

**Heart failure in dystrophin-deficient (*mdx*) mice:
Role of stretch-activated channels and reactive
oxygen species**

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University of Sydney**

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July 2007

ABSTRACT

Duchenne muscular dystrophy (DMD) is caused by deficiency of the cytoskeletal protein dystrophin. DMD patients develop a dilated cardiomyopathy. The first half of this thesis explores the possibility that dystrophin is involved in the regulation of a stretch-activated channel (SAC). Because cardiac failure only appears late in the progression of DMD, we examined age-related effects in the *mdx* mouse, an animal model of DMD. Old *mdx* (9-12 months) mice had raised resting intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), while cardiomyocytes from young (9 weeks) and old *mdx* mice displayed abnormal Ca^{2+} transients and Ca^{2+} handling protein expression. Old *mdx* mice had increased protein expression of TRPC1, a likely candidate protein for SACs, while the SAC inhibitors streptomycin and GsMTx-4 reduced resting $[\text{Ca}^{2+}]_i$ in old *mdx* mice. These observations suggest that SACs may be involved in the Ca^{2+} handling abnormalities in these animals. Thus, SACs may play a role in the pathogenesis of the heart failure associated with DMD.

The second half of this thesis investigated the effects of the antioxidant N-acetylcysteine (NAC) on myocardial function in young (9 weeks) *mdx* mice following 6 weeks of treatment. NAC had no effect on wild type mice. Experiments revealed that *mdx* mice had increased reactive oxygen species levels; NAC-treatment reduced ROS levels in *mdx* hearts. NAC-treatment attenuated abnormalities in *mdx* cardiomyocyte Ca^{2+} handling, reversed defects in *mdx* myofilament function, and prevented myocardial inflammation. *Mdx* hearts had increased NADPH oxidase activity, a ROS producing enzyme, suggesting it could be a possible source of augmented ROS in *mdx* mice. Therapies designed to reduce oxidative damage might be beneficial to DMD patients with heart failure.

DECLARATION

I hereby declare that all work presented in this thesis is my own, and to the best of my knowledge, it contains no material previously published or written by any other person, except where due reference or acknowledgement is made in the text



Iwan A. Williams

ACKNOWLEDGEMENTS

This thesis was completed under the supervision of Professor David Allen. I am grateful for his continued support, guidance and invaluable intellectual input. He had also been an excellent mentor and helped me to progress to the next stage in my career. He has always been more than generous with his time. I wish David, and his wife Trish, all the best for the future.

To Dr. Nicholas Whitehead, thanks for the plethora of scientific discussions and debates. Your knowledge of the Duchenne muscular dystrophy field has been immensely helpful. Your ability to play pool has also made the weekends memorable.

To the rest of the muscle research laboratory, including Dr. Ella Yeung, Catherine Pham, Jessica Tipene, Dr. Leah Cannon, Dr. Marie Ward, Dr. Othon Gervasio, Dr. Terence Moopanar, Trent Reardon, Dr. Xiao-Hui Xiao, Dr. Yue-Kun Ju and Dr. Yi Chu, thank you for all of your support, advice and chocolate brownies!

Lastly, I would like to thank my mum, dad and two sisters, and Emely McCord for their continued love and support. Without you I would not be where I am now, and for that I am forever indebted.

PUBLICATIONS AND ABSTRACTS

Refereed Publications

Containing material presented in this Thesis:

Williams, I.A. & Allen, D.G. (2007) The role of reactive oxygen species in the hearts of dystrophin-deficient (*mdx*) mice. *Am J Physiol Heart Circ Physiol.* **293**:H1969-1977

Williams, I.A. & Allen, D.G. (2007) Intracellular calcium handling in ventricular myocytes from *mdx* mice. *Am J Physiol Heart Circ Physiol.* **292**: H846-H855

Additional relevant publications:

Ward, M.L., Cooper, P., Chu, Y., Ju, Y-J., **Williams, I.A.**, & Allen, D.G (2007) Stretch-activated channels in the heart; contributions to length-dependence and to cardiomyopathy. *In preparation*

Williams, I.A., Xiao, X-H., Ju, Y-K. & Allen, D.A. (2007) Rise of $[Na^+]_i$ during ischemia in the rat heart – underlying mechanisms. *Pflugers Arch.* *In press*

Tsoutsman, T., Chung, J., Doolan, A., Nguyen L., **Williams, I.A.**, Tu, E., Lam, L., Bailey, C.G., Rasko, J., Allen, D.G. & Semsarian, C. (2006) Molecular insights from a novel cardiac troponin I mouse model of familial hypertrophic cardiomyopathy. *J Mol Cell Cardiol.* 41: 623-632

Abstracts

Williams, I.A. & Allen D.G. (2007) Improved Ca^{2+} handling and reduced inflammation in *mdx* (dystrophic) mice hearts following *in vivo* antioxidant treatment. XIX World Congress International Society for Heart Research, Bologna, Italy, June 22 - 26. *J Mol Cell Cardiol.* 42: S165

Williams, I.A. & Allen, D.G. (2006) Altered calcium handling in young and old *mdx* mice: The role of the Stretch-activated channel. Annual Scientific Meeting of the International Society for Heart Research, Perth, Australia, August 5 – 8. *J Mol Cell Cardiol.* 41: 750-751

Williams, I.A. & Allen, D.G. (2006) Altered calcium handling in young and old *mdx* mice: The role of the Stretch-activated channel. Annual Scientific Meeting of the International Society for Heart Research, Perth, Australia, August 5 – 8. *Heart, Lung Circ.* **15/S1**: S178

Tsoutsman, T., Chung, J., Doolan, A., Nguyen L., **Williams, I.A.**, Tu, E., Lam, L., Bailey, C.G., Rasko, J., Allen, D.G. & Semsarian, C. (2006) Mouse cardiac Troponin I model of hypertrophic cardiomyopathy: Phenotype associated with abnormal calcium handling. Annual Scientific Meeting of the International Society for Heart Research, Canberra, Australia, August 5 – 8. *Heart, Lung Circ.* **15/S1**: S181

Williams, I.A. & Allen, D.G. (2005) Abnormal calcium transients in cardiomyocytes from *mdx* (dystrophic) mice. Annual Scientific Meeting of the International Society for Heart Research, Perth, Australia, August 5 – 8. *Heart, Lung Circ.* **14**: S164

Williams, I.A., Xiao, X-H., Ju, Y-K. & Allen, D.A. (2004) Rise of intracellular sodium during ischaemia: the underlying mechanisms. XVIII World Congress International Society for Heart Research, Brisbane, Australia. August 7 – 11. *J Mol Cell Cardiol.* 37: 232

AWARDS RELATING TO THESIS

John Hunter Prize – Best Publication by a PhD student (2007)

Outstanding Poster Presentation – Bosch Institute Annual Scientific Meeting, Sydney
(2007)

Runner –up: Best Young Investigator Student Presentation – International Society for Heart
Research, Canberra (2006)

Best Oral Presentation by a Postgraduate Student - Bosch Institute Young Investigator
Symposium, Sydney (2006)

ABBREVIATIONS

ACF:	Aortacaval fistula
Ca ²⁺ :	Calcium
[Ca ²⁺] _i :	Intracellular calcium concentration
DCM:	Dilated cardiomyopathy
DMD:	Duchenne muscular dystrophy
DHPR:	Dihydropyridine receptor
EC coupling:	Excitation contraction coupling
ECG:	Electrocardiogram
EtBr:	Ethidium bromide
GFRC:	Growth factor-regulated channel
HRP:	Horse radish peroxidase
Mn ²⁺ :	Manganese
MnSOD:	Manganese superoxide dismutase
NADPH:	Nicotinamide adenine dinucleotide phosphate
NCX:	Sodium calcium exchanger
NF-κB:	Nuclear factor kappaB
NIH:	National Institute for Health
NO:	Nitric oxide
NOS:	Nitric oxidase synthase
PLA ₂ :	Phospholipase A ₂
PLN:	Phospholamban
ROS:	Reactive oxygen species

Abbreviations

RyR2:	Cardiac ryanodine receptor
SAC:	Stretch-activated channel
SERCA:	Sarcoplasmic reticulum calcium ATPase
SOD:	Superoxide dismutase
TGF- β :	Transforming growth factor beta
TnI:	Troponin I
T-tubule:	Transverse tubule
TRPC1:	Canonical transient receptor potential channel 1
VCAM-1:	Vascular cell adhesion molecule-1
WT:	Wild type
XO:	Xanthine oxidase

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CHAPTER ONE

General Introduction and Literature Review

1.1 Introduction

Duchenne muscular dystrophy (DMD) is a muscle wasting disease caused by the absence of the protein dystrophin that results in respiratory and cardiac failure. There are currently no effective therapies for this disease. The *mdx* mouse also lacks the protein dystrophin and has been extensively used to study the skeletal muscle manifestations of DMD.

One hypothesis to explain the DMD phenotype is that dystrophin is involved in regulating sarcolemmal channels and, in its absence, some channels are abnormally regulated; one channel of particular interest is the non-selective cation stretch-activated channel (SAC). SACs respond to mechanical stress with an increase in open probability, resulting in a raised resting intracellular calcium (Ca^{2+}) concentration ($[\text{Ca}^{2+}]_i$). A second hypothesis to account for the DMD phenotype is that loss of dystrophin leads to augmented levels of intracellular reactive oxygen species (ROS). ROS are raised in DMD and *mdx* skeletal muscle, and are also known to have many detrimental effects on cell function. However, the role of SACs and ROS in the development of *mdx* heart failure has not been explored.

It is widely recognised that failing hearts have defective Ca^{2+} handling (Lehnart *et al.*, 1998). However, no studies have investigated Ca^{2+} transients in *mdx* mouse hearts.

This thesis will explore the mechanisms of damage in *mdx* hearts, with a particular focus on changes in Ca^{2+} handling, excitation contraction (EC) coupling and cardiac inflammation. In particular, this thesis will investigate the role of SACs and ROS in the development of defective Ca^{2+} handling and inflammation in the *mdx* myocardium.

1.2 Duchenne Muscular Dystrophy

The term muscular dystrophy was first used by the German neurologist Wilhelm Erb at the turn of the 19th century, although credit for the first unequivocal clinical and pathological depiction of DMD goes to Edward Meryon (Meryon, 1852). However, Meryon's observations were overshadowed by the work of Duchenne (Duchenne, 1868) who gave a comprehensive description of the disease and from whom the disorder takes its name.

DMD is a muscle wasting disease that results in premature death. The disease is due to a mutation in the dystrophin gene, which results in the absence or drastically reduced levels of the protein. The disease is X-linked and is relatively common, affecting 1:3500 male births (Emery, 2002b). The main cause of death is respiratory failure, although cardiac failure accounts for at least 20% of deaths (Finsterer & Stollberger, 2003). The cardiomyopathy associated with DMD will be the focus of this thesis.

1.2.1 Clinical Presentation

DMD is characterised by general muscle weakness and wasting, which starts in the pelvic girdle and progresses towards distal and respiratory muscles. DMD patients are clinically normal at birth although plasma levels of the muscle isoform of creatine kinase are elevated. Symptoms first arise between the ages of 2 and 5 years when delays in motor milestone achievements become apparent (Jennekens *et al.*, 1991). Eventually, decreased lower level muscle strength results in boys becoming wheelchair bound around the age of 12 (Emery & Muntoni, 2003). Most patients die in their early twenties as a result of respiratory failure, due

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to intercostal muscle weakness and respiratory infection. The majority of patients also experience impaired cardiac function and/or cardiac conduction abnormalities, with 90% of patients presenting with subclinical or clinical cardiac involvement. Cardiac failure is the cause of death in 20% of patients (Farah *et al.*, 1980; Finsterer & Stollberger, 2003). Despite the high incidence, many patients remain cardiologically asymptomatic. This has been attributed to the reduced cardiac workload of wheelchair bound boys. However, with improvements in respiratory assistance the boys are surviving longer and it is probable that cardiac involvement will become increasingly common. Thus, detailed characterisation of the cardiac manifestations that occur in DMD are important.

The cardiomyopathy associated with DMD has the features of a dilated cardiomyopathy (DCM). DMD patients present with dilated cardiac cavities, valve abnormalities, decreased fractional shortening and ejection fraction, thrombus formation, myocardial fibrosis and reduced left ventricular systolic function (Finsterer & Stollberger, 2003). Electrocardiogram (ECG) abnormalities in DMD patients are attributed to progressive fibrosis of the cardiac conduction system and impairment of the autonomic nervous system. The most frequently observed cardiac abnormality is sinus tachycardia. It is unclear if diastolic dysfunction occurs (Finsterer & Stollberger, 2003; Mori *et al.*, 2004).

Unfortunately, there are no effective therapies available for DMD patients with DCM. Current treatment options are designed to slow disease progression rather than prevent the underlying pathology. In the absence of large clinical trials focused on DMD patients with DCMs, medical therapy is based on clinical trials incorporating patients from various

aetiologies, including DCMs. Based on the findings of these investigations, current treatments include angiotensin converting enzyme inhibitors, angiotensin II-type 1 receptor inhibitors, and various diuretics. Some patients take corticosteroids, although the potential cardiac benefits are unknown (Wagner *et al.*, 2006).

1.2.2 The protein dystrophin

In 1982 the gene for DMD was located on the short arm of the X chromosome (Xp21). This was the first inherited disorder in which the causative gene was located by linkage to a DNA marker (Emery, 2002a). Three years later, DMD was the first inherited disorder in which the defective protein was identified by positional cloning (Kunkel *et al.*, 1985; Ray *et al.*, 1985); the cloned gene was used to identify the protein, which was labelled 'dystrophin' (Hoffman *et al.*, 1987).

The dystrophin gene is the largest in the human genome, with a genomic sequence spanning 2.5 Mb (Coffey *et al.*, 1992; Monaco *et al.*, 1992), and is composed of 79 exons (Roberts *et al.*, 1993). Full length 14 kb mRNA transcribed from the DMD locus is predominantly expressed in muscle, with smaller amounts in brain. The large size of the dystrophin protein contributes towards its high rate of spontaneous mutation. Most mutations result in the absence or reduced levels of the dystrophin protein (Blake *et al.*, 2002).

The dystrophin protein (Fig. 1.1) is a 427 kDa cytoskeletal protein that is a member of the β -spectrin/ α -actin protein family (Koenig *et al.*, 1988). Dystrophin forms part of the

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dystroglycan complex, which is a group of tightly associated transmembrane and cytoskeletal proteins (Ervasti & Campbell, 1991).

The dystroglycan complex contains dystrophin, sarcoglycans, dystroglycans, dystrobrevins, syntrophins, caveolin 3, and neuronal nitric oxide synthase (nNOS; Lapidos *et al.*, 2004). It is thought that the dystroglycan complex acts as a signalling complex, while also providing a mechanical link from the intracellular cytoskeleton to the extracellular matrix (Lapidos *et al.*, 2004; Rando, 2001).

