx muscle histopathology examined (central nucleation, muscle hypertrophy, and mononuclear [phagocytic] cell infiltration). These improvements occurred despite the induction of a humoral response against murine dystrophin. Our results indicate that major therapeutic benefits of HDCBDysM are maintained for a long period of the animals' lifespan and suggest that HDCBDys holds promise for treating DMD by gene therapy.

6.3 INTRODUCTION

Duchenne muscular dystrophy (DMD) is a fatal X-linked recessive disorder affecting approximately 1 in 3500 male births (21, 3). DMD is caused by genetic mutations of the gene at locus Xp21, which result in the absence of a large (427 kDa) cytoskeletal protein known as dystrophin. Muscle pathology in DMD patients is characterized by progressive muscle destruction, fibrosis, and fatty cell infiltration, leading to muscle weakness and patient death around the age of 20. The mdx mouse model of DMD has a premature stop codon in the dystrophin gene and also lacks the protein product (20, 39). In normal muscle, dystrophin is present at the inner aspect of the muscle membrane (plasmalemma) where it associates with a group of proteins, including the dystroglycans, sarcoglycans, syntrophins, and dystrobrevin (8, 9). This dystrophin-glycoprotein complex (DGC) forms a physical link between extracellular matrix and the internal cytoskeleton of the muscle and is believed to mechanically stabilize the sarcolemma. Absence of dystrophin leads to a secondary loss or reduction in other members of the DGC from the muscle membrane in DMD patients and mdx mice (32, 33). At the physiological level, mdx mouse myofibers are abnormally vulnerable to damage caused by high mechanical stress, such as that associated with muscle contractions (46, 35, 29, 45). The latter can be revealed experimentally by greatly exaggerated force deficits and abnormal uptake of marker dyes into myofibers following bouts of forceful lengthening (eccentric) contractions (35, 29).

A promising approach to the treatment of DMD is to restore dystrophin expression by injecting muscles with viral vectors carrying an expression cassette for the dystrophin

cDNA (23). An ideal vector for such therapeutic intervention should be non-toxic and produce sufficient, long-term dystrophin expression in the targeted muscles. The use of recombinant adeno-associated virus (AAV) vectors has been shown to be an efficient means of gene transfer into skeletal muscle (40). However, these vectors have an insert capacity that is limited to only ~5 kb, which is far too small for the full-length dystrophin cDNA of approximately 12 kb. Although rAAV vectors carrying truncated versions of dystrophin (microdystrophins) have been successful in treating dystrophic features of mdx mouse skeletal muscles (44, 45, 10), the therapeutic efficacy of these microdystrophins in larger animal models and humans remains unknown.

Replication-deficient (E1-deleted) so-called first generation adenoviral vectors can also efficiently transduce muscle fibers in vivo (43, 5, 1, 47), but have two major drawbacks. Firstly, because most of the viral genome remains intact and the encoded protein products of these genes elicit deleterious host immune reactions, gene expression is quickly lost due to immune-mediated destruction of transduced fibers and/or silencing of transgene expression (48, 49). Secondly, the insert capacity of approximately 8 kb is once again insufficient to carry a full-length dystrophin cDNA.

In an attempt to overcome these problems, gutted or helper-dependent adenoviral vectors (HDAd) devoid of all viral sequences (except for the inverted terminal repeats and DNA packaging sequences) have been developed. These vectors have insert capacities of up to ~36 kb (11, 24, 25, 34), which is sufficient to accommodate not only one but two full-length dystrophin cDNAs (15). Another significant advantage of HDAd is a lesser degree of immunogenicity as compared to first generation adenoviral vectors,

which should allow for a greater longevity of transgene expression (30, 38, 4, 27, 50). Indeed, we recently reported substantial therapeutic efficacy in mdx mouse skeletal muscle using a novel HDAd (referred to henceforth as HDCBDysM), which contains two tandem full-length murine dystrophin transgenes under the control of a powerful hybrid CMV enhancer/β-actin promoter (16).

In our previous report, functional improvements in muscle physiology were only ascertained at two months post-treatment (16). Therefore, while HDCBDysM is able to significantly mitigate the dystrophic phenotype in mdx mice over the short-term, the extent to which such benefits can be sustained over the long-term has not been evaluated. Accordingly, the goal of the present study was to build upon our previous findings by studying mdx mice at one year after treatment with HDCBDysM. Our specific objectives were as follows: 1) to determine the long-term physiological benefits for contractile function conferred by HDCBDysM; 2) to examine the relationship between functional improvements and the long-term stability of transgene expression afforded by this vector; and 3) to ascertain the long-term effects of HDCBDysM treatment on characteristic histopathological features of the disease.

6.4 MATERIALS AND METHODS

HDCBDysM Construction and Growth

The HDCBDysM construct has previously been described in detail (16). This vector was propagated using the 293Cre-loxP system (34) and purified by two consecutive CsCl gradient centrifugations. The CsCl was removed from the viral preparation by chromatography on Sephadex G25 columns (Amersham Pharmacia Biotech Inc., Piscataway, NJ) and HDCBDysM was eluted with freezing buffer (50 mM HEPES pH 7.5, 2 mM MgCl2, 150 mM NaCl, 5% sucrose). The viral titre was determined by measuring the optical density at 260 nm (28) and HDCBDysM DNA structure was confirmed by Southern blot analysis. The relative potency of HDCBDysM was tested by infecting 293A cells with purified vector and by analyzing the amount of dystrophin produced 18 h later by western blot as described (14). The level of contamination by the E1/E3-deleted helper adenovirus ranged between 0.02 and 0.17%. This was determined by infecting 293A cells in 96-well plates with serial dilutions of HdCBDysM (purified by CsCl) as described (31). The contamination level was obtained by counting the number of wells that were cytopathic at 10 days post-infection. Known amounts of purified AdLC8cluc (helper virus) were used as a positive control.

Animals

All experiments were performed according to the McGill University guidelines for experimental animal care. Both the right and left tibialis anterior (TA) muscles of 2 to

4-day-old neonatal mdx mice (C57BL/10ScSn-mdx/J; The Jackson Laboratory, Bar Harbor, ME) were injected with 10 µl of 2.0 x 10^{12} HDCBDysM virus particles/ml as described previously (1). In addition, TA muscles from age-matched untreated mdx and normal C57BL/10 mice were used as controls.

In vivo measurement of muscle contractility parameters

At approximately 365 days post-injection, mice were anesthetized with ketamine (130mg/kg) and xylazine (20mg/kg), and immobilized in the supine position on a surgical platform. The skin of the anterior region of the lower hind limb was opened from the ankle to just above the knee to fully expose the TA. Two 27.5 gauge needles were used to secure the knee and ankle to the platform. The distal tendon of the TA was isolated and tied with 4-0 nylon suture to the lever arm of a force transducer/length servomotor system (model 305B dual mode; Cambridge Technology, Watertown, MA), which was mounted on a mobile micrometer stage to allow fine incremental adjustments of muscle length. Exposed portions of the TA were kept moist with a 37°C isotonic saline drip, and the TA was stimulated directly via an electrode placed on the belly of the muscle. Supramaximal stimuli with a monophasic pulse duration of 2 ms were delivered using a computer-controlled electrical stimulator (model S44; Grass Instruments, Quincy, MA, USA). Muscle force and length signals were displayed on a storage oscilloscope (Tektronix, Beaverton, OR, USA) and simultaneously acquired to a computer

(Labdat/Anadat software; RHT-InfoData, Montreal, Canada) via an analog-to-digital converter at a sampling rate of 1000 Hz.

After adjusting the TA to optimal muscle length (Lo, the length at which maximal twitch force is achieved), two twitch stimulations were performed. The mean value of force produced from these two contractions was considered as maximal isometric twitch force. Isometric tetanic force was then measured by stimulating the muscle at 10, 30, 60, 90, and 120 Hz for 300 ms. Maximal isometric tetanic force was usually achieved at a stimulation frequency of 120 Hz. Muscle cross-sectional area was determined by dividing muscle weight by its length and tissue density (1.06 g/cm3), which then allowed specific isometric force (i.e., force normalized to muscle cross-sectional area) to be calculated.