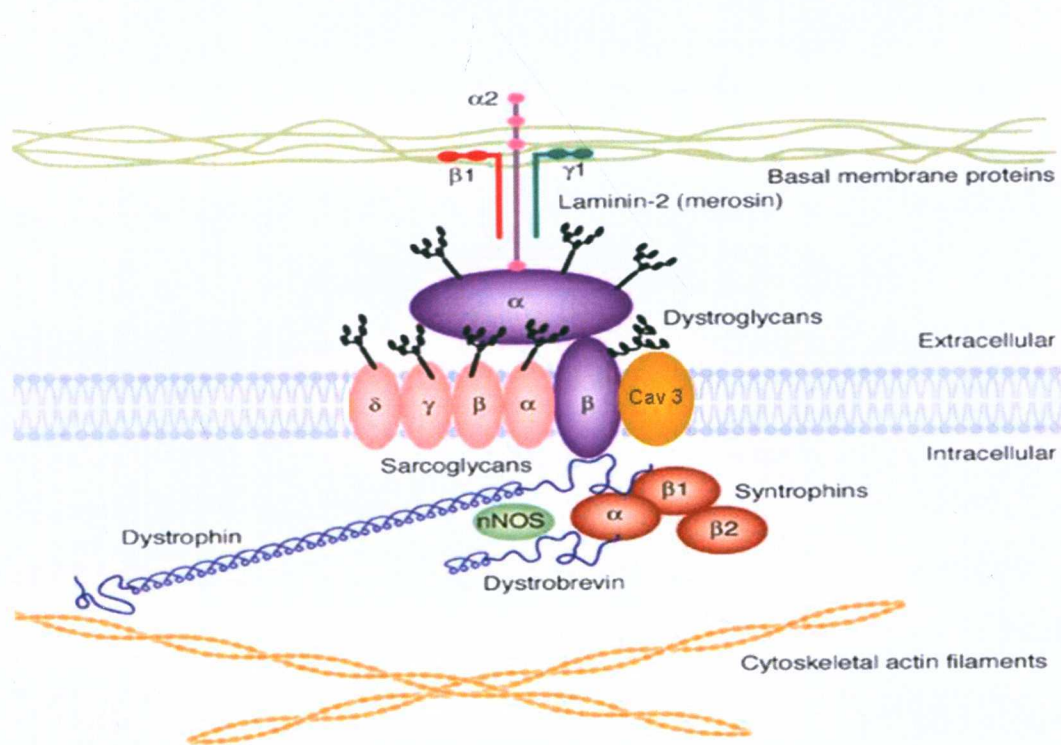


Figure 1.1 Location of dystrophin in relation to the dystroglycan complex

Dystrophin forms a link between the actin cytoskeleton and the extracellular matrix via the dystroglycan complex. The dystroglycan complex also includes neuronal nitric oxide synthase (nNOS), the syntrophins (α , β 1 and β 2), the dystroglycans (α and β), the sarcoglycans (α , β , δ , and γ), caveolin 3 (Cav 3) and dystrobrevin. Adapted from Wells *et al.* (2002).

Dystrophin is found as a continuous uniform layer on the cytoplasmic side of the sarcolemma in cardiac muscle (Byers *et al.*, 1991; Stevenson *et al.*, 1997). Dystrophin is absent from intercalated discs (Byers *et al.*, 1991) but extends from the peripheral plasma membrane along the transverse tubules (T-tubules; Kostin *et al.*, 1998). In contrast to rodent skeletal muscle, cardiac dystrophin does not preferentially associate with costameres (Stevenson *et al.*, 1997), although in human myocytes dystrophin does show concentrated bands at the site of the vinculin-rich costameres (Kaprielian *et al.*, 2000).

1.2.3 The *mdx* mouse model of Duchenne muscular dystrophy

The *mdx* mouse is the most commonly used animal model of DMD. The *mdx* mouse was discovered within a colony of C57Bl/10ScSn mice. In-breeding experiments led to the finding that the trait was X-linked recessive (Bulfield *et al.*, 1984). *Mdx* mice also lack the dystrophin protein due to a premature stop codon (Sicinski *et al.*, 1989).

The *mdx* mouse shares many of the skeletal and cardiac features of patients with DMD (Table 1.1.). Thus, *mdx* mice develop skeletal muscle damage, characterised by elevated total intracellular Ca^{2+} , necrotic foci and newly regenerated centrally nucleated myofibres (Coulton *et al.*, 1988; Gillis, 1999). Similar to DMD patients, the *mdx* cardiac phenotype progresses with age, with 9-12 month old mice displaying signs of heart failure (Quinlan *et al.*, 2004; Wehling-Henricks *et al.*, 2005). *Mdx* mice of 10-12 weeks of age do not show signs of heart failure (Quinlan *et al.*, 2004; Wilding *et al.*, 2005).

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	Human DMD	<i>Mdx</i> mice	Reference
Primary genetic defect	Loss of dystrophin expression	Loss of dystrophin expression	Gillis, 1999; Sicinski <i>et al.</i> , 1989
Severity of clinical phenotype	Severe	Mild	Gillis, 1999

Skeletal muscle

Muscle degeneration	Severe and constant	Initially severe followed by persistent low level degeneration	Coulton <i>et al.</i> , 1988; Jennekens <i>et al.</i> , 1991
Skeletal muscle inflammation	Yes	Yes	Coulton <i>et al.</i> , 1988
Elevated serum creatine kinase	Yes	Yes	Coulton <i>et al.</i> , 1988; Ozawa <i>et al.</i> , 1999
Necrotic foci	Yes	Yes	Coulton <i>et al.</i> , 1988

Cardiac Muscle

Develop dilated cardiomyopathy	Yes	Yes	Bia <i>et al.</i> , 1999; Finsterer & Stollberger, 2003
Decreased fractional shortening	Yes	Yes	Quinlan <i>et al.</i> , 2004; Finsterer & Stollberger, 2003
Electrocardiogram abnormalities	Yes	Yes	Bia <i>et al.</i> , 1999; Finsterer & Stollberger, 2003
Cardiac fibrosis	Yes	Yes	Finsterer & Stollberger, 2003; Wehling-Henricks <i>et al.</i> , 2005
Development of phenotype with age	Yes (rapidly)	Yes (slowly)	Bia <i>et al.</i> , 1999; Finsterer & Stollberger, 2003
Cardiac muscle inflammation	Yes	Yes	Wehling-Henricks <i>et al.</i> , 2005
Reduced life-span	Significant (33% of normal)	Marginal (80% of normal)	Chamberlain <i>et al.</i> , 2007; Eagle <i>et al.</i> , 2002
Cause of death	~ 80% respiratory failure ~ 20% cardiac failure	Unknown	Finsterer & Stollberger, 2003

Table 1.1 Comparison of genetic and clinical differences between DMD patients and *mdx* mice.

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However, even at this early age, *mdx* hearts are more vulnerable to mechanical stress and pressure overload (Danialou *et al.*, 2001; Kamogawa *et al.*, 2001), in addition to having decreased nNOS activity (Bia *et al.*, 1999), changes in autonomic receptor function (Lu & Hoey, 2000b), defective Ca^{2+} handling (Lohan & Ohlendieck, 2004; Woolf *et al.*, 2006) and altered metabolism (Khairallah *et al.*, 2007). Thus, even in young *mdx* mice, myocardial abnormalities are present which may play a role in their subsequent progression to heart failure.

Based on the above findings, *mdx* hearts appear to be a good model in which to study the DCM associated with DMD. However, as with many animal models of disease, the *mdx* mouse does not perfectly mirror the DMD phenotype. The major limitation of the *mdx* cardiac model is the finding that the 22 month lifespan of *mdx* mice is only marginally decreased compared to the 27 months of wild type (WT) mice (Chamberlain *et al.*, 2007). In comparison, the 25 year lifespan of DMD patients (Eagle *et al.*, 2002) is substantially reduced compared to the average human lifespan of approximately 75 years (Bowling, 1993). This suggests that the *mdx* disease phenotype is not as severe as DMD patients.

Another issue regarding the *mdx* mouse is the 9 months required for mice to develop heart failure (Quinlan *et al.*, 2004; Wilding *et al.*, 2005). The slow progression to heart failure results in a large time period before the outcome of therapeutic approaches is known.

1.3 Cardiomyopathy

Cardiomyopathy is a general diagnostic term designating myocardial disease, often of obscure or unknown aetiology. Cardiomyopathies can be classified by functional and morphological features into four categories: DCM, hypertrophic cardiomyopathy, restrictive cardiomyopathy and arrhythmogenic right ventricular cardiomyopathy. Cardiomyopathies can be primary myocardial disorders or develop as a secondary consequence of a variety of specific conditions, including myocardial ischaemia, valvular dysfunction, hypertension, myocarditis, metabolic disorders, systemic diseases, muscular dystrophies, neuromuscular disorders, toxic agents, and late pregnancy (Franz *et al.*, 2001; Richardson *et al.*, 1996). Patients with DMD develop cardiac abnormalities that closely resemble the characteristics of a DCM.

1.4 Dilated cardiomyopathy

The cardiomyopathy associated with DMD closely mirrors that of a DCM. DCMs are characterised by cardiac dilation with increased end-systolic and end-diastolic volumes and a decrease in cardiac output. Poor systolic pressure generation results in a decreased left ventricular ejection fraction causing a self-induced volume overload to take place, accompanied by a marked increase in diastolic wall stress. There is usually some degree of compensatory hypertrophy, inadequate to normalise wall stress (Opie, 2004). DCMs are frequently associated with conduction defects. Histomorphological findings include extensive fibrosis as well as hypertrophic and atrophic myofibres (Franz *et al.*, 2001). The

prevalence of DCMs in the general population is 40 - 50 cases per 100,000 (Codd *et al.*, 1989; Franz *et al.*, 2001).

Clinical presentation of DCMs range from symptomless to heart failure, stroke from thromboembolism, arrhythmias and sudden cardiac death. However, patients more typically present with signs and symptoms of pulmonary congestion and/or low cardiac output, often on a background of exertional symptoms and fatigue for many months or years before their diagnosis (Elliott, 2000).

1.5 Pathophysiological mechanisms in Duchenne muscular dystrophy

The underlying pathology in DMD is progressive muscle damage resulting in a decrease in skeletal and cardiac muscle function. As previously mentioned, DMD is caused by absence of the cytoskeletal protein dystrophin; this raises the question, how does absence of dystrophin lead to the phenotype associated with DMD? Several different mechanisms have been proposed to answer this question. This thesis will explore two of these mechanisms, namely:

1. The raised resting $[Ca^{2+}]_i$ hypothesis
2. The raised intracellular ROS hypothesis

It is worth noting that the pathogenesis of DMD is most likely to be multi-factorial, and that many different mechanisms probably contribute towards disease progression. It is also

important to note that the majority of the research into the underlying disease processes in DMD has been performed on the *mdx* mouse. Most of the research involving the *mdx* mouse has focused on skeletal muscle damage, with considerable less attention spent on cardiac muscle function. As a result, this thesis will frequently refer to observations relating to findings from *mdx* skeletal muscle. In addition, as DMD patients develop a DCM, findings relating to DCMs will also be reviewed.

1.6 Raised resting $[Ca^{2+}]_i$ hypothesis

The role of intracellular Ca^{2+} and muscle damage has been studied as early as 1978, when Duncan (1978) showed that increasing $[Ca^{2+}]_i$ produced muscle damage, as measured by loss of soluble muscle proteins and myofibrillar degeneration. There is good evidence that raised resting $[Ca^{2+}]_i$ plays a role in the pathogenesis of DMD muscle damage, although there is not universal consensus (Gillis, 1999). Furthermore, among those who support the raised resting $[Ca^{2+}]_i$ hypothesis in DMD, dispute exists over the mechanism/s accounting for increased Ca^{2+} influx, and how raised resting $[Ca^{2+}]_i$ promotes muscle damage and loss of function (Fig. 1.2).

No investigators have evaluated total Ca^{2+} or resting $[Ca^{2+}]_i$ in cardiac tissue from DMD patients. However, *mdx* hearts have been shown to have raised total Ca^{2+} (Dunn & Radda, 1991) and resting $[Ca^{2+}]_i$ (Alloatti *et al.*, 1995). In DMD (Oberc & Engel, 1977) and *mdx* skeletal muscle (McArdle *et al.*, 1992; Reeve *et al.*, 1997), it is generally agreed that total Ca^{2+} is increased. However, agreement has not been reached on whether resting $[Ca^{2+}]_i$ is raised in DMD and *mdx* skeletal muscle (for review see Gillis, 1999). Possibilities for the discrepancies between research groups include methodological differences relating to the type of fluorescence indicators used to

measure $[Ca^{2+}]_i$ and the effect of manually dissected versus collagenase dispersed fibres. With regard to collagenase dispersed fibres, De Backer et al. (2002) found that resting $[Ca^{2+}]_i$ homeostasis was tightly maintained in 90% of *mdx* muscle fibres studied; however, the remaining 10% showed evidence of compromised resting $[Ca^{2+}]_i$ homeostasis. The investigators concluded that the findings fit well with the classical histological observations that *mdx* muscle fibres comprise discrete foci of degenerated fibres, surrounded by otherwise healthy looking fibres. The findings also provide a possible explanation as to why many investigators are unable to find evidence for raised $[Ca^{2+}]_i$ in collagenase dispersed *mdx* muscle fibres.

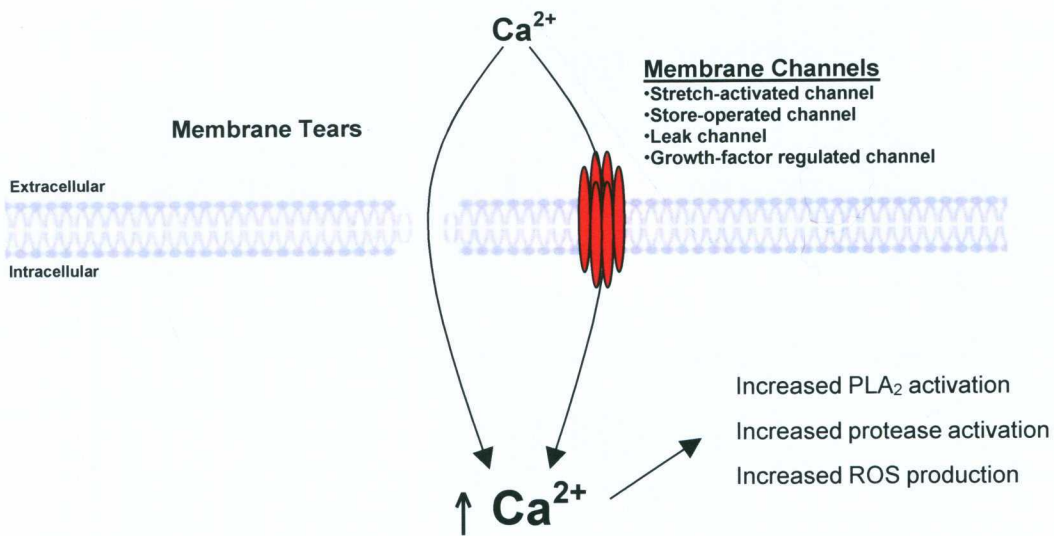


Figure 1.2 Ca^{2+} entry pathways and intracellular effects in DMD/*mdx* muscle

In the absence of dystrophin, DMD and *mdx* muscle has raised resting $[Ca^{2+}]_i$. Potential routes through which Ca^{2+} enters into myocytes include: membrane tears, stretch-activated channels, leak channels, store-operated channels and growth factor-regulated channels. Raised resting $[Ca^{2+}]_i$ may result in myocyte damage by increasing activation of proteases, phospholipase A_2 (PLA_2) or augmenting production of reactive oxygen species (ROS).

Further support for the role of raised resting $[Ca^{2+}]_i$ in the pathogenesis of DMD originates from experiments studying the extraocular muscles of *mdx* mice (Khurana *et al.*, 1995). In both DMD patients and *mdx* mice, extraocular muscles are not affected by necrosis. Furthermore, *mdx* extraocular muscles are more resistant to necrosis induced by pharmacologically elevated resting $[Ca^{2+}]_i$ compared to *mdx* pectoral muscle (Khurana *et al.*, 1995). Investigators concluded that extraocular muscles are spared in DMD due to their ability to better maintain resting $[Ca^{2+}]_i$ homeostasis compared to other striated muscles.

1.6.1 Calcium entry pathways

Two main hypotheses have been proposed to explain why resting $[Ca^{2+}]_i$ is raised in *mdx* muscle (Fig. 1.2). The first suggests that dystrophin stabilises the cell membrane and in its absence membrane tears occur, leading to increased Ca^{2+} entry. The second hypothesis proposes that dystrophin is involved in the aggregation of ion channels involved in Ca^{2+} fluxes across the sarcolemma; in the absence of dystrophin these channels become dysfunctional allowing augmented Ca^{2+} entry. These two hypotheses are explored below.

1.6.2 Membrane tears hypothesis

The membrane tears hypothesis in muscle cell damage pre-dates the discovery of dystrophin (Rowland, 1980) and is supported by several research groups (Clarke *et al.*, 1993; Petrof *et al.*, 1993; Porter *et al.*, 1992). The membrane tears hypothesis proposes that dystrophin provides structural integrity to the muscle cell membrane. In the absence of dystrophin the sarcolemma is thought to be prone to damage from mechanical stress, resulting in contraction and stretch-induced transient membrane tears. It is believed that

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membrane tears would allow rapid equilibrium of ions between the intracellular and extracellular spaces. The large Ca^{2+} gradient that exists in myocytes between the extracellular and intracellular environments would result in increased Ca^{2+} influx. In addition, membrane tears would allow the loss of soluble intracellular proteins to the extracellular milieu, such as creatine kinase.