Following a two minute recovery period, the ability of the TA to withstand a series of high-stress eccentric (lengthening) contractions was determined. Each contraction involved supramaximal stimulation at 120 Hz for a total of 300 ms; the muscle was held at Lo during the initial 100 ms (isometric component), and then lengthened through a distance of 25% of Lo during the last 200 ms (eccentric component). Peak muscle length was maintained for an additional 100 ms after cessation of the stimulation, followed by a return to Lo during the next 100 ms. A total of five such contractions were imposed on the muscle, each being separated by a 30 second recovery period. Lastly, a 120 Hz stimulation was performed at Lo to determine the final level of isometric force production following the eccentric contraction protocol. Because the level of damage and dysfunction due to eccentric contractions is directly correlated with the magnitude of

the peak stress imposed on the muscle (35), the isometric force deficit induced by each eccentric contraction was normalized to peak stress as previously described (7, 16).

Histological evaluation of sarcolemmal damage following eccentric contractions

Evans blue dye (EBD), a low molecular weight tracer compound that binds tightly to serum albumin, is excluded from normal myofibers but enters into fibers with a damaged or leaky sarcolemma (42). Therefore, we used this dye to determine the extent to which HDCBDysM treatment could protect the sarcolemma of mdx muscle from damage induced by our protocol of eccentric contractions. EBD (5 µg/µl solution in PBS; 5 µl/g body wt) was injected into the jugular vein of anaesthetized mice approximately 30 minutes before the first eccentric contraction was performed. At the end of each experiment, the TA was quickly removed and snap-frozen in isopentane precooled with liquid nitrogen. Transverse sections of the TA were fixed in methanol for 5 minutes and visualized by fluorescence microscopy to identify EBD-positive fibers. A digital camera was then used to capture images to computer, and quantitative analysis was performed using a commercial software package (Image-Pro Plus, Media Cybernetics, Silver Springs, MD).

Immunohistochemistry and western blotting

Transverse cryostat sections were stained for dystrophin using a rabbit polyclonal antibody raised against the C-terminus of dystrophin (1) and visualized with either horseradish peroxidase- or Cy-3-conjugated streptavidin (Jackson Immuno Research Laboratories Inc., West Grove, PA). For each HDCBDysM-injected muscle, we determined the total number of transduced fibers by counting dystrophin-positive fibers on a single muscle section that spanned the entire TA cross-section. The percentage of dystrophin-positive fibers was calculated by dividing this value by the total number of fibers on the section. Dystrophin expression was also analyzed by western blotting as previously described (15). The membranes were processed using the ECF western blot kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's recommendations. The signal was visualized and quantified using a PhosphorImager system (STORM, Molecular Dynamics Inc, Sunnyvale, CA). The blots were also stained with a monoclonal antibody against vinculin (V2638, Sigma-Aldrich, St. Louis, MO) as loading control. In muscle sections, which had been counterstained with hematoxylin, we also assessed the percentage of myofibers with central nuclei, which is a quantitative cumulative index of antecedent myofiber necrosis and subsequent regeneration (22). Counterstaining with hematoxylin also permitted evaluation of the numbers of inflammatory cells (mostly macrophages) (41) present in transduced regions of HDCBDysM-treated as compared to untreated mdx muscles. Restoration of the DGC was assessed by immunostaining with antibodies specific for β-dystroglycan

(NCL-43DAG; Novocastra, Newcastle-upon-Tyne, UK), α-sarcoglycan (NCL-50DAG; Novocastra), and utrophin (NCL-DRP2; Novocastra) as previously described (12).

Real-time PCR for quantification of adenoviral vector DNA

Real-time PCR was performed on DNA from HDCBDysM-injected mdx TA muscles at 10, 180, and 365 days post-injection. DNA was extracted from muscle cryosections (about 70 sections per muscle) obtained from the transduced regions of HDCBDysM-treated muscles by overnight digestion at 50°C in 0.5 ml of lysis buffer (100mM Tris-HCl pH 8.5, 5mM EDTA, 0.2% SDS, 200mM NaCl) containing 20 µg/ml proteinase K, followed by phenol/chlorophorm extraction and isopropanol precipitation. The DNA was washed with 70% ethanol, dried and resuspended in TE (10mM Tris-HCl pH 8, 1mM EDTA). Real-time PCR was carried out using the QuantiTectTM Probe PCR Kit (Qiagen, Valancia, CA) with primers and detection probes (TaqMan probe, from Qiagen, Valancia, CA) specific for the CB promoter region (5-cccacttggcagtacatcaa-3, 5-atggggagagtgaagcagaa-3, 5-FAM-cgtcaatgacggtaaatggcccgcct-TAMRA-3) of HDCBDysM, and the murine adipsin gene (5-acgtgagacccctacccttg-3, 5-gactaccccgtcatggtacg-3, 5-TET-tcacccatgcaggacgcaggcctgatgt-TAMRA-3), which served as a DNA input control. To quantify vector and adipsin DNA content, real-time PCR reactions were performed in two separate tubes, both of which contained DNA extracted from the same muscle. PCR was performed in 25 µl volumes containing 100 ng of sample DNA, 12.5 µl 2x QuantiTect Probe PCR Master Mix, 100 nM TaqMan

Probe, and 200 nM of each primer. All reactions were performed in the SmartCyclerR (Cepheid, Sunnyvale, CA) using the following parameters: 50°C for 2 min and 95°C for 15 min followed by 45 cycles of the amplification step (94°C for 15s, 58°C for 30s, and 72°C for 30s). The copy number of HDCBDysM DNA (CB primers) was determined using a standard curve made by spiking 100 ng of untreated muscle DNA with known amount of plasmid encoding the CB promoter (pCBDysFl, ref. 15). All the data were normalized to the amount of adipsin DNA, which was determined using a standard curve made of untreated muscle DNA of 7 week-old mice.

Host humoral immune response to HDCBDysM

293A cells were transfected with a plasmid encoding the full-length murine dystrophin cDNA (pCBmdys5'ITR) (16) using Cytofectene (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's recommendations. The next day, the cells were lysed and 50 µg of protein was separated on a 5% gel and processed for western blot by enhanced chemiluminescence as described previously (14). Dilution (200X) of sera of HDCBDysM-injected mdx or of uninjected mdx mice were used as a primary antibody. The detection of a dystrophin band on the blot indicated the presence of antibodies against dystrophin in the tested serum.

Statistical analysis

Data are expressed as the mean values ± SEM unless stated otherwise. Most data were analyzed using Student's unpaired two-tailed t-test. Comparisons of the response to eccentric contractions were analyzed by two-way ANOVA, with both treatment status and the number of eccentric contractions being incorporated into the ANOVA model. Statistical significance was set at $P < 0.05$.

6.5 RESULTS

HDCBDysM confers long term improvement of mdx muscle function

When compared to normal muscles, dystrophin-deficient mdx muscles display: 1) decreased specific isometric force production, and 2) an increased susceptibility to force deficits following forceful lengthening (eccentric) contractions (35, 45). Therefore, these two functional outcome measures were evaluated at one year following treatment with HDCBDysM. As shown in Fig. 1A, both maximal isometric twitch force and maximal isometric tetanic force were significantly improved at one year following treatment. The effects of HDCBDysM treatment on the resistance to force deficits caused by eccentric contractions are displayed in Fig. 1B. As can be seen, the magnitude of the force deficit following eccentric contractions in the HDCBDysM treated muscles was significantly less than observed in untreated mdx muscles. On average, the dysfunction caused by eccentric contractions in the HDCBDysM-treated mdx muscle was intermediate between that seen in untreated mdx and wild-type C57BL10 muscles.

The increased vulnerability of dystrophin-deficient mdx muscles to mechanical stress-induced damage is also reflected by abnormal sarcolemmal hyperpermeability to low molecular weight tracer dyes (35, 29). In order to assess whether HDCBDysM could protect against such sarcolemmal damage, we examined EBD uptake in myofibers of treated and untreated mdx muscles following the imposition of eccentric contractions. The percent increase in EBD-positive fibers after eccentric contractions in the HDCBDysM-treated and untreated muscles (relative to the baseline level of EBD-positive fibers in mdx muscles not subjected to eccentric contractions) was determined. As shown in Fig. 2, the percent increase in EBD-positive fibers in HDCBDysM-treated muscles was significantly lower than observed in the untreated mdx group.