Most of the evidence in support of the membrane tears hypothesis originates from experiments in which *mdx* skeletal muscles are subjected to eccentric contractions. Eccentric muscle contractions, or stretched contractions as they are also known, occur when muscles are stretched during a contraction. In both humans and animals, eccentric muscle contractions causes immediate muscle weakness, followed by soreness, swelling and stiffness that normally becomes apparent 1-2 days after the contractions (Proske & Morgan, 2001). Of particular interest to the study of DMD is the finding that *mdx* muscles are more prone to eccentric muscle contraction-induced damage (Head *et al.*, 1992; Petrof *et al.*, 1993).

When *mdx* and WT skeletal muscles were subjected to eccentric muscle contractions, investigators found that *mdx* skeletal muscle fibres take up membrane impermeant dye and/or release muscle-specific proteins (Moens *et al.*, 1993; Vilquin *et al.*, 1998). Investigators concluded that eccentric muscle contractions had produced membrane tears in muscle fibres accounting for the uncontrolled flux of ions and proteins. Recent experiments using cardiac muscle from *mdx* mice has been presented in support of the membrane tears hypothesis. Yasuda *et al.* (2005) attached microcarbon fibres to isolated *mdx*

cardiomyocytes, allowing investigators to measure force production while controlling myocyte length. It was shown that *mdx* cardiomyocytes were more susceptible to stretch-induced Ca^{2+} overload and that application of the membrane sealant poloxamer 188 reduced the defect. In addition, *in vivo* administration of poloxamer 188 improved acute heart failure in *mdx* mice. Investigators concluded the poloxamer improved *mdx* muscle function via its ability to stabilise the cardiomyocyte cell membrane, thus preventing membrane tears. The above findings provide circumstantial evidence in support of the membrane tears hypothesis. However, little direct evidence supports the concept that *mdx* muscle membranes are more susceptible to tears. Furthermore, there is data which argues against the membrane tears hypothesis.

The sarcolemma of *mdx* skeletal muscle is more elastic than non-dystrophic membranes implying that the *mdx* sarcolemma would have more protection against stretch-induced tears (Pasternak *et al.*, 1995). The tensile strength (Franco, Jr. & Lansman, 1990; Hutter *et al.*, 1991) and pressure required to rupture the sarcolemma of *mdx* and WT myotubes is similar (Franco, Jr. & Lansman, 1990). These findings suggest that the surface membrane of *mdx* myocytes is not more likely to tear than WT.

The membrane tears theory predicts that local ruptures in the membrane would lead to localised increases in ion influx. However, imaging studies in WT skeletal muscle failed to detect areas of increased ion concentration following eccentric muscle contractions (Balnave *et al.*, 1997; Yeung *et al.*, 2003a). McArdle's group have demonstrated that isolated *mdx* muscle does not have increased susceptibility to damage caused by either

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isometric or eccentric muscle contractions (McArdle *et al.*, 1991; McArdle *et al.*, 1992). They suggest that augmented permeability is not the primary pathogenic process, as pre-necrotic *mdx* mice (14 days old) have normal levels of creatine kinase and no increased permeability to procion orange (McArdle *et al.*, 1994).

Studies demonstrating that eccentric muscle contractions lead to increased membrane permeability to fluorescent dyes do not necessarily support the hypothesis that fluorescent dye entry is via membrane tears. It is possible that following stretch, intracellular pathways are activated that lead to increased membrane permeability. In support of this hypothesis, Whitehead *et al.* (2006) found that following eccentric muscle contractions the intracellular concentration of the membrane impermeant dye procion orange continued to increase for up to an hour after stretch. It was concluded that if dye uptake was the result of membranes tears it should have occurred rapidly at the time of the stretch, and would not be expected to occur progressively over time. Furthermore, dye uptake was reduced by the SAC blockers streptomycin and GsMTx-4, suggesting that stretch resulted in increased SAC opening and the activation of Ca^{2+} -dependent damage pathways which then lead to increased membrane permeability (Whitehead *et al.*, 2006).

Finally, although poloxamer 188 prevented stretch-induced increases in resting $[\text{Ca}^{2+}]_i$ (Yasuda *et al.*, 2005), conclusive evidence was not presented to support the premise that the poloxamer was having its effect by preventing membrane tears. Thus, it is possible that poloxamer 188 improved function by reducing the activity of stretch-activated channels.

Furthermore, recent work by Quinlan et al. (2006) found that poloxamer 188 failed to prevent exercise-induced membrane breakdown in *mdx* skeletal muscle fibres.

1.6.3 Membrane channel aggregation hypothesis

The membrane aggregation theory suggests that dystrophin is involved in the aggregation and regulation of membrane channels (Fig. 1.2). Of particular interest are those channels involved in regulating Ca^{2+} ion movements across the sarcolemma. It is hypothesised that in the absence of dystrophin these channels allow increased or un-controlled Ca^{2+} entry into myocytes. In the following section the following four candidate ion channels will be reviewed: 1) Mechanosensitive channels; 2) Leak channels; 3) Store-operated channels; 4) Growth factor-regulated channels.

1.6.3.1 Mechanosensitive channels

Mechanosensitive channels, which include stretch-inactivated and stretch-activated channels, are a group of channels that respond to mechanical stress, one class of which are non-selective cation channels (SACs). SACs were first discovered in tissue-cultured chick pectoral muscle in 1984 by Guharay & Sachs (1984). SACs role in normal cardiomyocyte physiology include the response to cell volume changes and the response to increased myocardial mechanical strain/stress (Kohl *et al.*, 2005). For many years, the molecular identity of the SAC was unknown; however, recent work by Maroto et al. (2005) suggests that the canonical transient receptor potential 1 (TRPC1) protein forms the mammalian SAC. TRPC channels are a family of transmembrane ion channels, many of which are permeable to Ca^{2+} (Vennekens *et al.*, 2002). The potential role of other TRPC channels as

SACs is still unknown, but it is possible that SACs are composed of heterodimers of the different TRPC channel isoforms (Spasova *et al.*, 2006).

1.6.3.2 SACs and DMD skeletal muscle: Electrophysiological evidence

The first electrophysiological evidence implicating mechanosensitive channels in the pathogenesis of DMD were performed by Franco & Lansman (1990). Patch-clamp investigations identified increased frequency of a Ca^{2+} -permeable mechanosensitive channel in *mdx* myotubes compared to WT. It was hypothesised that in WT mice, dystrophin binds to the channels providing a counterforce to the resting membrane tension and that in muscle lacking dystrophin, the resting tension may be sufficient to keep some of these channels open. Although these early findings gave weight to the importance of stretch-inactivated channels, later work by the same group found that the distinction between stretch-inactivated and stretch-activated channels is not as clear as originally envisaged, with channels having the ability to switch between the two gating types (for review see Lansman & Franco-Obregon, 2006). In addition, although electrophysiological experiments play an important role in identifying the presence of mechanosensitive channels, the actual physical deformations that occur in the sarcolemma upon suction/pressure application is complex (Suchyna & Sachs, 2007). However, Franco and Lansmann's experiments introduced the idea that the properties of mechanosensitive channels are abnormal in *mdx* skeletal muscle compared to WT. Supporting this hypothesis are recent electrophysiological findings demonstrating that the occurrence and open probability of mechanosensitive channels is greater in *mdx* skeletal muscle (De Backer *et al.*, 2002; Vandebrouck *et al.*, 2002b). Furthermore, electrophysiological experiments in

DMD myotubes have shown increased activity of a Ca^{2+} permeant cationic mechanosensitive channel. (Vandebrouck *et al.*, 2001; Vandebrouck *et al.*, 2002a). Thus, good evidence exists to suggest that mechanosensitive channels, and in particular SACs, have abnormal properties in *mdx* and DMD skeletal muscle. It is therefore possible that increased Ca^{2+} entry via these channels accounts for the raised resting $[\text{Ca}^{2+}]_i$ found in DMD and *mdx* skeletal muscle.

1.6.3.3 SACs and DMD skeletal muscle: Physiological evidence

Patch-clamp experiments play an important role in identifying membrane channel abnormalities. However, this technique is often un-physiological, as patch-clamping can induce membrane deformities that do not occur under normal conditions (Suchyna & Sachs, 2007). Thus, it can be argued that physiological experiments which explore the response of cardiac/skeletal muscle to stretch better represent the *in vivo* scenario. In the next section findings from physiological investigations relating to the role of SACs in the pathology of DMD and *mdx* skeletal muscle damage will be presented.

A significant component of the force reduction following eccentric muscle contractions in *mdx* skeletal muscle fibres can be reduced by the SAC inhibitors gadolinium and streptomycin (Yeung *et al.*, 2003b). Furthermore, the rise in resting $[\text{Ca}^{2+}]_i$ in *mdx* skeletal muscle following eccentric muscle contractions can be blocked by the SAC inhibitors, streptomycin and GsMTx-4 (Yeung *et al.*, 2005). GsMTx-4 is found in tarantula toxin and is the most potent and specific blocker of SACs known to date (Suchyna *et al.*, 2000). It

was concluded that SAC blockers prevented Ca^{2+} influx and thereby reduced Ca^{2+} -induced muscle damage.

Tutdibi et al. (1999) performed experiments in which isolated *mdx* and WT skeletal muscles were loaded with the Ca^{2+} -sensitive dye, fura-2. Manganese (Mn^{2+}) was added to the bath solution and the rate of fura-2 quenching recorded. Extracellular Mn^{2+} enters cells via the same routes as Ca^{2+} . Thus the rate of fura-2 quenching by Mn^{2+} can be used to estimate membrane Ca^{2+} permeability. Investigators found that the fura-2 quench rate was twice as high in *mdx* muscles, and that fura-2 quenching could be prevented by the SAC inhibitor, gadolinium. Furthermore, nifedipine, which blocks the dihydropyridine receptor (DHPR) and Ca^{2+} leak channels (Hopf *et al.*, 1996) had no effect on quench rates. It was concluded that mechanosensitive, rather than Ca^{2+} leak channels, were responsible for the increased Ca^{2+} influx in *mdx* muscles.

Finally, Kumar et al. (2004) found that in response to stretch, *mdx* mice had increased activation of extracellular signal-regulated kinase 1/2 which could be prevented by removal of extracellular Ca^{2+} and by pre-treatment with the SAC inhibitor gadolinium.

1.6.3.4 SACs and cardiac muscle: Electrophysiological evidence

The role of SACs in DMD and *mdx* cardiac muscle has not been investigated. However, it has long been known that the electrophysiological properties of cardiomyocytes are sensitive to stretch, a process referred to as mechanoelectric feedback (Lab, 1996). SACs are present in embryonic ventricular myocytes (for review see Kohl *et al.*, 2005) and at least one report has

presented single-channel recordings from a SAC in neonatal ventricular myocytes (Craelius *et al.*, 1988). However, no investigators have reported single-channel recordings from SACs in adult ventricular myocytes, although stretch-induced whole cell currents have been recorded (Kamkin *et al.*, 2000; Zeng *et al.*, 2000). Explanations for the inability to obtain single-channel recordings from SACs in adult ventricular myocytes include the possibility that channels become inactivated by the mechanics of patch formation (Franco-Obregon, Jr. & Lansman, 1994). Alternatively, SACs may be located on internal membranes, such as the T-tubules. Neonatal cell T-tubules are not fully developed, explaining why single-channel recordings of SACs might be possible from neonatal ventricular myocytes (Craelius *et al.*, 1988).

1.6.3.5 SACs and cardiac muscle: Physiological evidence

Strong physiological evidence exists to support the presence of SACs in cardiac muscle. Stretching isolated adult cardiomyocytes induces a rise in resting $[Ca^{2+}]_i$ and an inward current which can be blocked by the SAC inhibitor streptomycin (Gannier *et al.*, 1994) or by removal of extracellular Ca^{2+} (Belus & White, 2003; Gannier *et al.*, 1996). Similar experiments have demonstrated the presence of a gadolinium-sensitive stretch-induced rise in resting $[Ca^{2+}]_i$ in neonatal cardiomyocytes (Ruwhof *et al.*, 2001; Tatsukawa *et al.*, 1997), and of a swelling-induced, gadolinium-sensitive cation current in adult cardiomyocytes (Clemo & Baumgarten, 1997). SACs have also been implicated in several cardiac pathologies. Gadolinium and GsMTx-4 both block a swell-induced current in the rapid-pacing canine (Clemo *et al.*, 1998; Clemo *et al.*, 1999) and aortic regurgitation-induced rabbit (Suchyna *et al.*, 2000) models of DCM. Furthermore, Nicolosi *et al.* (2004) have shown that streptomycin and gadolinium restore contractile function in papillary muscles from a hamster model of DCM.

In addition to DCMs, SACs have also been implicated in the pathology of cardiac arrhythmias (Bode *et al.*, 2000; Eckardt *et al.*, 2001), ventricular fibrillation secondary to non-penetrating chest wall impacts (Garan *et al.*, 2005) and cardiac ischemia (Takano & Glantz, 1995). Thus, it is clear that SACs play an important role in both the normal physiology and pathophysiology of the myocardium.

1.6.3.6 Calcium leak channels

Fong *et al.* (1990) were the first to report that the open probability of a Ca^{2+} -specific leak channel was greater in DMD and *mdx* skeletal muscle. Leak channels appeared within a few μm of artificially induced membrane ruptures, (McCarter & Steinhardt, 2000), contractile activity increased the activity of the channel (Turner *et al.*, 1993) and their appearance was inhibited by the calpain inhibitor, leupeptin (McCarter & Steinhardt, 2000). Interestingly, the Ca^{2+} leak channel has been shown to behave as a store-operated channel (Hopf *et al.*, 1996; Pozzan *et al.*, 1994), and its activity is enhanced by the DHPR blocker nifedipine but blocked by the nifedipine analogue AN 1043 (Alderton & Steinhardt, 2000a). Activation of Ca^{2+} -dependent proteases is thought to be essential for leak channel activation. Thus, it was hypothesised that membrane tears lead to the insertion of Ca^{2+} leak channels into the sarcolemma. However, as little direct evidence supports the membrane tears hypothesis, the source of Ca^{2+} required to increase leak channel activity probably comes from an alternative Ca^{2+} entry route.

1.6.3.7 Store-operated calcium channels

Depletion of intracellular Ca^{2+} stores triggers Ca^{2+} entry in a process known as store-operated Ca^{2+} entry. The mechanism linking intracellular stores to the sarcolemma is still not fully understood. However, it is believed that Ca^{2+} enters cells through a class of channels known as store-operated channels. Several groups have found increased store-operated channel activity in *mdx* skeletal muscle (Balghi *et al.*, 2006; Boittin *et al.*, 2006; Vandebrouck *et al.*, 2002b). Store-operated Ca^{2+} entry appears to be under the control of Ca^{2+} -independent phospholipase A_2 , as Ca^{2+} influx through store-operated channels can be attenuated by inhibitors of this enzyme (Boittin *et al.*, 2006). Increased store-operated Ca^{2+} entry required the presence of TRPC1 and TRPC4 protein expression in skeletal muscle cells (Vandebrouck *et al.*, 2002b), raising the possibility that SAC and store-operated channel activity arise from the same channels, which are encoded for by a combination of different TRPC proteins.

1.6.3.8 Growth factor-regulated calcium channels

Growth factor-regulated channels (GFRC) belong to the TRP family and were originally identified as Ca^{2+} -permeable cation channels that are activated by IGF-1 and other growth factors (Kanzaki *et al.*, 1997; Kanzaki *et al.*, 1999). Iwata *et al.* (2003) found that GFRC expression was elevated in cardiac and skeletal muscle of the BIO14.6 hamster and skeletal muscle of *mdx* mice. The GFRC was activated in response to stretch, was blocked by gadolinium, and was responsible for increased Ca^{2+} influx and creatine phosphokinase efflux. Recent work by Rolland *et al.* (2006) provide further support for the involvement of GFRCs in the *mdx* pathology. However, the importance of this mechanism in the DMD pathology is still unclear.

1.7 Raised resting $[Ca^{2+}]_i$ damage pathways

In the previous section, evidence was presented in support of the idea that augmented Ca^{2+} entry through membrane channels results in the increased resting $[Ca^{2+}]_i$ found in DMD and *mdx* muscle. Furthermore, alternative Ca^{2+} entry routes have been discussed. The following section will explore the mechanism by which raised resting $[Ca^{2+}]_i$ results in myocyte dysfunction.

1.7.1 Activation of proteases

In 1988 Turner et al. (1988) demonstrated that *mdx* muscle fibres had increased Ca^{2+} -dependent protein degradation; this is now thought to be due to activation of an enzyme known as calpain (Goll *et al.*, 2003; Zaidi & Narahara, 1989). Calpains are Ca^{2+} -dependent cysteine proteases. Two ubiquitous forms of calpain have been identified, μ - and m-calpain. The Ca^{2+} concentration required for half-maximal activation of μ and m-calpains are 2-75 μ M and 200-1000 μ M, respectively (Friedrich, 2004). Although these Ca^{2+} concentrations are not usually found in cardiomyocytes, it is possible that lower concentrations of Ca^{2+} result in partial activation of calpains, or that resting $[Ca^{2+}]_i$ reaches high enough concentrations within specific intracellular compartments (van der Laarse, 2002). Support for this idea has recently been presented by Gailly et al. (2007) who measured calpain activity in resting skeletal muscle which had a $[Ca^{2+}]_i$ range of 20-80 nM, suggesting that calpains can be activated at low $[Ca^{2+}]_i$. Calpain activity has been shown to be increased in both DMD (Kar & Pearson, 1976; Kumamoto *et al.*, 2000) and *mdx* skeletal muscle (Bonuccelli *et al.*, 2003; Combaret *et al.*, 1996; Spencer *et al.*, 1995). Intramuscular injection of the calpain inhibitor leupeptin, or

overexpression of the endogenous calpain inhibitor, calpastatin, attenuated muscle degeneration and necrosis in *mdx* mice (Badalamente & Stracher, 2000; Spencer & Mellgren, 2002). Interestingly, mechanical stress induced a large increase in $[Ca^{2+}]_i$ and calpain activity in *mdx* compared to WT skeletal muscle (Gailly *et al.*, 2007); this effect was suppressed by GsMTx-4, suggesting a role for SACs in the stress-induced increase in enzyme activity.

No studies have explored Ca^{2+} -activated calpains in DMD or *mdx* hearts. However, in other cardiac pathologies there is evidence that Ca^{2+} -activated calpains contribute towards disease progression. In cardiac ischemia it has been suggested that raised $[Ca^{2+}]_i$ results in Ca^{2+} -activated calpains degrading troponin I (TnI; Gao *et al.*, 1997), which produces contractile dysfunction due to decreased maximal Ca^{2+} -activated force and decreased Ca^{2+} -sensitivity (Carrozza, Jr. *et al.*, 1992; Kusuoka *et al.*, 1987; Papp *et al.*, 2000). In support of this theory, overexpression of calpastatin, an endogenous inhibitor of calpain, prevents TnI degradation and contractile dysfunction (Maekawa *et al.*, 2003). There is evidence that calpain-induced degradation of TnI occurs in heart failure, as TnI proteolysis products are present in the blood of heart failure patients (Missov *et al.*, 1997). Although increased calpain expression has been shown to occur in an animal model of DCM (Kawada *et al.*, 2005), calpain activity *per se* has yet to be measured in heart failure.

1.7.2 Activation of phospholipase A_2

DMD patients have increased activity of phospholipase A_2 (PLA₂) (Lindahl *et al.*, 1995; Tagesson & Henriksson, 1984). PLA₂ is an enzyme that catalyzes the hydrolysis of fatty acid ester bonds, leading to the formation of various inflammatory mediators (Chakraborti, 2003).

Thus, increased PLA₂ activation maybe involved in the DMD inflammatory response. PLA₂ has been shown to be activated by Ca²⁺, raising the possibility that the raised [Ca²⁺]_i found in DMD/*mdx* muscle leads to augmented activation of the enzyme (Jackson *et al.*, 1984; Qiu *et al.*, 1998). Interestingly, in DMD patients the highest levels of PLA₂ activity precede the phase of disease in which muscle function deteriorates, implying that increased PLA₂ activity maybe a primary damage pathway in DMD (Lindahl *et al.*, 1995). Unfortunately, the role of PLA₂ in the pathogenesis of DMD is understudied, leaving many questions unanswered, such as, is PLA₂ activity increased in *mdx* muscle? If so, does inhibiting PLA₂ improve the *mdx* muscle phenotype?

1.7.3 Increased levels of intracellular reactive oxygen species

Another possible consequence of raised resting [Ca²⁺]_i is an increase in the production of ROS. This topic will be covered in detail below.

1.8 Reactive oxygen species

It is becoming increasingly clear that reactive oxygen species (ROS) mediated oxidative damage plays an important role in the pathogenesis of DMD skeletal muscle damage. Furthermore, a plethora of studies have implicated oxidative damage in the pathogenesis of DCMs. However, there is little specific evidence for the role of ROS in the heart failure associated with DMD. The following section will review the literature surrounding the role of ROS in DMD and *mdx* skeletal and cardiac muscle damage.

ROS are important oxygen metabolites formed during oxidative phosphorylation and other electron transfer reactions. ROS are oxygen molecules in different states of reduction or oxidation, as well as compounds of oxygen with hydrogen and nitrogen (Halliwell & Gutteridge, 1998). ROS are of interest as they are reactive and can thus be deleterious to cell function due to oxidative protein damage (Commoner *et al.*, 1954). ROS can induce disulphide bridge formation, oxidise proteins and cleave polypeptide chains (Giustarini *et al.*, 2004). Oxidative damage occurs when there is an imbalance between ROS production and the antioxidant capacity of cells.

1.8.1 ROS production and metabolism

ROS are produced in most cells of the body, including cardiac muscle. During normal cellular metabolism, the primary source of ROS is leakage of superoxide from the mitochondrial electron transport chain (Fig. 1.3). However, superoxide can also be produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, NOS, and xanthine oxidase (XO; Hool, 2006).

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Superoxide, *per se*, is thought to be the starting point for biological ROS. Superoxide can be converted to additional ROS which have both physiological and pathophysiological roles. Superoxide reacts with nitric oxide to form peroxynitrite (Fig. 1.3). Alternatively, superoxide can be dismutated by superoxide dismutase (SOD) to hydrogen peroxide. There are two forms of SOD, cytoplasmic Cu,Zn-SOD and mitochondrial MnSOD. Hydrogen peroxide can be dehydrated to water by either glutathione peroxidase or catalase. Hydrogen peroxide can also be converted into hydroxyl radicals via Fenton chemistry (Halliwell & Gutteridge, 1998).

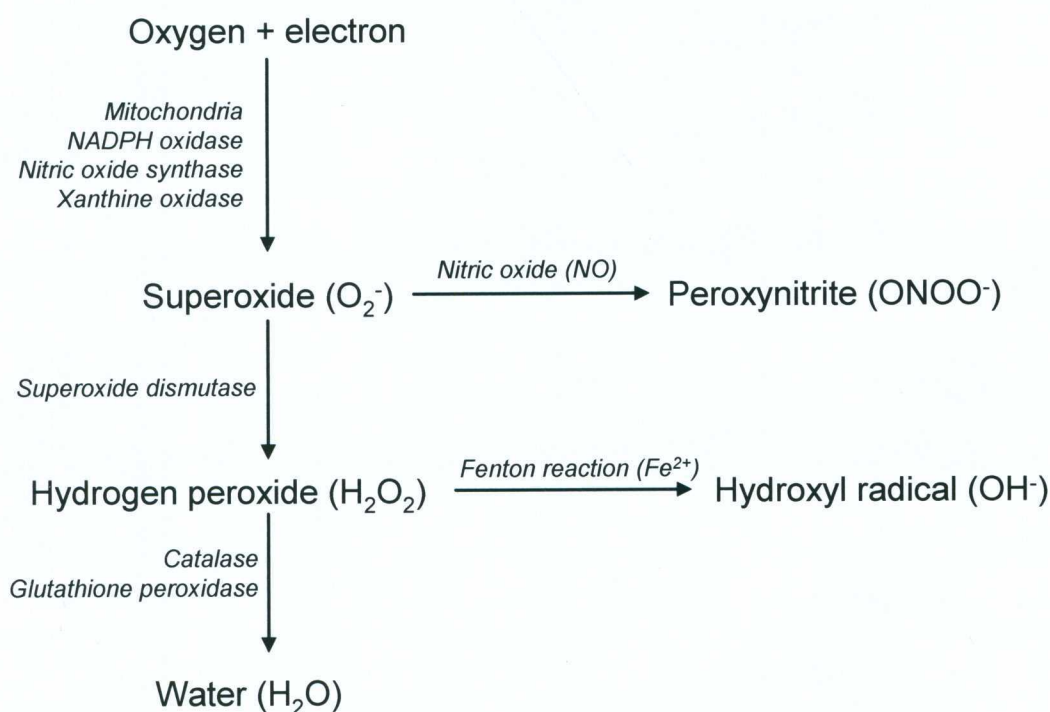


Figure 1.3 Sources and metabolism of reactive oxygen species

1.8.2 Normal physiology of ROS

Although ROS are often considered deleterious to cell function they play an important role in regulating the redox state of proteins, and are thus important physiological molecules. ROS are able to interact with, and alter, key signalling molecules, such as second messengers, tyrosine and mitogen activated protein kinases, nuclear factor kappaB (NF- κ B), and AP-1 (for review see Valko *et al.*, 2007). ROS are produced by inflammatory cells in order to defend against micororganisms (Hool, 2006). However, as discussed below, altered ROS levels can induce many pathological changes.

1.8.3 Pathophysiology of ROS

1.8.3.1 ROS and DMD/*mdx* skeletal muscle damage

A growing body of evidence supports the hypothesis that ROS are a pathological mediator in DMD skeletal muscle damage. Oxidative stress results in skeletal muscle pathologies that resemble dystrophic changes (for review see Rando, 2002). ROS are increased in DMD skeletal muscle, as shown by increased lipid peroxidation (a marker of oxidative stress; Mechler *et al.*, 1984), increased nitrotyrosine levels (a by-product of peroxynitrite; Buchwalow *et al.*, 2006), increased oxidative protein damage (Haycock *et al.*, 1996) and changes in antioxidant enzymes (Austin *et al.*, 1992; Kar & Pearson, 1979). *Mdx* skeletal muscle has elevated ROS production (Hauser *et al.*, 1995; Lafoux *et al.*, 2006; Rando *et al.*, 1998), altered antioxidant capacity (Faist *et al.*, 1998; Ragusa *et al.*, 1996; Rando *et al.*, 1998), and increased susceptibility to oxidative stress induced death (Disatnik *et al.*, 2000; Rando *et al.*, 1998). Interestingly, ROS have been implicated in skeletal muscle changes

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prior to the onset of the degenerative process, suggesting that increased oxidative stress may be an early event in the development of the *mdx* skeletal muscle pathology (Disatnik *et al.*, 1998).

Studying ROS levels in muscles that are minimally affected or unaffected in DMD has provided further support for the involvement of ROS in the underlying pathology. Thus, a correlation exists between the antioxidant capacity of cells and the degree of damage they experience. For example, in DMD patients (Wang *et al.*, 1999) and *mdx* mice (Carnwath & Shotton, 1987), type II fibres have a greater susceptibility to damage and a lower antioxidant capacity than type I fibres (Asayama *et al.*, 1986; Austin *et al.*, 1988; Salminen & Vihko, 1983). Furthermore, the extraocular muscles are spared in DMD patients (Porter, 1998) and have a high antioxidant capacity (Ragusa *et al.*, 1996).

The pathological role of ROS in DMD is further supported by findings that *in vivo* antioxidant treatments provide beneficial effects. *Mdx* muscle damage is reduced following treatment with antioxidants (Bornman *et al.*, 1998; Buetler *et al.*, 2002; Hnia *et al.*, 2007). Unfortunately, clinical trials of antioxidants in DMD patients have shown no clear benefits (Fenichel *et al.*, 1988; Stern *et al.*, 1982). However, these trials have been limited in size and duration. Furthermore, the studies were mainly performed on patients who had already undergone severe muscle degeneration which may limit the effectiveness of antioxidant treatment. Thus, the negative results do not necessarily refute the oxidative stress hypothesis of DMD muscle damage.

1.8.3.2 Why are ROS increased in DMD/*mdx* skeletal muscle?

Increased ROS in DMD patients and *mdx* mice may occur secondary to raised resting $[Ca^{2+}]_i$. An increase in resting $[Ca^{2+}]_i$ could result in mitochondrial Ca^{2+} overload leading to augmented mitochondrial ROS production (Brookes *et al.*, 2004; Nethery *et al.*, 2000); this hypothesis is supported by the observation that mitochondrial function is impaired in *mdx* skeletal muscle (Kuznetsov *et al.*, 1998). However, no direct evidence has demonstrated that mitochondrial ROS production is increased in DMD or *mdx* skeletal muscle. An increase in resting $[Ca^{2+}]_i$ could also lead to increased XO activity (Baker & Austin, 1989) or increased NADPH oxidase activity (Meier, 1996); however, no direct evidence exists to support that these sources of ROS are raised in DMD and *mdx* skeletal muscle. A second possible cause of increased ROS in DMD and *mdx* skeletal muscle relates to the altered antioxidant capacity of muscles. However, it is unclear whether changes in antioxidant capacity are primary events or occur secondary to increased ROS production. It is also unknown why absence of dystrophin leads to changes in endogenous antioxidant activity. One possibility is that signalling molecules within the dystroglycan complex may be involved in antioxidant enzyme expression; in DMD these signalling pathways may become abnormal leading to changes in antioxidant enzyme expression (Rando, 2002).

1.8.3.3 ROS and dilated cardiomyopathies

The above section reviewed evidence supporting a role for ROS in the pathology of DMD skeletal muscle damage. No studies have explored the involvement of ROS in relation to the DCM that occurs in DMD. However, an extensive literature surrounds the role of ROS in other forms of DCMs. This literature will be the focus of the following section.

Electroparamagnetic resonance studies have demonstrated that ROS formation is increased in explanted human hearts with DCM (Sam *et al.*, 2005) in addition to alterations in antioxidative enzyme levels (Baumer *et al.*, 2000; Dieterich *et al.*, 2000; Sam *et al.*, 2005). Furthermore, lipid peroxidation levels in patients correlate with their clinical class of heart failure (Keith *et al.*, 1998).

ROS are also elevated in animal models of DCM, with increased superoxide production (Ide *et al.*, 2000) increased lipid peroxidation (Srivastava *et al.*, 2002) and alterations in antioxidant levels (Mailer *et al.*, 1991) all reported. Transgenic mice deficient in the endogenous antioxidant MnSOD develop a DCM that is reversed by administration of an exogenous SOD (Nojiri *et al.*, 2006). Finally, as proof that increased ROS can promote myocardial dysfunction, artificially raising ROS in cardiac muscle reduces active muscle tension (Blaustein *et al.*, 1986) and causes apoptosis (Cheng *et al.*, 1995; Siwik *et al.*, 1999).

Trials with antioxidant treatments also suggest that ROS are involved in DCMs. Clinical trials with the selective XO inhibitor, allopurinol, improved ventricular efficiency (Cappola *et al.*, 2001). At present a large clinical trial is underway to evaluate the XO inhibitor oxypurinol in patients with heart failure (Freudenberger *et al.*, 2004). In animal models of DCM, *in vivo* treatments aimed at reducing ROS production attenuated cardiac damage, increased cardiac function and prevented myofibrillar oxidative damage (Duncan *et al.*, 2005; Minhas *et al.*, 2006).

1.8.4 Sources of increased ROS in dilated cardiomyopathies

The cellular sources and underlying mechanisms for increased ROS levels in DCMs are not well understood. There are several potential sources of ROS production in the myocardium, including NADPH oxidase, XO, mitochondria, NOS, decreased antioxidant capacity, as well as endothelial cells and phagocytes.