High-level full-length dystrophin expression one year after treatment with HDCBDysM despite a decline of the number of vector DNA

After confirming the functional efficacy of HDCBDysM in mdx muscles one year post-injection, we next examined the level of expression of the dystrophin transgene in these same muscles by immunohistochemistry (Fig. 3A). At 365 days post-injection, we found the average number of dystrophin-positive fibers per muscle to be 1381, which corresponds to approximately 52% of the total fibers in the mdx TA at this age (Fig. 3B). Western blotting revealed that the HDCBDysM-treated muscles expressed, on average, 64 ± 24% (mean ± SEM; n=3) of the amount dystrophin found in age-matched, wild-type C57BL10 mouse muscles (Fig. 3C).

Real-time PCR was used to determine the number of copies of vector DNA at different time points up to 365 days after HDCBDysM injection. On average, more than 2800 copies of vector DNA per 100 ng of muscle DNA were detected at 10 days post-injection, whereas the average copy number decreased significantly at 180 and 365 days post-injection (Fig. 3D).

Correlation between transduction level and functional benefits in mdx muscles

In order to determine whether the number of dystrophin-positive fibers in treated muscles correlated with the level of correction of muscle function parameters, linear regression analysis was performed . The number of dystrophin-expressing fibers ranged from 26% to 76% in the HDCBDysM-treated muscles. Approximately 2% of total fibers in the six untreated mdx muscles were revertant fibers, which expressed dystrophin (20); these muscles were also included in the linear regression analysis. A significant correlation was found between the percentage of dystrophin-positive fibers and protection against the force deficit induced by eccentric contractions ($R=0.58$; $p=0.02$). On the other hand, no significant correlation was found between the percentage of dystrophin-expressing fibers and the magnitude of isometric force production.

Treatment with HDCBDysM ameliorates dystrophic histopathology and restores DGC

Central nucleation of muscle fibers occurs after myofiber necrosis and regeneration, and can thus be used as an index of all prior necrosis (22). To evaluate whether HDCBDysM-mediated dystrophin expression protects mdx muscle fibers from necrosis up to 365 days post-injection, the percentage of fibers with central nuclei was compared in dystrophin-expressing and dystrophin-negative fibers within the same muscles. Transduced fibers expressing dystrophin had strikingly fewer central nuclei than dystrophin-negative fibers (Fig. 4A).

Compensatory hypertrophy is another characteristic feature of mdx skeletal muscles and, accordingly, these muscles weigh more than those of age-matched normal wild-type C57BL10 mice (18). Therefore, we examined whether HDCBDysM treatment caused a reduction in TA muscle weight relative to the untreated muscles. The average weight of the treated muscles was approximately 38% less than that of untreated group (Fig. 4B).

The mdx dystrophic pathology is also characterized by infiltration into the muscle of mononuclear immune cells (mostly macrophages destined for phagocytosis of necrotic fibers) (41). To examine if HDCBDysM treatment could reduce this infiltration, we counted the number of infiltration cells in dystrophin-positive regions (containing ~200 myofibers) of treated muscles and compared this to untreated mdx muscles.

Interestingly, the number of inflammatory cells in HDCBDysM-transduced regions was approximately 4 times less than found in non-treated mdx muscles (Fig. 4C).

Skeletal muscles of DMD patients and mdx mice display a dramatic reduction of other members of the DGC, such as the sarcoglycans and dystroglycans, from the sarcolemma (32, 33). To ascertain whether HDCBDysM treatment in neonatal muscle was able to maintain the DGC at the sarcolemma after one year, we stained serial sections for α-sarcoglycan and β-dystroglycan (Fig. 5). Except for the rare revertant fibers, staining for these two members of the complex was absent or greatly reduced in the untreated mdx TA. In contrast, dystrophin-expressing fibers in sections from the HDCBDysM-treated muscles exhibited α-sarcoglycan and β-dystroglycan staining at the sarcolemma which was comparable to wild-type C57BL10 controls, indicating that HDCBDysM-mediated restoration of the DGC is maintained for at least 365 days post-injection. Treatment with HDCBDysM did not alter extrasynaptic utrophin expression (data not shown).

Humoral immune response to murine dystrophin in mdx mice after HDCBDysM treatment

Full-length dystrophin is not expressed in mdx mice. Therefore, we examined whether HDCBDysM treatment generated a humoral response against murine dystrophin. Sera from HDCBDysM-treated mdx mice were collected 365 days post-injection, and evaluated for the presence of antibodies against murine dystrophin

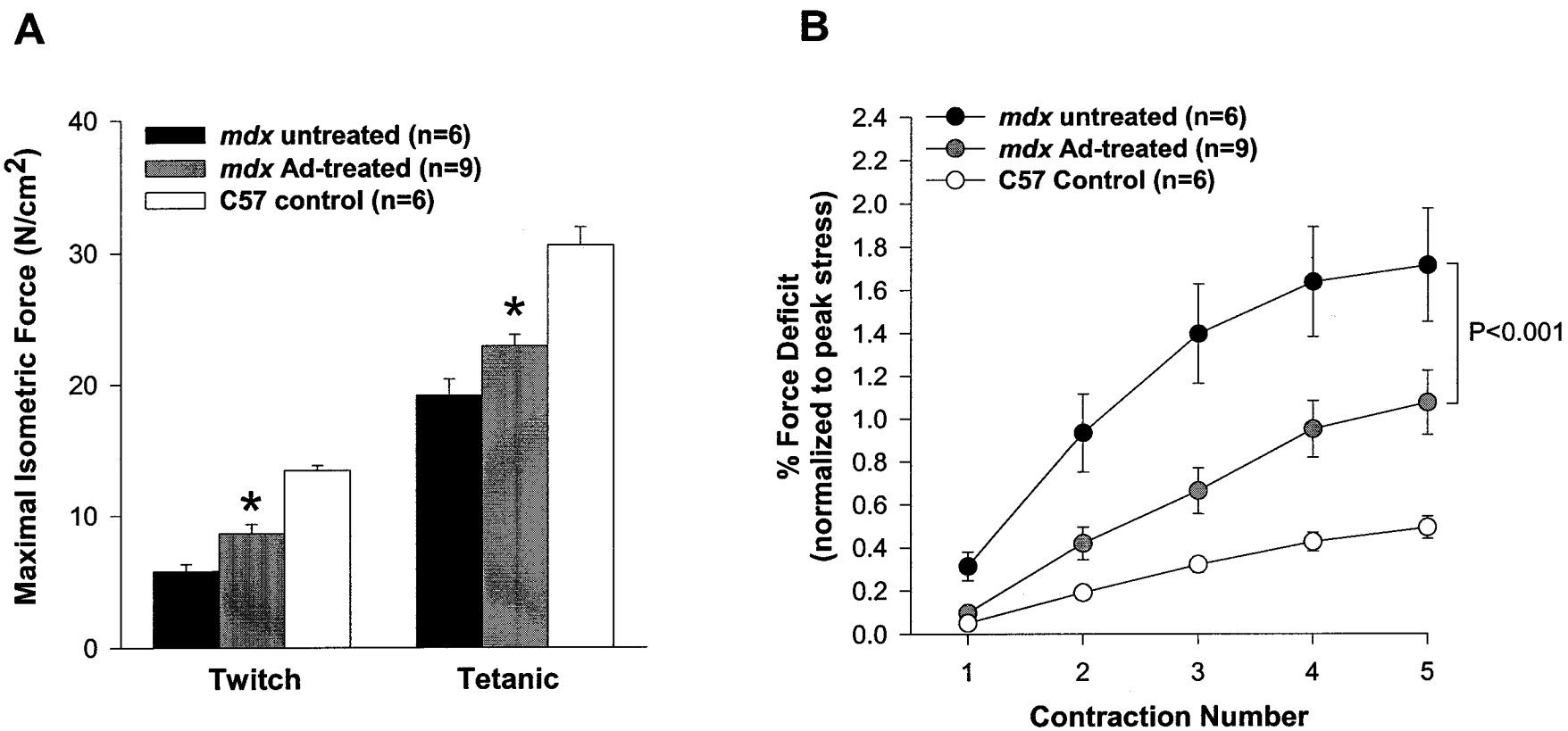

Figure 1. Treatment of neonatal mdx muscles with HDCBDysM significantly improves muscle function. (**A**) Isometric force production; and (**B**) Resistance to eccentric contraction-induced force deficits, at 365 days post-injection. Values are group means ± standard error. n, number of muscles analyzed. *P<0.05 vs. untreated mdx.

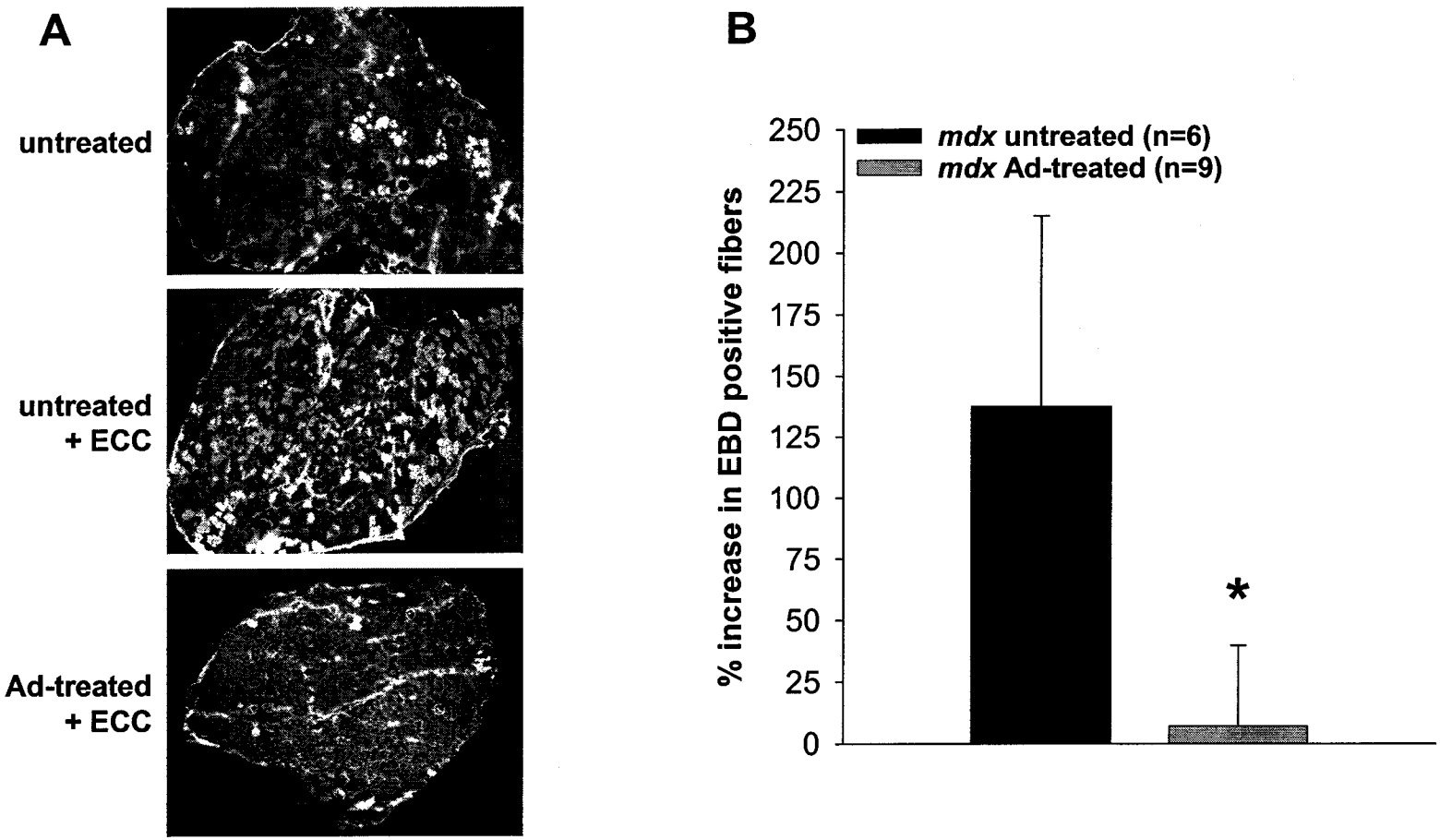

Figure 2. Treatment of neonatal mdx muscles with HDCBDysM results in a lower level of contraction-induced sarcolemmal injury. (**A**) Representative micrographs demonstrating EBD uptake in untreated mdx muscle not subjected to eccentric contractions, as well as HDCBDysM-treated and untreated mdx muscles subjected to eccentric contractions. (**B**) The percent increase in EBD-positive fibers following eccentric contractions (relative to muscles not subjected to eccentric contraction) was significantly lower in the HDCBDysM-treated muscles. Values are means ± standard error. n, number of muscles analyzed. *$P<0.05$ vs. untreated mdx.