1.8.5 NADPH Oxidase

NADPH oxidase activity and its subunit expression have been shown to be increased in human DCMs (Heymes *et al.*, 2003; Maack *et al.*, 2003). NADPH oxidase is also activated by stretch (Hishikawa & Luscher, 1997), and requires intact caveolae to function (Yang & Rizzo, 2007). As abnormal responses to stretch (Yasuda *et al.*, 2005) and an increase in caveolae are known to occur in DMD (Repetto *et al.*, 1999). Thus, NADPH oxidase appears to be an attractive potential source of ROS in the DCM that occurs in DMD.

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Although early work focused on the neutrophil NADPH oxidase, it is now clear that NADPH oxidase plays an important role in both the physiology and pathophysiology of non-phagocytic cells, including cells of the cardiovascular system. In the following section, where possible, reference will be made to cardiomyocytes; however, where information is lacking reference will be made to findings originating from work relating to neutrophils and the vasculature.

NADPH oxidases are intracellular membrane-associated multimeric enzymes that catalyze the 1-electron reduction of oxygen using NADH or NADPH as the electron donor:



Cardiac NADPH oxidase ROS production is mainly intracellular, as opposed to neutrophil ROS production which is mostly extracellular, correlating with the host defence function of phagocytic cells (Griendling *et al.*, 1994; Griendling *et al.*, 2000; Zafari *et al.*, 1998). Additional differences between neutrophil and cardiovascular NADPH oxidases include the finding that the capacity of the vascular enzyme is only a third of neutrophils (Griendling & Ushio-Fukai, 1998) and has constitutive activity which is absent in neutrophils (Pagano *et al.*, 1997).

1.8.5.1 NADPH oxidase structure and expression

The phagocytic NADPH oxidase contains a membrane-associated flavocytochrome composed of one 22 kDa p22^{phox} (for phagocyte oxidase) subunit and one gp91^{phox} subunit with a molecular weight of 65 kDa (Fig. 1.4). Although the flavocytochrome contains all the catalytic machinery necessary for superoxide production, activation requires the translocation of several cytosolic regulatory subunits p40^{phox}, p47^{phox}, p67^{phox} and the small G protein, rac1 (Quinn *et al.*, 1993) or rac2 (Cave *et al.*, 2006).

Early studies found that although NADPH oxidase activity was present in the myocardium, gp91^{phox} protein expression was not. These observations led to the discovery of a new family of gp91^{phox} homologues, termed Noxs (for NADPH oxidase), with gp91^{phox} known as Nox2 in the new terminology (Cave *et al.*, 2006). Currently 5 Noxs have been described (Murdoch *et al.*, 2006). Both Nox2 and Nox4 have been shown to be present in the myocardium (Bendall *et al.*, 2002; Byrne *et al.*, 2003) and in isolated cardiomyocytes (Li *et al.*, 2002; Peng *et al.*, 2005). The membrane-bound p22^{phox} subunit and the cytosolic regulatory subunits, p40^{phox}, p47^{phox}, p67^{phox} and rac1 are present in cardiac muscle (Heymes *et al.*, 2003; Li *et al.*, 2002).

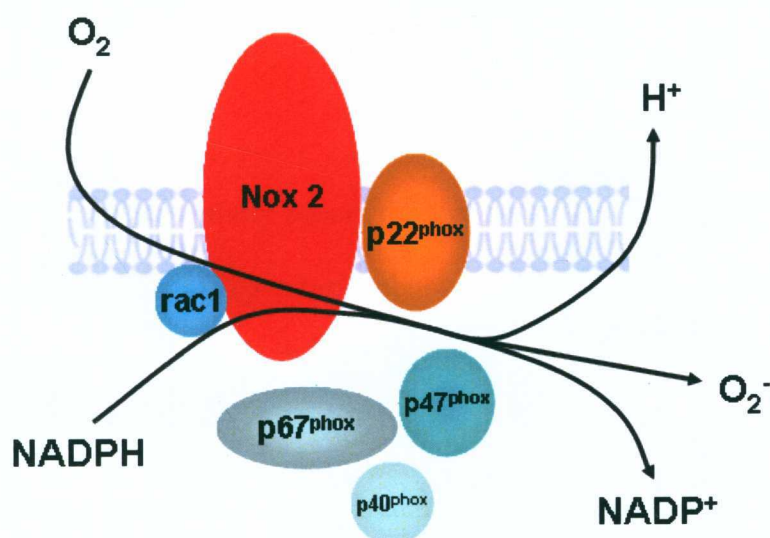


Figure 1.4 Schematic showing the structure of NADPH oxidase under basal and activated conditions.

Activation of NADPH oxidase requires the translocation of the cytosolic subunits p47^{phox}, p40^{phox}, p67^{phox}, and the small G protein, rac1 to the membrane bound Nox2/p22^{phox} complex.

1.8.5.2 Activation of NADPH oxidase

Upon stimulation, p47^{phox} becomes phosphorylated allowing the cytosolic complex (of p40^{phox}, p47^{phox}, p67^{phox}) to migrate to the membrane, where it binds to the membrane-associated flavocytochrome to assemble into the active complex (Fig. 1.4; Cave *et al.*, 2006). Rac1 translocation is also implicated in the activation of NADPH oxidase (Quinn *et al.*, 1993). Although the importance of p67^{phox} (Pagano *et al.*, 1997) and p22^{phox} (Ushio-Fukai *et al.*, 1996; Yang & Rizzo, 2007) for correct function of NADPH oxidase has been demonstrated via antisense/immunodepletion studies, their role in acute/chronic activation of the enzyme remains unclear. Likewise, the role of p40^{phox} is also unknown. Although many different stimuli have been shown to activate NADPH oxidase (for review see Cave

et al., 2006), in the following section only those triggers that may be activated in the DMD pathology will be discussed.

Chronic NADPH oxidase activity occurs through increased expression of subunit proteins or increased translocation of regulatory subunits (Griendling *et al.*, 2000). With regard to DMD, one hypothesis to account for chronic activation of NADPH oxidase relates to the structural properties of dystrophin. Thus, the increased elasticity (Pasternak *et al.*, 1995) and susceptibility of *mdx* myocytes to stretch may lead (Yasuda *et al.*, 2005) to increased NADPH oxidase activity. Stretch increases both NADPH oxidase activity (Hishikawa *et al.*, 1997; Hishikawa & Luscher, 1997; Hwang *et al.*, 2003b) and augments gp91^{phox} protein expression (Hwang *et al.*, 2003a). Increased translocation of regulatory subunits may also be involved in NADPH oxidases response to stretch. Rac1 is stimulated by stretch and requires the presence of caveolae (Kawamura *et al.*, 2003), which are known to be altered in DMD (Galbiati *et al.*, 2001).

Another potential mechanism linking increased NADPH oxidase activity to DMD relates to the raised resting $[Ca^{2+}]_i$ found in diseased muscle. Intracellular Ca^{2+} has been shown to activate NADPH oxidase ROS production in human fibroblasts (Meier, 1996); thus, the raised resting $[Ca^{2+}]_i$ found in the *mdx* myocardium might increase NADPH oxidase activity. However, the effect of Ca^{2+} on myocardial NADPH oxidase is yet to be explored. Finally, increased NADPH oxidase activity in DMD may relate to the finding that caveolae are required for enzymatic activity (Kawamura *et al.*, 2003; Yang & Rizzo, 2007). This hypothesis is explored in more detail below.

1.8.6 Mitochondria

The high density of mitochondria (Bers, 2001) and high rate of oxidative phosphorylation in cardiomyocytes (Sheeran & Pepe, 2006), coupled with the finding that ROS can leak out of mitochondria during respiration (Boveris *et al.*, 1976; Brookes *et al.*, 2004) support the argument that any defect in mitochondrial function could lead to substantial myocardial ROS production. Evidence suggests that mitochondrial ROS production is increased in DCMs (Ide *et al.*, 1999). In addition, knocking out mitochondrial MnSOD produces a DCM in mice (Li *et al.*, 1995). Although the mechanism for increased mitochondrial ROS production in DCMs is not fully understood (Sawyer & Colucci, 2000), in the setting of DMD, mitochondrial Ca^{2+} overload (Brookes *et al.*, 2004) or mechanical stretch may be involved (Gunning *et al.*, 1973; Strauer *et al.*, 1977).

1.8.7 Xanthine oxidase

The enzyme XO can produce both superoxide and hydrogen peroxide (Harrison, 2002). XO activity is increased in experimental heart failure (Ekelund *et al.*, 1999) and in DCM patients serum levels of uric acid are augmented, suggesting increased XO activity (Leyva *et al.*, 1997). In the spontaneous hypertensive/heart failure rat model of DCM, acute XO inhibition improves cardiac contractility (Kogler *et al.*, 2003), while clinical trials of XO inhibitors in both human and animal models of DCM have been mostly successful (see Chapter 1, Section 1.8.3.3). It is still unclear why XO activity is increased in DCMs; one possibility is that raised activity occurs in response to the augmented cardiac energy demands of failing heart (Duncan *et al.*, 2005). Another possibility is that XO activity is

augmented secondary to increased NADPH oxidase activity (McNally *et al.*, 2003) although this theory is disputed (Maytin *et al.*, 2004; Minhas *et al.*, 2006).

1.8.8 Nitric oxide synthase

In addition to synthesising NO, NOS can also produce superoxide and peroxynitrite (Vasquez-Vivar *et al.*, 1998; Xia *et al.*, 1996); this process is known as NOS uncoupling. Exogenous peroxynitrite induces apoptosis in cardiomyocytes (Arstall *et al.*, 1999) while endogenously produced peroxynitrite produces a loss of cardiac efficiency in working hearts (Ferdinandy *et al.*, 1999). In *mdx* mice hearts, although nNOS is downregulated, inducible NOS (iNOS) activity is increased (Bia *et al.*, 1999). Thus, uncoupled iNOS could potentially be a source of increased ROS production in *mdx* cardiomyocytes. It is unclear why NOS becomes uncoupled; one possibility is that ROS derived from NADPH oxidase induces NOS uncoupling (Landmesser *et al.*, 2003).

1.8.9 Altered antioxidant capacity in dilated cardiomyopathies

In DCMs increased oxidative damage may result from a reduction in myocardial antioxidant capacity. Support for this hypothesis as a primary pathology is supported by findings showing that mutations in the targeting signal of the MnSOD gene results in a DCM in humans (Hiroi *et al.*, 1999; Valenti *et al.*, 2004). In addition, transgenic mice deficient in MnSOD develop a DCM that is reversed by administration of a mimetic SOD (Nojiri *et al.*, 2006). However, in other forms of DCM it is unclear whether changes in antioxidant expression and/or activity are primary causes or secondary consequence of increased oxidative stress (Baumer *et al.*, 2000; Fukuchi *et al.*, 1991; Sam *et al.*, 2005).

1.9 Excitation contraction coupling in DMD

1.9.1 Excitation contraction overview

The process whereby electrical excitation of a cardiomyocyte leads to myocyte contraction is known as EC coupling (for review see Bers, 2001). Cardiomyocytes are excited via the repeated electrical signal generated from the sino atrial node. The electrical signal generated at the sino atrial node spreads throughout the heart, depolarising the sarcolemma, resulting in the opening of voltage-sensitive Na^+ channels. Further depolarisation of the sarcolemma leads to activation of DHPRs (Fig. 1.5). Opening of the DHPR allows extracellular Ca^{2+} to move down its concentration gradient (2 mM extracellular, 100 nM intracellular) and into the intracellular environment. Some of this Ca^{2+} contributes directly to the activation of myofilament contraction. However, in a process known as Ca^{2+} -induced Ca^{2+} release, some of the Ca^{2+} that enters the myocyte activates the cardiac ryanodine receptor (RyR2), which is found in the sarcoplasmic reticulum (SR) membrane. Upon activation, the RyR2 opens allowing Ca^{2+} to move out of the SR, resulting in a further increase in $[\text{Ca}^{2+}]_i$. The increase in $[\text{Ca}^{2+}]_i$ allows myofilament cross-bridge formation, allowing coordinated and vigorous contraction of the myofilaments resulting in the generation of force.

In order to terminate contraction, $[\text{Ca}^{2+}]_i$ is returned to resting levels. This allows Ca^{2+} to dissociate from the myofilaments inducing relaxation. $[\text{Ca}^{2+}]_i$ is returned to resting levels by two main pathways. The first pathway involves the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX), which is found in the sarcolemma and returns Ca^{2+} to the extracellular space. The second pathway

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involves the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA), which is found in the SR membrane, and pumps Ca^{2+} back into the SR.

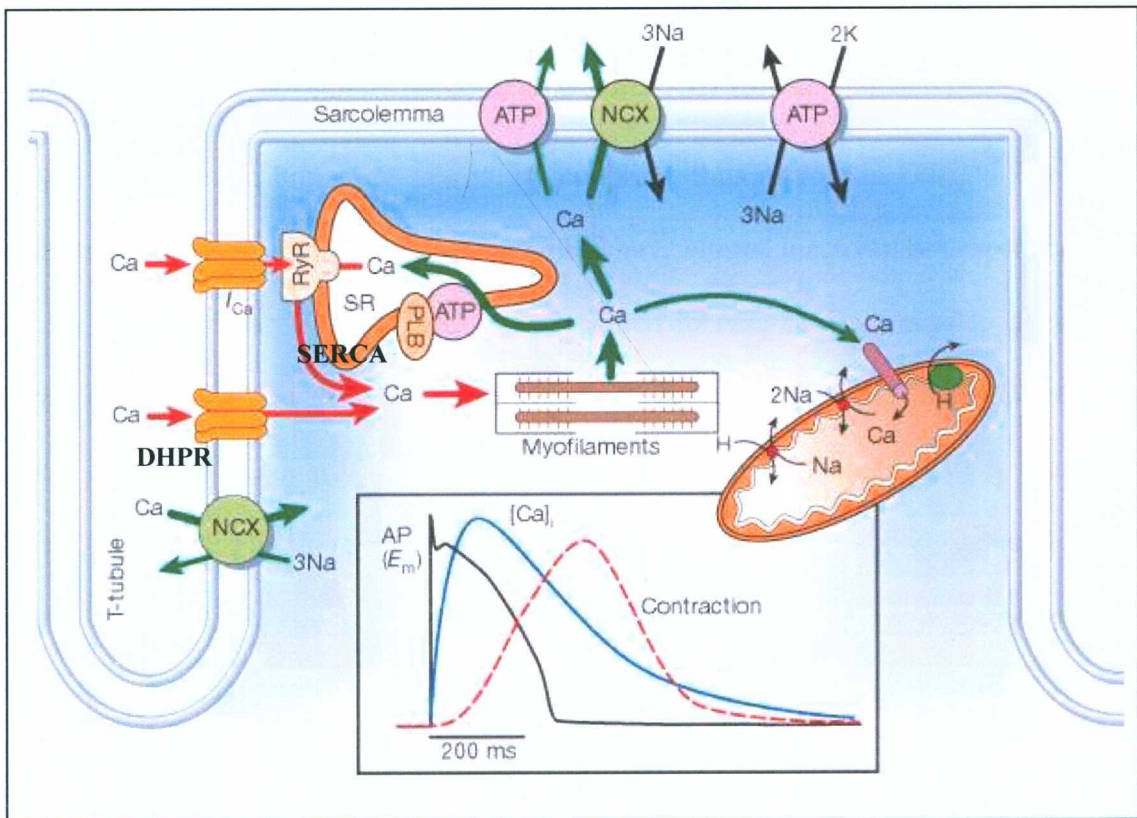


Figure 1.5 Ca^{2+} handling in a cardiomyocyte

Ca^{2+} handling proteins and Ca^{2+} pathways utilized during excitation contraction coupling. Red and green arrows indicate Ca^{2+} pathways taken during excitation and relaxation, respectively. Inset shows the time course of an action potential, Ca^{2+} transient and contraction measured in a rabbit ventricular myocyte at 37°C. ATP, ATPase; DHPR, dihydropyridine receptor; NCX, $\text{Na}^+/\text{Ca}^{2+}$ exchanger; PLB, phospholamban; SERCA, sarcoplasmic reticulum ATPase; SR, sarcoplasmic reticulum. Adapted from Bers (2002).