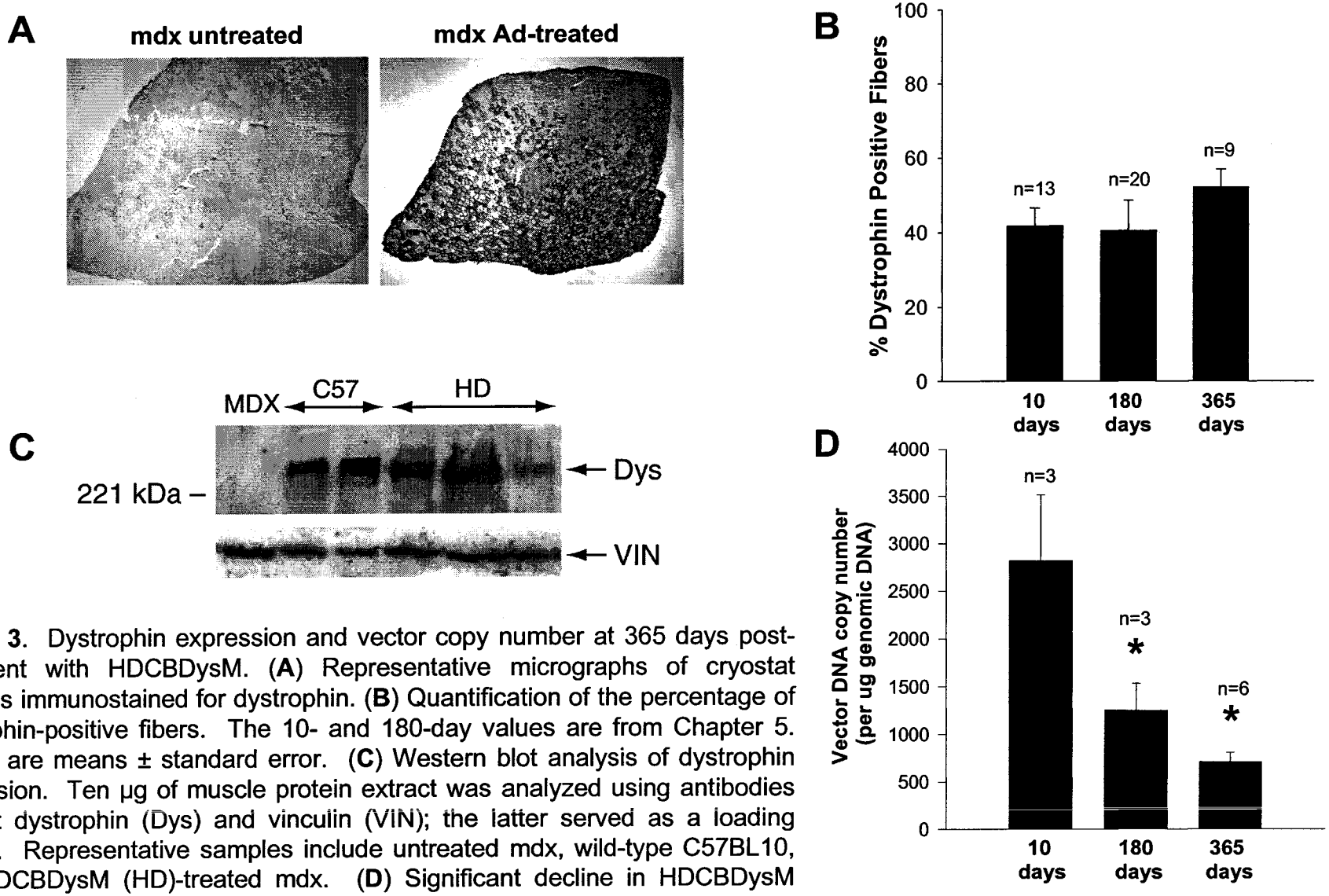

Figure 3. Dystrophin expression and vector copy number at 365 days post-treatment with HDCBDysM. (**A**) Representative micrographs of cryostat sections immunostained for dystrophin. (**B**) Quantification of the percentage of dystrophin-positive fibers. The 10- and 180-day values are from Chapter 5. Values are means ± standard error. (**C**) Western blot analysis of dystrophin expression. Ten µg of muscle protein extract was analyzed using antibodies against dystrophin (Dys) and vinculin (VIN); the latter served as a loading control. Representative samples include untreated mdx, wild-type C57BL10, and HDCBDysM (HD)-treated mdx. (**D**) Significant decline in HDCBDysM vector copy number over time (from 10 to both 180 and 365 days). Total vector copy number detected in each muscle was normalized to the adipsin (Adi) gene DNA content. Values are means ± standard error. *P<0.05 vs. 10 days.

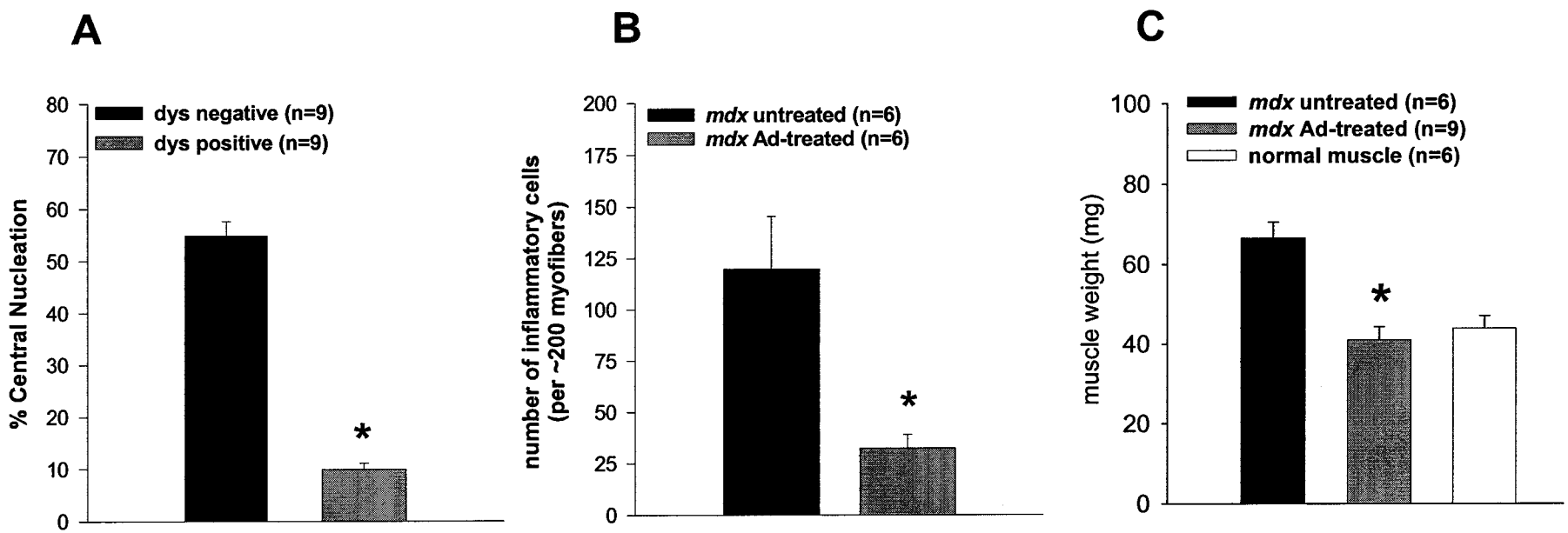

Figure 4. HDCBDysM treatment mitigates the dystrophic histopathological phenotype after one year. (**A**) HDCBDysM-transduced fibers had significantly fewer central nuclei than non-transduced fibers. (**B**) The weights of HDCBDysM-treated muscles were significantly lower than those of untreated muscles, but were comparable to normal mouse muscles of the same age. (**C**) The number of mononuclear phagocytic cells invading or surrounding transduced fibers of HDCBDysM-treated mdx was significantly lower than in untreated mdx muscles. Values are means ± standard error. n, number of muscles analyzed. *P<0.05.