The following section will explore the changes that occur in the Ca^{2+} handling proteins of the myocardium in DMD patients with DCMs. The effects of ROS on myocardial Ca^{2+} handling proteins will also be discussed. Where possible, evidence will be given from studies involving DMD patients or *mdx* mice. However, when this information is unavailable or limited, evidence from DCMs and other models of heart failure will be presented.

1.9.2 DHPR regulation and expression in DMD

The DHPR plays an important role in EC coupling, thus its role in DMD will be briefly reviewed. DHPRs from DMD patient myotubes have altered inactivation properties (Imbert *et al.*, 2001). Similar findings have been reported in *mdx* cardiac muscle, where patch-clamp studies revealed delayed DHPR inactivation (Sadeghi *et al.*, 2002; Woolf *et al.*, 2006); interestingly, delayed inactivation might result from increased ROS (Coetzee & Opie, 1992). The DHPR response to exogenous reagents appears to be defective in *mdx* hearts, although defects depend on both the type and age of cardiac tissue (Sadeghi *et al.*, 2002; Woolf *et al.*, 2006). Ventricular tissue from *mdx* hearts has increased DHPR mRNA and protein expression (Woolf *et al.*, 2006).

1.9.3 Ryanodine receptor regulation and expression in DMD

There are three known isoforms of the RyR receptor, RyR1 (Inui *et al.*, 1987b) RyR2 (Inui *et al.*, 1987a) and RyR3 (Hakamata *et al.*, 1992). RyR2 is the predominant isoform expressed in cardiac tissue (Inui *et al.*, 1987a; Lai *et al.*, 1988). The RyR traverses the SR membrane and extends toward the sarcolemma, where it is in close proximity to the DHPR

(Fig. 1.5). RyR2 protein and mRNA expression has not been measured in DMD patients. RyR2 mRNA is decreased in *mdx* cardiac tissue (Rohman *et al.*, 2003). In DCMs it is debated as to whether RyR2 expression is altered (Lehnart *et al.*, 1998).

The RyR2 is redox sensitive (Marengo *et al.*, 1998); the oxidising conditions produced by ROS are thought to increase both the activity (for review see Meissner, 2004; Zima & Blatter, 2006) and the leak through the RyR2 (Menshikova & Salama, 2000). It is unclear whether the effects of ROS on the RyR2 are due to a direct action on the channel or on the proteins associated with the RyR2, as is the case for RyR1 (Aracena *et al.*, 2005).

1.9.4 SERCA regulation and expression in DMD

SERCA pumps Ca^{2+} from the cytoplasm back into the lumen of the SR in order to reduce $[\text{Ca}^{2+}]_i$ and allow muscle relaxation. There are 3 known SERCA genes; SERCA2a is the primary isoform expressed in the heart (Wu *et al.*, 1995). *Mdx* cardiac muscle has decreased SERCA2a mRNA levels (Rohman *et al.*, 2003). In *mdx* skeletal muscle there is a change in the ratio of the slow SERCA2a isoforms and the fast SERCA1 isoform (Divet *et al.*, 2005), which may account for the slow rate of Ca^{2+} uptake reported in *mdx* skeletal muscle (Kargacin & Kargacin, 1996). However, others have found no change in SERCA activity in *mdx* mice (Divet *et al.*, 2005).

In DCMs it is widely accepted that the rate of removal of cytosolic Ca^{2+} following a Ca^{2+} transient is impaired, and that this is due to decreased SERCA function (Lehnart *et al.*, 1998; Schwinger *et al.*, 1995). The decrease in SERCA function has been shown to be the

result of both decreased SERCA protein expression and/or decreased activity (Lehnart *et al.*, 1998). However, some groups have reported no differences in these parameters (Movsesian *et al.*, 1989; Schwinger *et al.*, 1995). Alternatively, decreased SERCA function may be due to changed activity/expression of SERCA's endogenous inhibitory protein, phospholamban (PLN). The role of PLN is discussed below.

ROS depress SERCA function (Kaneko *et al.*, 1989a; Kaneko *et al.*, 1989b; Morris & Sulakhe, 1997), possibly by altering SERCA's ATP-binding site (Xu *et al.*, 1997). Interestingly, SERCA activity in *mdx* skeletal muscle is more susceptible to oxidative stress induced injuries compared to WT (Lafoux *et al.*, 2006).

1.9.5 Phospholamban regulation and expression in DMD

PLN is a SR membrane protein that interacts and inhibits SERCA by lowering its apparent affinity for Ca^{2+} (Fujii *et al.*, 1987; Simmerman *et al.*, 1986). Under normal conditions PLN binding to SERCA is not saturated (Kadambi *et al.*, 1996). In its dephosphorylated state, PLN inhibits SERCA function (Cantilina *et al.*, 1993). β -adrenergic stimulation results in protein kinase A mediated phosphorylation of PLN, which reverses the inhibitory effect of PLN on SERCA (Simmerman & Jones, 1998; Tada & Katz, 1982). PLN exists as either a monomer or polymer. It is thought that the PLN monomer is responsible for binding to SERCA and inhibiting it while the pentamer acts as a pool of PLN (Autry & Jones, 1997; Jones *et al.*, 2002; Kimura *et al.*, 1997).

The role of PLN in DMD or *mdx* muscle is unknown. However, in human and animal models of heart failure, altered PLN function has been shown to be involved in the pathogenic process. Alterations in PLN function result in increased inhibition of SERCA leading to impaired removal of cytosolic Ca^{2+} . In DCM patients mutations have been found that increase PLN's inhibition of SERCA (Haghighi *et al.*, 2006; Schmitt *et al.*, 2003). In support of these findings, PLN ablation has been shown to rescue a mouse model of DCM (Minamisawa *et al.*, 1999), while PLN inhibition attenuated heart failure progression in cardiomyopathic hamsters (Hoshijima *et al.*, 2002). PLN phosphorylation is also decreased in humans with DCMs, which results in increased inhibition of SERCA (Schwinger *et al.*, 1999a).

There is limited data surrounding the role of ROS on PLN function. It has been postulated that PLN oxidation prevents phosphorylation of PLN, thus locking it in the form bound to SERCA (Bigelow & Squier, 2005; Sulakhe *et al.*, 1997). In support of this finding are results showing that the antioxidant N-acetylcysteine (Cailleret *et al.*, 2004) and inhibition of the ROS producing enzyme XO (Saliaris *et al.*, 2006), both increases PLN phosphorylation.

1.9.6 Sodium calcium exchanger regulation and expression in DMD

The NCX is an ion exchanger found in the sarcolemma of cardiomyocytes. Following a Ca^{2+} transient the NCX's main function is to extrude one Ca^{2+} ion in exchange for 3 Na^{+} ions. There are 3 isoforms of the NCX; NCX1 is the predominant isoform expressed in the heart (Bers, 2001). No studies have explored NCX function and/or expression in DMD or

mdx hearts. However, an investigation into the role of the NCX in DMD skeletal muscle found an increase in both NCX function and expression (Deval *et al.*, 2002). In patients and animal models of DCM, NCX mRNA, protein expression and activity are generally found to be increased (for review see Bers, 2001), although these findings are not universal (Kubo *et al.*, 2001; Schwinger *et al.*, 1999b). It is unclear why NCX function is increased in heart failure.

ROS have been shown to both increase and decrease NCX function (Kourie, 1998). Thus, XO derived superoxide and hydrogen peroxide both stimulate NCX (Kato & Kako, 1988; Reeves *et al.*, 1986), while the strong oxidant HOCl inhibited NCX (Kato & Kako, 1988); the nature of the inhibition or stimulation is not clear.

1.10 Inflammation and fibrosis in DMD and DCMs

Mdx mice develop cardiac and skeletal muscle inflammation and fibrosis. Increased inflammation may lead to fibrosis which reduces cardiac contractility and promotes ECG defects and arrhythmias. In the following section, possible causes and sources of cardiac inflammation and fibrosis in DMD will be discussed.

The extracellular matrix is a fibrillar network that embeds myofibers and the whole cardiac structure. Under normal conditions, the extracellular matrix, and in particular collagen, forms the physical connection between myocytes, preventing myocyte slippage during contraction, passively contributing towards diastolic stiffness and ensuring coordinated transduction of myocyte generated force to the ventricular chamber during systole. The two main forms of collagen found in the myocardium are collagen type I and III (See *et al.*, 2005; Swynghedauw, 1999).

Cardiac fibrosis is an increase in the collagen concentration within the myocardium. It is generally accepted that two forms of cardiac fibrosis occur; reparative and reactive. Reparative fibrosis occurs in response to a loss of myocardial material (due to necrosis or apoptosis) and is mainly interstitial. In contrast, reactive cardiac fibrosis occurs in the absence of cell damage as a reaction to inflammation and is primarily perivascular, before subsequently extending into the interstitial space (Swynghedauw, 1999). Cardiac fibrosis increases extracellular electrical resistance resulting in a decrease in conduction velocity (Fenoglio, Jr. *et al.*, 1983; Sanyal *et al.*, 1978; Weber *et al.*, 1994). Cardiac fibrosis may also enhance myocardial stiffness leading to poor diastolic filling (Kass *et al.*, 2004).

DMD patients (Finsterer & Stollberger, 2003) and *mdx* mice (Quinlan *et al.*, 2004; Wehling-Henricks *et al.*, 2005) develop cardiac fibrosis. *Mdx* hearts also develop inflammation (Bridges, 1986; Spencer *et al.*, 2001; Wehling-Henricks *et al.*, 2005). It has been proposed that *mdx* cardiac fibrosis is the result of the inflammatory process, i.e. reactive fibrosis. In support of this idea is the finding that *mdx* mice bred on a nu/nu background (i.e. lack T-cells; Morrison *et al.*, 2000) or procedures that reduce macrophage concentrations in muscle tissue, reduce cardiac and skeletal muscle fibrosis (Wehling *et al.*, 2001; Wehling-Henricks *et al.*, 2005). However, inflammation may not be an initiating factor in the *mdx* pathology, as macrophages appear late in the process of skeletal muscle breakdown (Cullen & Fulthorpe, 1975).

In both cardiac and skeletal muscle, inflammatory cells may induce fibrosis by secreting cytokines that induce connective tissue production by cells such as fibroblasts. One such cytokine is transforming growth factor (TGF)- β (Hartel *et al.*, 2001; Van Erp *et al.*, 2006), which is known to be increased in DMD (Bernasconi *et al.*, 1995) and *mdx* (Gosselin *et al.*, 2004) skeletal muscle, and in various heart failure models (Nicoletti & Michel, 1999).

The above findings suggest that inflammation may induce fibrosis in DMD. However, why are inflammatory cells activated in DMD? One possibility is that increased oxidative stress activates inflammatory mediators; one such mediator is NF- κ B.

1.10.1 Role of NF- κ B and ROS in inflammatory cell activation

NF- κ B is a transcription factor that regulates a battery of inflammatory genes. NF- κ B exists as a dimer, most commonly as p50/p65 (Fig. 1.6). In resting cells NF- κ B resides in the cytoplasm in an inactive form due to binding of the inhibitory protein, inhibitor- κ B (I κ B). The initial step in activation of NF- κ B is the phosphorylation of I κ B, which facilitates ubiquitination of I κ B, leading to its degradation. The initial phosphorylation step is catalyzed by the inhibitor- κ B kinases (IKKs) which are found as a macromolecular complex (for review see Hall *et al.*, 2006). The loss of I κ B unmasks a nuclear localization sequence within the NF- κ B dimer which promotes translocation to the nucleus where the transcription factor binds to specific NF- κ B response sequences within promoters or enhancer regions of specific target genes. In addition to nuclear translocation of NF- κ B, post-translational modification of NF- κ B, such as phosphorylation, is important for efficient transcriptional activation. With regard to the p65 NF- κ B subunit, phosphorylation at S276 (Okazaki *et al.*, 2003), S529 (Wang & Baldwin, Jr., 1998), and S536 (Jiang *et al.*, 2003) have all been shown to be important for agonist-induced stimulation of the NF- κ B pathway.

NF- κ B localisation to the nucleus is greater in both DMD (Monici *et al.*, 2003) and *mdx* skeletal muscle compared to control muscle (Kumar & Boriek, 2003), suggesting it may have a role in the dystrophic inflammatory response. In accord with this hypothesis, agents that prevent NF- κ B activation also reduce markers of damage in *mdx* skeletal muscle (Carlson *et al.*, 2005; Messina *et al.*, 2006b). With regards to NF- κ B and ROS, it has been shown that *mdx* skeletal muscle damage and NF- κ B DNA binding and levels are reduced by antioxidant

treatment (Hnia *et al.*, 2007; Kumar & Boriek, 2003; Messina *et al.*, 2006a). Thus, it appears that ROS-induced activation of NF- κ B is an important step in initiating the pathological inflammatory response in DMD skeletal muscle. No studies have explored the role of NF- κ B in *mdx* cardiac inflammation. However, DCM patients have increased nuclear expression of NF- κ B (Frantz *et al.*, 2003; Wong *et al.*, 1998) while in animal models of DCM, NF- κ B binding to DNA has been shown to be activated by ROS (Eisner *et al.*, 2006).

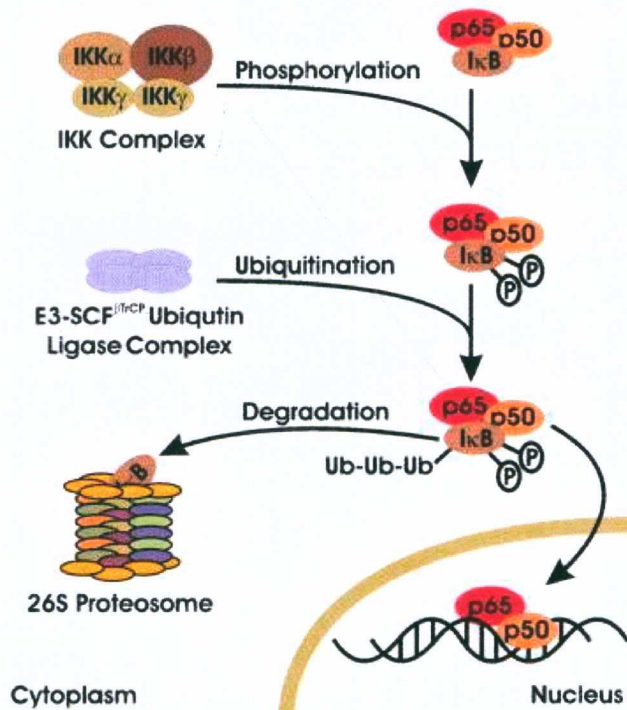


Figure 1.6 Canonical NF- κ B activation pathway

Stimulus-induced activation of the multimeric IKK complex leads to the phosphorylation and proteasome-mediated degradation of I κ B. This process liberates NF- κ B dimers and facilitates their translocation from the cytoplasm to the nucleus. DNA-binding of NF- κ B dimers facilitates the activation or repression of gene transcription. Taken from Hall *et al.* (2006).

1.11 Caveolae

Caveolae ('little caves') are flask-shaped plasma membrane invaginations that participate in vesicular transport, cellular cholesterol homeostasis and signal transduction (for review see Razani *et al.*, 2002). With regard to DMD, caveolae may provide a link between absence of dystrophin and increased activity of the ROS producing enzyme, NADPH oxidase. Caveolin is a small molecular weight protein (21-24 kDa) that is a principal component of caveolae. The mammalian gene family consists of caveolin 1, 2 and 3. Caveolin 1 and 2 are co-expressed in many cells types, whereas caveolin 3 is muscle specific (Scherer *et al.*, 1996; Scherer *et al.*, 1997; Tang *et al.*, 1996). Caveolin 1 and 3 share high sequence structural similarities (Razani *et al.*, 2002). In the following literature review, wherever possible reference will be made to caveolin 3; however, when no findings for caveolin 3 exist, reference will be made to caveolin 1.

Dystrophin associates with caveolin 3 (Song *et al.*, 1996) and in DMD/*mdx* skeletal muscle, where dystrophin is absent, caveolin 3 levels are augmented (Repetto *et al.*, 1999; Vaghy *et al.*, 1998) leading to increased numbers of caveolae (Galbiati *et al.*, 2000). Caveolin 3 and caveolae are also increased in human and animal models of DCMs (Damy *et al.*, 2004; Hare *et al.*, 2000). Caveolins play an important role in both the formation of caveolae and on the assembly of signalling complexes with intracellular enzymes, such as NADPH oxidase and NOS. In accord with other modular protein domains, caveolin has a scaffolding domain for interaction with other proteins (Couet *et al.*, 1997a; Couet *et al.*, 1997b; Li *et al.*, 1996).

Chapter 1

Caveolae are important in the spatial regulation and agonist stimulation of NADPH oxidase. When caveolae become disrupted, NADPH oxidase enzyme subunits shift out of caveolae and into other membranous and intracellular compartments (Yang & Rizzo, 2007). Disruption of caveolae also prevents activation of the cytosolic protein rac1 (Kawamura *et al.*, 2003). Furthermore, caveolin 1 has been shown to be essential for the translocation of rac1 to caveolae and its subsequent activation in vascular smooth muscle cells (Zuo *et al.*, 2005).

1.12 Hypothesis and Aims

This thesis is based on the hypotheses that altered SAC function and increased ROS levels are involved in the pathogenesis of the DMD cardiac phenotype. The aims of this thesis are as follows:

1. To explore cardiomyocyte Ca^{2+} handling in WT and *mdx* mice;
2. To study the potential role of SACs in *mdx* cardiomyocyte Ca^{2+} handling defects;
3. To ascertain if *mdx* hearts have raised ROS levels;
4. To examine potential sources of augmented ROS production in *mdx* hearts;
5. To investigate the role of ROS on *mdx* cardiomyocyte Ca^{2+} handling and myofilament dysfunction;
6. To explore the role of ROS in the *mdx* cardiac inflammatory response.

CHAPTER TWO

General Methods

2.1 Animals

Male wild type control (WT; C57BL/10ScSn) and *mdx* mice, which occur in this strain of mice, of either 2-3 months or 9-12 months of age (young and old, respectively) were obtained from The Animal Resource Centre (Perth, Australia). All experiments were approved by the Animal Ethics Committee of the University of Sydney.

2.2 Isolation of cardiomyocytes

Isolation of single ventricular myocytes was based on the methods of Ju et al. (1996). Mice were anaesthetised with an intra-peritoneal injection of pentobarbitone (6.5 mg/g) and anti-coagulated with heparin (400 U). Hearts were rapidly excised and mounted on a Langendorff perfusion column (Fig. 2.1) and perfused retrogradely at 37°C with a physiological salt solution (PSS; mM: 140 NaCl, 5 HEPES, 1 MgCl₂, 5.4 KCl, 0.33 NaH₂PO₄, 5.5 glucose, 1 CaCl₂, pH 7.4 ± 0.05 with NaOH) and equilibrated with 100% oxygen. The perfusate was then changed to a Ca²⁺-free PSS. After 5 minutes of perfusion with Ca²⁺-free PSS the perfusate was changed to a PSS solution containing 20 µM Ca²⁺, 0.65 mg/ml Type 2 collagenase (Worthington, Lakewood, NJ, USA) and 0.0065 mg/ml protease (Sigma, St. Louis, MO, USA). Three sections of left ventricle were removed following 14 - 20 minutes of enzyme perfusion. The sections were washed, minced and titrated for 1 minute in Ca²⁺-free PSS, before being exposed to increasing concentrations of Ca²⁺-containing PSS until a final level

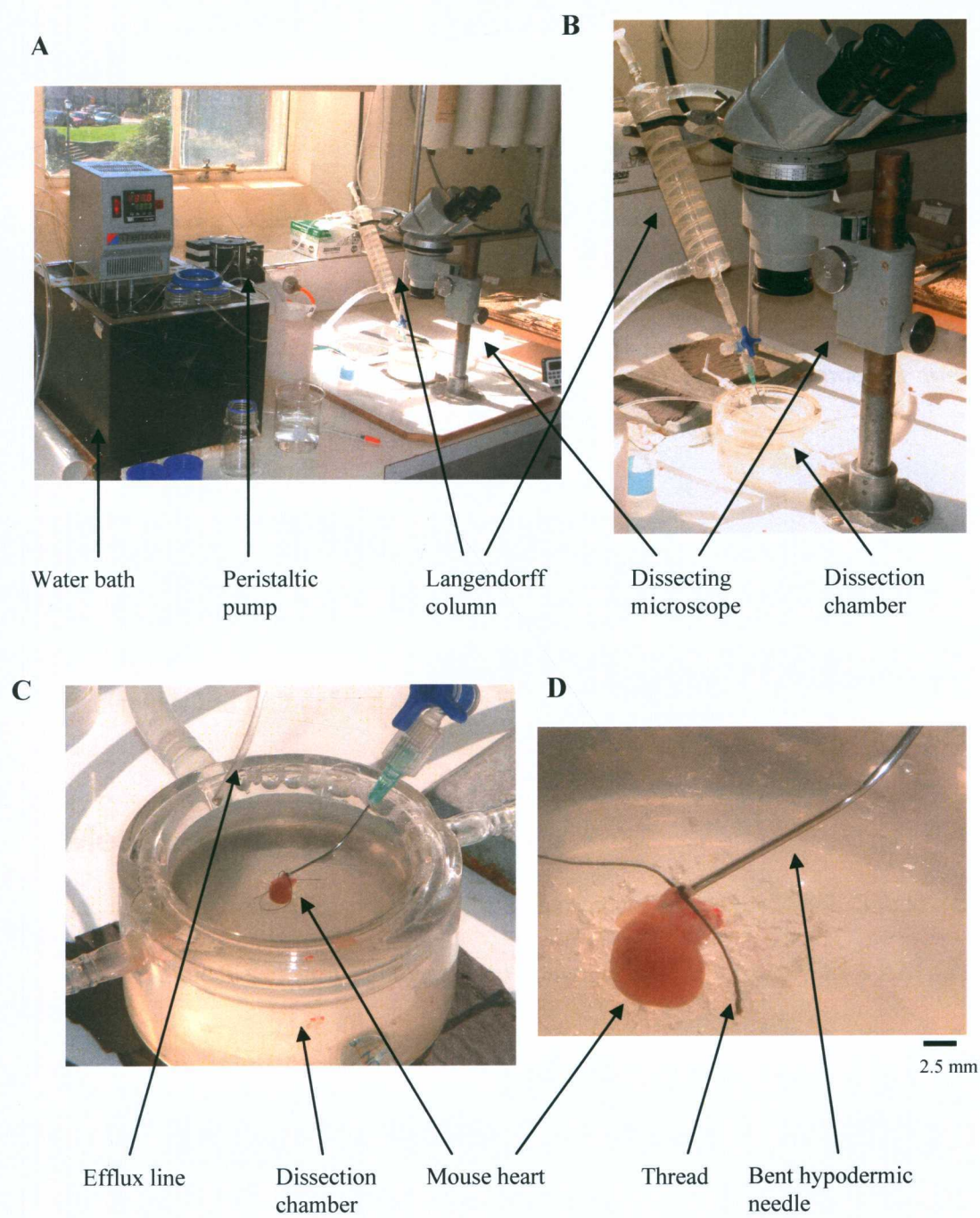


Figure 2.1 Apparatus used during isolation of cardiomyocytes

A: Overview of apparatus. **B:** Dissection microscope and Langendorff column. **C:** The aorta of mice hearts were cannulated with a hypodermic needle and perfused retrogradely with a physiological salt solution. **D:** Cannulated mouse heart. Bar = 2.5 mm.

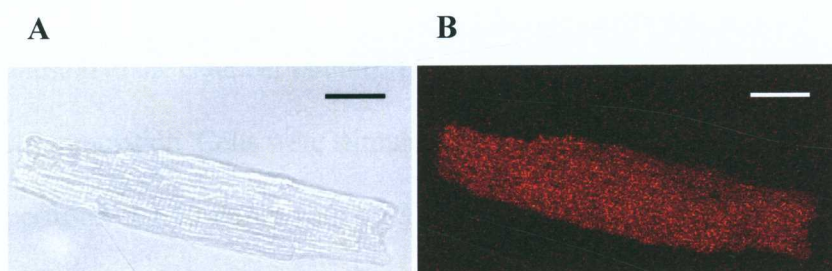


Figure 2.2 Light and fluorescent images of a cardiomyocyte

A: Light image of a cardiomyocyte. **B:** Image of fluo-4 loaded cardiomyocyte. Fluo-4 loaded cardiomyocytes were examined with a 488 nm illumination line and recorded at wavelengths between 510-560 nm. Bar = 20 μm .

of 1 mM Ca^{2+} was obtained. This was the standard experimental Ca^{2+} concentration. All experiments on cardiomyocytes were performed within 2 - 8 hours after isolation. All myocytes used in this study had well-defined striations and did not spontaneously contract (Fig. 2.2).

2.3 Measurement of $[\text{Ca}^{2+}]_i$

Changes in $[\text{Ca}^{2+}]_i$ were measured using the fluorescent indicator fluo-4. Single ventricular myocytes were incubated in a light protected container with 1.35 μM fluo-4 AM (Molecular Probes, Carlsbad, CA, USA) for 25 minutes at room temperature. Cells were then allowed to settle to the bottom of the container. The fluo-4 containing solution was removed and replaced with fresh PSS twice over a 20 minute period. This washing process removed excess fluo-4 containing solution and allowed time for intracellular esterases to cleave the fluo-4 AM group, trapping the dye intracellularly.

Fluo-4 loaded cells were placed in an experimental chamber coated with poly-L-lysine, which aided cell adhesion to the chamber bottom. Two electrodes either side of the chamber allowed for electrical field stimulation. Cells were stimulated at 10% above threshold, at a frequency of 1 Hz. Cells were perfused at 1.5 ml/min with a PSS. The experimental protocol was performed on the stage of an inverted confocal microscope (Leica, Wetzlar, Germany). Myocytes were examined with a x16, numerical aperture 1.2, water immersion objective using a 488 nm illumination line and recording at wavelengths between 510-560 nm. Data was analysed using Scion Image software (National Institute of Health (NIH) Bethesda, MD, USA). Ca^{2+} transient time constants were calculated by fitting exponential decay curves to data.

2.4 Calibration of fluo-4

Calibration of fluo-4 was based on the method of Ward et al. (2003). At the end of an experiment, cells were exposed to a Ca^{2+} -free PSS that contained in mM: 10 caffeine (to release SR Ca^{2+}), 10 2,3-butanedione monoxime (BDM; to prevent muscle contraction), 10 ethylene glycol tetraacetic acid (EGTA; to buffer free Ca^{2+}), 0.1 2,5-di(tert-butyl)-1,4-benzohydroquinone (TBQ; to block Ca^{2+} uptake into the SR via SERCA), 1 ouabain (to block the Na^+ - K^+ -ATPase), and 0.005 ionomycin (a Ca^{2+} ionophore). The resulting fluorescence was designated $F_{\min}$. Cells were then exposed to a Na^+ -free, high Ca^{2+} PSS solution that contained in mM: 140 LiCl (to replace Na^+), 2 CaCl_2 , 10 caffeine, 10 BDM, 0.1 TBQ, 1 ouabain, and 0.005 ionomycin. The resulting fluorescence was designated $F_{\max}$. The following equation was then used to convert fluo-4 fluorescence readings into $[\text{Ca}^{2+}]_i$ concentrations:

$$[\text{Ca}^{2+}]_i = K_d(F - F_{\min})/(F_{\max} - F)$$

A K_d of 770 mM was assumed (Woodruff *et al.*, 2002).

2.5 Measurement of cardiomyocyte Ca^{2+} -sensitivity and fractional shortening

Measurement of cardiomyocyte Ca^{2+} -sensitivity was based on the work of Spurgeon et al. (1992) who found that $[\text{Ca}^{2+}]_i$ and the myofilaments achieved a dynamic equilibrium during the relaxation phase of a twitch contraction. Thus, the position of the relaxation trajectory reflects the relative myofilament response to Ca^{2+} , i.e. Ca^{2+} -sensitivity. Measurement of fractional shortening was based on the work of Delbridge et al. (1989) and Gomez et al. (1997), who used line-scan images through cardiomyocytes to estimate cell length during contractions.

In the current study, cell length was determined using the fluorescent signal provided by fluo-4 and measured using repeated confocal scans (400 Hz) along the long axis of cardiomyocytes; this was termed an *xt* scan (Fig. 2.3). Each *xt* scan generated was analysed off-line to obtain cell length data using customized boundary identification algorithms. The start and end boundary of a cardiomyocyte was determined as the point when the fluo-4 fluorescent signal was 20% of the maximum fluo-4 signal (Fig. 2.3). The value 20% was chosen as it gave reliable results and correlated well with the visually-determined cell end using normal microscopy.

To confirm that estimates of cell length using the fluo-4 fluorescent signal were comparable to cell length estimates using conventional transmission light microscopy, cell length versus time traces were plotted using data required from both imaging techniques. As seen

in Figure 2.4, traces of cell length versus time from both imaging techniques produced identical cell length traces during a contraction.

Ca^{2+} -sensitivity was calculated by plotting $[\text{Ca}^{2+}]_i$ versus cell length during a contraction; the $[\text{Ca}^{2+}]_i$ - length trajectory during the relaxation phase was used to determine $[\text{Ca}^{2+}]_i$ at 50% cell shortening which was used to estimate Ca^{2+} -sensitivity. In this thesis the rate of cell shortening and relaxation were not investigated.

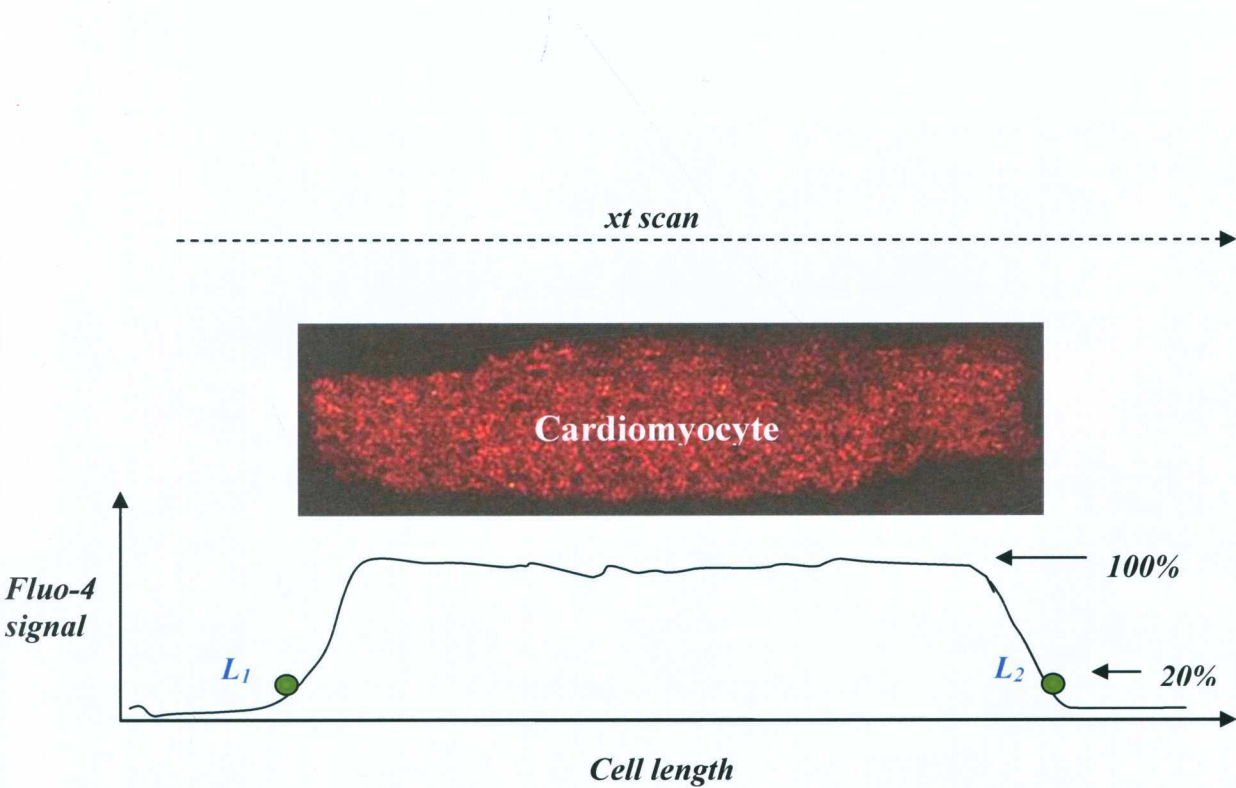


Figure 2.3 Diagram illustrating adjustment used to estimate cardiomyocyte length

When measuring along the long axis (*xt scan*) of a cardiomyocyte the cell boundary is often unclear. To reduce error, the start (L_1) and end (L_2) of the cardiomyocyte (green circles) was determined as the point when fluo-4 fluorescence was 20% of maximum.