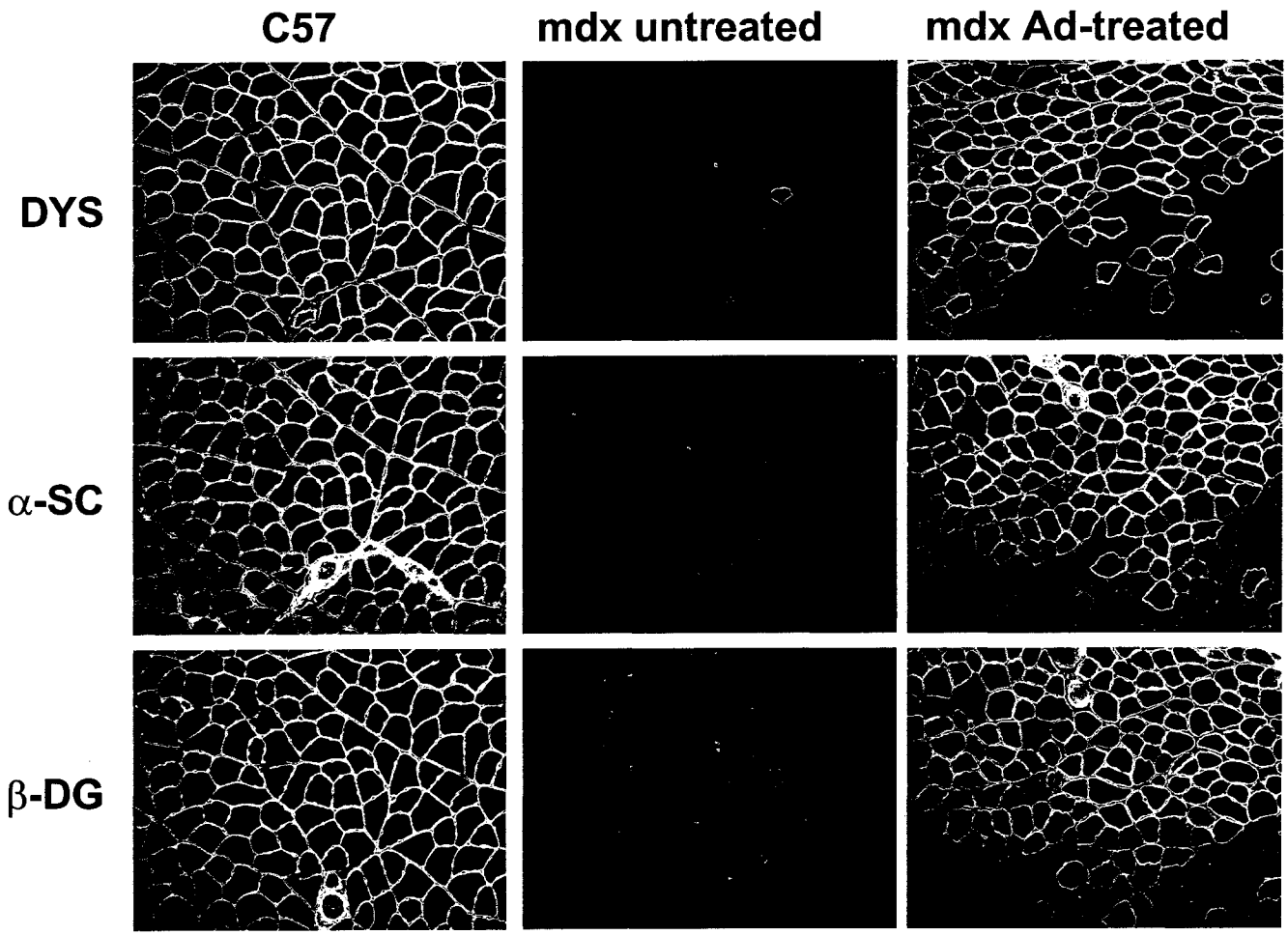

Figure 5. HDCBDysM maintains the dystrophin-glycoprotein complex at the sarcolemma after one year. Serial cryostat sections of HDAd-treated mdx, untreated mdx and C57BL10 wild-type muscles were immunostained for dystrophin (DYS), α-sarcoglycan (α-SG), β-dystroglycan (β-DG). Dystrophin-positive fibers of HDCBDysM-treated muscles showed a similar pattern of immunostaining for α-SG and β-DG.

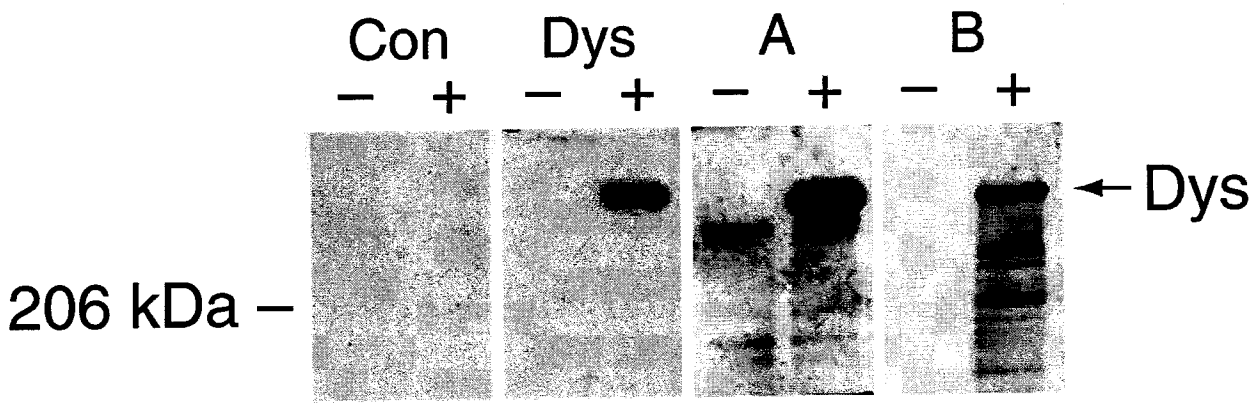

Figure 6. HDCBDysM treatment induces a significant humoral response to murine dystrophin. 293A cells were mock-transfected (-) or transfected with plasmid encoding murine dystrophin (+). The cell lysate was analyzed by western blot using one of the following as primary antibody: normal mdx mouse serum (CON), a commercially available antibody against dystrophin (Dys), or the sera of two different mdx mice injected 365 days earlier with HDCBDysM (A, B). Arrow indicates full-length dystrophin.

using the lysate of cells transfected with a murine dystrophin cDNA as the source of antigen. Western blotting revealed that all sera tested (five treated animals) demonstrated the presence of antibodies against murine dystrophin (Fig. 6).

6.6 DISCUSSION

In comparison to earlier-generation adenoviral vectors, HDAd are far less immunogenic and also permit delivery of the full-length dystrophin cDNA. Previously, we described the development and shorter-term therapeutic benefits of one such vector, referred to as HDCBDysM, which encodes two tandem full-length isogenic dystrophin transgenes under the control of the powerful hybrid CMV enhancer/β-actin promoter (15). In the present study, we have performed a detailed investigation of the long-term (365 days) efficacy of HDCBDysM treatment in mdx mice, with particular focus on the functional improvements afforded by this vector. The principal findings of our study are the following: (1) HDCBDysM injection in mdx neonatal skeletal muscle led to a long-term improvement of isometric force production; (2) HDCBDysM protected against the force deficits as well as sarcolemmal damage associated with eccentric contractions; (3) protection against contraction-induced force deficits was significantly correlated with the degree of muscle transduction; and (4) HDCBDysM provided long-term, high-level dystrophin expression and corrected characteristic features of dystrophic histopathology. A comparable reduction of the centronucleation index as reported in the present study was also observed in a previous investigation of dystrophin gene transfer into neonatal

mdx mice using HDAd (17). To our knowledge, our study is the first report of such long-term amelioration of multiple features of the dystrophic phenotype by dystrophin replacement, including muscle contractility parameters, using adenoviral vectors. In addition, these benefits occurred despite some loss of vector DNA over time and the induction of a humoral immune response against the isogenic murine dystrophin protein. A humoral immune response against the isogenic murine dystrophin was also observed at 90 and 180 days after treatment of neonatal mdx mice with the same vector (16).

Several studies have described the varying degrees of success with which viral vectors containing dystrophin or utrophin gene cassettes improve functional deficits in mdx muscle (5, 47, 13, 7, 45). However, few studies have addressed this issue using newer generation HDAd. Recently, DelloRusso et al. (6) tested the ability of HDAd carrying a single full-length murine dystrophin cDNA to correct functional deficits in adult mdx muscles injected at one year of age. At one month post-injection, this vector protected against eccentric contraction-induced force deficits, but had no beneficial effects on isometric force production (6). Our group reported similar findings, i.e., improvements in resistance to eccentric contraction-induced force deficits but not in maximal isometric force production, at 60 days following injection of HDCBDysM into younger adult mdx mice (16). In contrast, when administered to neonatal mdx mice, HDCBDysM improved both isometric force and resistance to eccentric contractions at 2 months post-injection (16). In the present study, we greatly extend these findings by demonstrating that these benefits are sustained for up to at least one year (approximately half of the animal's lifespan) after therapeutic dystrophin gene transfer

with HDCBDysM. Our observation that dystrophin expression correlates more closely with protection against eccentric contractions than with isometric force generation is consistent with dystrophin's role as a cytoskeletal structural protein. Hence dystrophin is not directly involved in the process of muscle contraction and attendant force production.

In addition to eccentric contraction-induced force deficits, we also examined the ability of full-length dystrophin gene transfer to protect the sarcolemma from contraction-induced damage using a tracer technique. Deconinck et al. (5) examined whether injection of a first generation adenoviral vector containing a human mini (6.3 kb)-dystrophin cDNA into neonatal mdx mouse gastrocnemius muscles could protect against sarcolemmal damage. In their study, injected muscles were subjected to a bout of eccentric contractions at approximately 3.5 months post-treatment, and sarcolemmal permeability to a low molecular weight tracer dye was found to be approximately three times lower in transduced muscle fibers (5). Although some important differences in the eccentric contraction protocols (e.g., 7% stretch by Deconinck et al. (5) versus 25% stretch in our study) and the tracer molecules (Procion orange versus EBD) employed makes direct comparison difficult, the data from our study suggest a similarly high level of protection against eccentric contraction-induced sarcolemmal injury one year post-injection of HDCBDysM. Indeed, the percent increase in EBD-positive fibers following eccentric contractions was approximately 10 times lower in the HDCBDysM-treated group than in untreated mdx muscles.

It should be noted that although HDCBDysM improved isometric force production and resistance to mechanical stress-induced force deficits, it did not restore these

contractile parameters to the levels found in normal wild-type mice. This seems to be at variance with previously reported findings in dystrophin-expressing transgenic mdx mice, in which only about 20% of the normal wild-type level of dystrophin was needed to normalize force production (36). This apparent discrepancy may be due, at least in part, to the fact that direct muscle injection with adenoviral vectors does not generally result in homogeneous transgene expression throughout the entire tendon-to-tendon length of transduced muscles. Therefore, only certain segments of a transduced muscle fiber may contain sufficient dystrophin expression to offer complete protection against the dystrophic process. In addition, it may be that certain regions of a muscle fiber require more dystrophin than others. For instance, the myotendinous junction is a site of dystrophin enrichment in normal muscle, and is also particularly vulnerable to injury when muscles are subjected to high levels of mechanical stress (37, 26). Inhomogeneous expression of dystrophin within individual muscle fibers could also help to explain why the correlation between the percentage of transduced fibers and protection against eccentric contraction, while statistically significant, was far from unity. These observations further underscore the importance of developing an efficient means of delivery via the vascular bed, not only in order to carry the dystrophin transgene to multiple muscles, but also to achieve a more homogeneous distribution of transgene expression within each transduced muscle fiber in all regions of the treated muscles.

In the present study, the vector copy number (by real-time PCR) decreased by approximately four-fold from 10 to 365 days. Normally, the dystrophin protein is extremely stable at the sarcolemma, with persistent expression for up to 26 weeks after

dystrophin gene inactivation (2). This may explain why the reduction of dystrophin expression (as monitored by western blot) appears to lag behind the loss of vector DNA, which decreased by two-fold at 180 days post-injection but without a corresponding reduction of dystrophin expression (16). Gilchrist et al (17) reported that injection of HDAd encoding both full-length murine dystrophin and E. coli LacZ into neonatal LacZ/ mdx mice results in stable expression of dystrophin for up to 20 weeks only, with a subsequent decline at 40 and 53 weeks post-treatment (17). However, the mechanisms responsible for this reduction (e.g., loss of vector DNA, promoter shut-down, etc.) were not determined. In other studies employing HDAd carrying non-immunogenic transgenes, no significant loss of vector DNA was demonstrated for several months following intramuscular injection of these vectors in mouse muscle (4). However, it is important to note that the above studies were accomplished in non-dystrophic muscle. Because mdx muscles go through cycles of necrosis followed by regeneration, vector DNA may have been lost from fibers which were not completely protected from necrosis due to insufficient and/or inhomogeneous dystrophin expression, as alluded to earlier. In addition, some of the vector DNA loss could also reflect turnover of non-myogenic cells within the muscle (e.g., inflammatory cells) which were coincidentally transduced during direct intramuscular injection of the vector.

We previously reported that HDCBDysM injection in neonatal mdx mice resulted in dystrophin expression within approximately 40% of the TA muscle fibers, and that this transduction level was maintained for the 180-day duration of the study (16). In the same study, western blotting revealed that HDCBDysM-treated muscles contained about

twice the amount of dystrophin protein found in normal wild-type control muscles, which indicates that the transduced muscle fibers contained levels of dystrophin which are substantially higher than normal. Intriguingly, we found an even higher (52%) percentage of dystrophin-positive fibers at one year post-injection in the present study, but the total amount of dystrophin protein (once again by western blotting) had decreased to 64% of normal wild-type control levels at the same time point. There are at least two possible explanations for this finding. First, it may reflect a gradual loss of promoter activity within the transduced fibers. Second, it could be due to the previously mentioned loss of vector DNA over time, but by a mechanism, which does not entail destruction of the transduced muscle fiber. For instance, the loss of dystrophin expression within transduced fibers could be due to instability and continued degradation of the extra-chromosomal vector DNA as an alien molecule. If this is indeed the case, a better understanding of this process could allow the development of methods to stabilize episomal vectors such as HDAd, thereby further increasing the longevity of therapeutic transgene expression.

In conclusion, we have shown that HDCBDysM treatment in neonatal mdx mouse muscle results in high-level dystrophin expression and significant amelioration of multiple aspects of the dystrophic phenotype, including functional deficits, one year post-injection. These results suggest that if isogenic, full-length dystrophin is delivered by HDAd at an early point in life, the beneficial effects of such therapy may be maintained for a prolonged period of time.

6.7 ACKNOWLEDGMENTS

I thank Jun-Ichi Miyazaki (Osaka University Medical School, Osaka, Japan) for the generous gift of plasmid pCAGGS, as well as Merck and Co., Inc. (West Point, PA, USA) for the 293Cre cells and AdLC8cluc; Carol Allen, Mylène Bourget, Duska Gvosdic, Stephen Prescott, and Klara Rostworowski for their expert technical assistance; Yifan Lu, Stefan Matecki, Renald Gilbert for their important contributions to this experiments presented here; and finally the supervisors of this project Josephine Nalbantoglu, George Karpati, and Basil J. Petrof for their invaluable guidance and advice

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CHAPTER 7.

CONCLUSIONS

7.1 SUMMARY

ALS and DMD are two invariably fatal neuromuscular disorders for which there is little effective treatment. Furthermore, the pathogenesis of these disorders have not been fully elucidated. There has been greater progress in DMD in this regard as genetic and molecular mechanisms of dystrophin-deficiency are now better understood. Despite this, the exact means by which the absence of dystrophin leads to muscle cell degeneration has not been precisely determined. ALS, on the other hand, is thought to be a multifactorial disease and, aside from the small percentage (~2%) of patients with SOD1 mutations, no exact genetic cause is known. These short-comings in our understanding of these diseases have certainly hampered progress toward finding effective therapies. The broad purpose of this thesis was to explore the mechanisms of muscle dysfunction in animal models of these disorders, and to evaluate the efficacy of a new gene transfer vector as a potential treatment for DMD.

ALS and DMD patients eventually die due to respiratory failure because of dysfunction of the diaphragm and other respiratory muscles. These two disorders also share in common the fact that oxidative/nitrosative stress has been implicated in pathogenesis. In ALS, damage due to reactive oxygen and reactive nitrogen species is believed to occur, for the most part, in motor neurons. In recent years, reports had suggested that mitochondrial dysfunction might occur in skeletal muscles of ALS patients, and that ubiquitous expression of mutant SOD1 could induce muscle damage in addition to denervation changes (1-3).

In Part I of this thesis (Chapter 2), we tested the effect of the ALS-linked SOD1-G37R mutation overexpression on skeletal muscle properties in transgenic mice. In particular, we examined contractile properties and markers of oxidative (protein carbonyls) and nitrosative (3-nitrotyrosine formation) stress in soleus and diaphragm muscles, and examined these muscles for classic signs of a primary myopathic process. Muscles from SOD1-G37R transgenic mice displayed abnormal contractile properties, but showed no evidence of a primary myopathy. Furthermore, we did not see any significant increases in protein carbonyls, although 3-nitrotyrosine formation in SOD1-G37R muscles tended to be increased. We concluded that the altered contractile function in SOD1-G37R mice was most likely due to motor neuron disease alone, but we could not rule out the possibility that the mutant enzyme also alters muscle function by some other means without inducing an overt myopathic process.

In Part II of this thesis (Chapters 3 and 4), we examined the role of oxidative/nitrosative stress in dystrophin-deficient muscles. In contrast to ALS, the target of free radical-mediated damage in DMD is believed to be the skeletal muscles themselves. The literature suggests both that oxidative stress occurs in dystrophin-deficient muscle (4-7) and that these muscles are abnormally susceptible to this form of pathophysiological stress (8,9). In regards to the latter, it had been shown that cultured dystrophin-deficient myotubes are more vulnerable to oxidative stress than normal myotubes, but this abnormal susceptibility had not been demonstrated in vivo. In addition, it had recently been established that dystrophin-deficient muscle suffers functional ischemia (10,11). Therefore, the purpose of Chapter 3 was to test if mdx

muscles are abnormally susceptible to I/R injury, an in vivo model of oxidative/nitrosative stress, which is of great relevance to DMD. We found that mdx muscles were indeed more susceptible to I/R-induced 3-nitrotyrosine formation, lipid peroxidation, and membrane fragility. Importantly, we found also that I/R predisposes mdx muscles to greater sarcolemmal injury when subjected to mechanical stress, which is a hallmark of dystrophin-deficient muscles. By linking the concepts of functional ischemia, oxidative/nitrosative stress, and the well described abnormal susceptibility to mechanical stress-induced sarcolemmal injury, Chapter 3 provides the possibility of a new conceptual framework for understanding the pathogenesis of DMD, with several complex interactions taking part.

Chapter 3 adds to the literature which implicates oxidative/nitrosative stress in the pathogensis of DMD. However, it remained unclear why the redox balance of dystrophin-deficient cells is disturbed, and why these muscles are abnormally susceptible to oxidative/nitrosative stress. In Chapter 4, we tested the possibility of a deficiency in the glutathione antioxidant system in mdx muscles. On the contrary, we found that GPx and GR activities were actually increased in these muscles, which suggests that a deficiency of glutathione-associated antioxidants is not the basis for an increased vulnerability to oxidative/nitrosative damage in mdx muscles. Despite this antioxidant upregulation, we observed an altered glutathione status and further evidence for oxidative/nitrosative damage (ICDH and aconitase inhibition) in mdx muscles under baseline conditions, suggesting that this potential compensatory mechanism is not fully protective.

In Chapter 4, we also used our I/R model to dynamically stress the antioxidant systems, and found that this form of oxidative/nitrosative stress could induce a glutathione antioxidant profile in normal C57 muscles which was very similar to that found in mdx muscles at baseline. This supports the idea that recurrent ischemia and reperfusion could be a cause of the altered glutathione status in mdx muscles under baseline conditions. However, in contrast to normal muscles, we found that mdx muscles were partially protected against further negative effects of I/R, perhaps due to a form preconditioning resulting from chronic recurrent ischemia in these muscles. Nonetheless, this upregulation of antioxidant enzymes in mdx muscles was not sufficient to protect against the protein modifications and membrane damage seen in Chapter 3. Therefore, the results of Chapter 4 (and Chapter 3) suggest that while upregulation of antioxidant enzymes may offer partial protection against acute I/R insults, this compensatory mechanism may eventually be overwhelmed by the chronic nature of the ongoing oxidative/nitrosative stress caused by repeated episodes of ischemia and reperfusion with exercise and rest, respectively.

While these results suggest that antioxidant therapies in DMD might be beneficial, trials of antioxidant treatments, such as vitamin E supplementation, have been disappointing (12). This may be due to the complicated nature of antioxidant interactions. For instance in Chapter 4, we showed that total GSH is reduced in mdx muscle. Among other antioxidant functions, GSH is known to recycle vitamin E. This might explain why vitamin E supplementation has not proved beneficial in dystrophin deficiency. In addition, oxidative/nitrosative stress, while tremendously important, is

probably only one of the factors which contributes to myofiber destruction in DMD. Therefore, since the loss dystrophin is fundamentally responsible for all cellular disturbances in dystrophin-deficient muscle, a more complete therapy would be the replacement of functional dystrophin genes in these muscles. Accordingly, in Part III (Chapters 5 and 6) we turned our attention to the analysis of a new fully-gutted adenoviral vector to achieve dystrophin gene transfer therapy in dystrophin-deficient muscles.

Gilbert et al (13) constructed a HDAd encoding two tandem human dystrophin cDNAs regulated by a powerful hybrid CMV enhancer/β-actin (CB) promoter, which induced a high level dystrophin expression in mdx mouse muscle. However, the use of this vector only resulted in transient dystrophin expression, which was thought to be due to the fact that the dystrophin transgene used was non-isogenic (i.e., human). Therefore, in Chapter 5, we tested the efficacy of a fully deleted HDAd (HDCBDysM) encoding two, tandem isogenic (i.e., murine) dystrophin cDNAs, each regulated by the CB promoter in neonate and juvenile mdx mouse muscles. In neonatal mice, which have an immature immune system, HDCBDysM induced unabated dystrophin expression to a level and duration not achieved in prior studies using a HDAd. However, in treated juvenile mdx muscles dystrophin expression decreased substantially between 30 and 60 days. Moreover, despite the use of an isogenic transgene, an inflammatory response was detected in the juvenile (but not in the neonatal-injected) muscles. The transient nature of dystrophin expression in juveniles may have been due to this inflammatory response. Marked improvement of muscle histology and physiology was nonetheless

achieved in both animal groups up to 60 days (the duration of the study). However the extent to which such benefits could be sustained over the long-term was not evaluated.

Therefore, the purpose of Chapter 6 was to build upon our previous findings by studying mdx mice at one year after neonatal treatment with HDCBDysM. We found that HDCBDysM treatment provided long-term, high-level dystrophin expression and corrected characteristic features of dystrophic histopathology. Importantly, this was associated with long-term improvement of isometric force production and protection against eccentric contraction-induced force deficits and sarcolemmal damage. The results of Chapter 6 suggest that if isogenic, full-length dystrophin is delivered by HDAd at an early point in life, beneficial effects may be maintained for a prolonged period of time. Together, Chapters 5 and 6 suggest HDCBDys is a very promising vector for treating DMD by gene therapy. However, we recognize that further studies are required to fully characterize the factor(s) triggering the inflammatory response in juvenile-injected animals, which might reduce the long-term effectiveness of this vector in fully immunocompetent subjects.

In conclusion, the work of thesis has contributed to the body of knowledge concerning skeletal muscle dysfunction and damage in the neuromuscular disorders ALS and DMD. In particular, we have further defined the role of oxidative/nitrosative stress in these pathophysiological processes. These findings may help to unravel the remaining mysteries of the pathogenesis of these devastating diseases. In addition, our studies which tested a new fully-gutted adenoviral vector for dystrophin gene transfer may represent a significant step towards an effective treatment for DMD.

7.2 CLAIMS OF ORIGINALITY

The work presented in this thesis has provided the following important original contributions to the existing body of scientific knowledge:

1). Skeletal muscles from the mutant SOD1-G37R transgenic mouse model of ALS display decreased isometric force production and altered contractile kinetics relative to non-transgenic littermates, but no overt myopathic process. In addition, the level of disease progression is accelerated in diaphragm compared to soleus.

2). Skeletal muscles from the dystrophin-deficient mdx mouse model of DMD display an abnormal susceptibility to ischemia/reperfusion-induced 3-nitrotyrosine production, lipid peroxidation, and sarcolemmal fragility.

3). Skeletal muscles from the dystrophin-deficient mdx mouse model of DMD display an abnormal glutathione status, which is characterized by an increased GSSG/GSH ratio, and upregulation of the antioxidant enzymes GPx and GR. An increased GSSG/GSH ratio is also seen in pre-necrotic mdx muscles.

4). Skeletal muscles from the dystrophin-deficient mdx mouse model of DMD display decreased enzymatic activity of the citric acid cycle enzymes, isocitrate dehydrogenase and aconitase.

5). The upregulation of antioxidant enzymes, GPx and GR, in skeletal muscles of the dystrophin-deficient mdx mouse model of DMD is associated with partial protection against further negative effects induced by acute I/R-injury, but is not able to prevent 3-nitrotyrosine production, lipid peroxidation, and membrane fragility induced by I/R in these muscles.

6). In juvenile mdx muscles, injection of a helper-dependent adenoviral (HDAd) vector encoding two, tandem murine dystrophin full-length cDNAs, each regulated by the powerful hybrid CMV enhancer/β-actin promoter, is able to provide marked improvement of muscle histology and physiology up to 60 days post-injection. However, dystrophin expression induced by this HDAd vector in juvenile mdx mice is only transient and is associated with an inflammatory response.

7). In neonatal mdx mice muscles, injection of a helper-dependent adenoviral (HDAd) vector encoding two, tandem murine dystrophin full-length cDNAs, each regulated by the powerful hybrid CMV enhancer/β-actin promoter, is able to provide long-term, high-level

dystrophin expression and correction of dystrophic histopathology and functional deficits up to one year following treatment.

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Facility Committee: RVH

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tle (must match the title of the funding source application): Strategies for therapy of respiratory muscle failure in muscular 'strophy

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University Veterinarian:		**Date:** 5/09/02
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Facility Committee: RVH

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Approval Signatures:

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