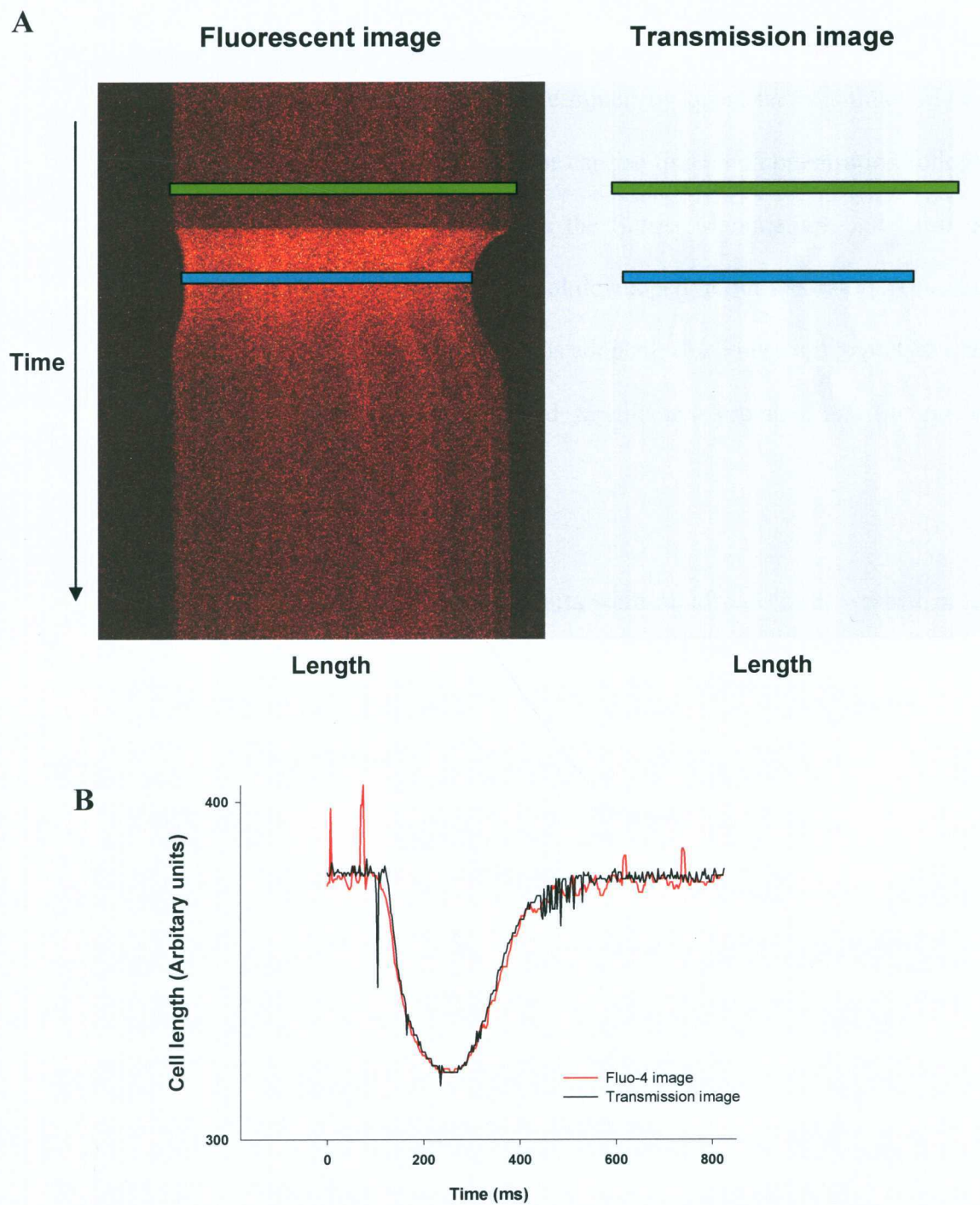


Figure 2.4 Analysis of cell shortening during a contraction using light and fluorescent microscopy

A: *xt* scan through fluo-4 loaded cardiomyocyte. The length of the green and blue bar indicates cardiomyocyte length at rest and during a contraction, respectively. **B:** Graph showing cardiomyocyte length versus time during a contraction. Traces represent cell length calculated from a transmission (black trace) and fluorescent (red trace) *xt* scan.

2.6 Preparation of cardiac tissue homogenate

The relative levels of specific proteins were determined by quantitative immunoblotting (Meyer *et al.*, 1995). Initially, the methodology for cardiac tissue homogenisation followed the protocol, and used the reagents, included in the Sigma Mammalian Cell Lysis Kit (Sigma, St. Louis, MO, USA). However, a protocol developed in our laboratory (described below) produced higher protein yields and was thus adopted. It is important to note that this procedure was designed to harvest cytosolic and sarcolemma proteins, and not nuclear proteins.

Mice were anaesthetised as described above. Hearts were rapidly excised, washed in ice-cold phosphate buffered solution (PBS; mM: 137 NaCl, 2 KCl, 10 phosphate buffer, pH 7.4 $\pm$ 0.05 with NaOH) and palpated gently to remove blood from the heart, before the left ventricle was removed and minced. 20 mg/ml of minced tissue was added to ice-cold cell lysis solution containing in mM: 150 NaCl, 0.05 Tris-HCl, 0.025 EGTA, 0.025 ethylenediamine tetraacetic acid (EDTA), 1% Triton X-100 (v/v) and the following inhibitors to prevent protein degradation: 1% protease inhibitor cocktail, 0.5% phosphatase inhibitor and 0.35% calpain inhibitor 1 (all w/v; from Sigma, St. Louis, MO, USA) and homogenised for 15 seconds with an electric tissue homogeniser (Polytron, Kinematica, Lucerne, Switzerland). Lysis buffer solution was incubated for 30 minutes on an orbital shaker at 4°C before being homogenised for 30 seconds. Homogenate was centrifuged at 12,000 g at 4°C for 10 minutes. Supernatant was removed, aliquoted and stored at -80°C for subsequent use. The homogenate was quantified for protein content using a Bradford assay (Bio-Rad, Hercules, CA, USA).

2.7 Immunoblotting

Heart sample homogenates (prepared as above) were solubilized in Laemmli sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS (w/v), 25% glycerol (w/v), 0.01% bromophenol blue (w/v), 5 % β -mercaptoethanol (v/v)) and heated (see relevant chapters for immunoblotting conditions used for specific proteins) before being separated by SDS-PAGE in a Mini-Protean III electrophoresis system (Bio-Rad, Hercules, CA, USA). According to the weight of the protein of interest, appropriate molecular weight markers were used to estimate the size of blotted proteins (PageRuler Prestained Protein Ladder, Fermentas, Burlington, Canada; High-Range Rainbow, Amersham BioSciences, Uppsala, Sweden; HiMark Pre-Stained High Molecular Weight Marker Protein Standard, Invitrogen, Carlsbad, CA, USA). Following electrophoresis, protein was transferred to a nitrocellulose membrane in a Mini Trans-Blot Transfer Cell (Bio-Rad, Hercules, CA, USA). To prevent non-specific antibody binding, membranes were incubated in PBS-Tween block (PBST-block; PBS, 0.1% Tween (v/v), 5% non-fat dried milk powder (w/v)) for a minimum of 4 hours. Membranes were incubated with primary antibodies (see relevant chapters for antibody incubation conditions), before being washed with PBST for 30 minutes. PBST solution was changed four times during the washing procedure. Membranes were then incubated with horse radish peroxidase (HRP)-conjugated secondary antibodies (see relevant chapters for antibody incubation conditions). Blots were then washed for 30 minutes in PBST using the same washing procedure described above. Blots were developed with the use of an enhanced chemiluminescence system (Amersham BioSciences, Uppsala, Sweden). Band densities were evaluated by densitometry. Films were scanned using Epson scanning software and analysed with ImageJ software (NIH, Bethesda, MD, USA). Each individual value represents the mean of two different bands. Data points are made up of the area of the band multiplied by the density of the

band. All bands were normalised to the housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH; for methodology see below). All antibodies were diluted in PBST.

2.7.1 Stripping of membranes and immunoblotting for GAPDH

For each protein of interest, immunoblotting bands were normalised to GAPDH. Once bands had been evaluated for the protein of interest (see above), membranes were stripped in order to determine GAPDH protein expression. Stripping removed both primary and secondary antibodies. Membranes were stripped by placing them, protein side down, in stripping solution for 30 minutes at 50°C. Stripping solution contained: 62.5 mM Tris pH 6.8, 0.8% β -Mercaptoethanol (v/v) and 1% Sodium disulphate (w/v) dissolved in Milli-Q water. Following stripping, Milli-Q water was continuously washed over membranes for 5 minutes to remove stripping solution. Membranes were then blocked in PBST-block for at least 2 hours before being probed with primary antibody against GAPDH (rabbit anti-mouse IgG; Chemicon; Temecula, CA, USA) at room temperature for 15 minutes. The concentration of GAPDH primary antibody varied (1:15,000 – 1:60,000) depending on the amount of protein used during the first immunoblot (i.e. the initial protein of interest). Membranes were then washed with PBST for 30 minutes. The PBST solution was changed four times during the washing procedure. Membranes were then incubated with anti-mouse IgG HRP-conjugated secondary antibody (1:4,000; Santa Cruz, Santa Cruz, CA, USA) for 20 minutes at room temperature. Membranes were washed with PBST for 30 minutes. The PBST solution was changed four times during the washing procedure. Band densities were then evaluated using the same procedure as discussed above. All antibodies were diluted in PBST.

2.8 Whole heart freezing procedure

The following apparatus was used to suspend hearts before freezing. A hypodermic needle was inserted into a piece of cork (2 cm x 2 cm x 0.3 cm). The top of the hypodermic needle was bent, and a piece of Blu-tack™ attached (Fig. 2.5) A plastic pasteur pipette, with a gauge wide enough to accommodate a mouse heart, was cut to form a cylinder; the plastic cylinder was secured to the cork with several small pieces of Blu-tack™. This piece of equipment was termed the 'heart suspension apparatus'.

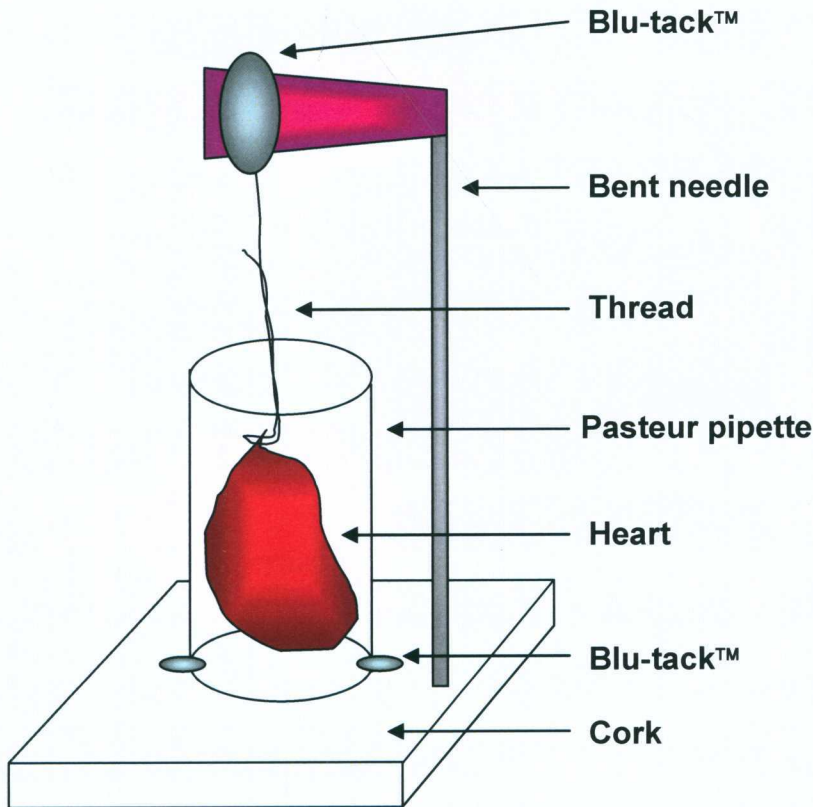


Figure 2.5 Apparatus used to freeze mouse hearts

Mouse hearts were suspended in a cylinder formed from a plastic pasteur pipette. Hearts were suspended by attaching one end of a piece of thread to the aorta and the other end to the head of a bent hypodermic needle. The whole structure was formed on a piece of cork.

Mice were anaesthetised as described above, and hearts rapidly excised and washed in ice-cold PBS. A piece of thread was attached to the aorta; the other end of the thread was attached to the head of the hypodermic needle (in the heart suspension apparatus) using Blu-Tack™. This procedure suspended the heart in the plastic cylinder. Tissue-Tek (ProSciTech, Thuringowa, Australia) was poured over the heart until the plastic cylinder was full. Bubbles were removed from the solution, and care was taken to ensure that the heart did not touch the sides of the plastic cylinder. A glass beaker containing 2-methylbutane (isopentane; Sigma, St. Louis, MO, USA) was partially submerged in liquid nitrogen until crystals began to form on the base of the beaker. At this point, the heart suspension apparatus was submerged in the isopentane until the Tissue-Tek surrounding the heart had solidified (~ 10-15 seconds). The heart suspension apparatus was removed from the isopentane solution and placed in a sealable plastic container and stored at -80°C for subsequent use.

2.9 Immunohistochemistry

Hearts were first allowed to equilibrate to the temperature inside the cryostat (-20°C; Leica, Wetzlar, Germany). The plastic cylinder formed around the hearts during freezing (see above) was carefully removed. Transverse heart cross-sections (10 µm) were cut on the cryostat and placed on poly-lysine coated slides (Sigma, St. Louis, MO, USA) which were then stored at -20°C for subsequent use. All heart cross-sections were used within 2 weeks of sectioning. All sections were allowed to equilibrate with room temperature before application of specific antibodies or dyes (see relevant chapters for details). Upon completion of the dye/antibody incubation procedure heart sections were mounted using Fluorsave (Calbiochem, Darmstadt,

Germany) in order to preserve fluorescent signal. *In situ* fluorescence was assessed using fluorescence microscopy (Zeiss, NY, USA).

2.10 *In vivo* treatment of mice with the antioxidant N-acetyl cysteine

Two N-acetyl-cysteine (NAC) treatment protocols were utilized during the course of this thesis.

2.10.1 Protocol 1

Protocol 1 was a pilot study. Mice were divided into 3 groups, WT control, *mdx* control, and *mdx* mice that received 1% NAC in their drinking water from 6 until 9 weeks of age (i.e. 3 weeks of treatment). Mice were sacrificed at 9 weeks of age, which was the age that all investigations were performed. With regard to protocol 1, only the effect of NAC-treatment on *mdx* cardiomyocyte Ca^{2+} handling was investigated (Chapter 6).

2.10.2 Protocol 2

NAC-treatment using protocol 1 appeared to have beneficial effects on Ca^{2+} handling in *mdx* cardiomyocytes (Chapter 6). The pathogenic process starts from an early age in *mdx* mice (McGeachie *et al.*, 1993). Thus, in order to prevent activation of ROS-dependent pathways, antioxidant treatment in protocol 2 was commenced as soon as practicable (i.e. when animals had been weaned). In protocol 2, mice were divided into 4 groups; WT and *mdx* control mice that received tap water, and WT and *mdx* mice that received 1% NAC (WT-NAC, *mdx*-NAC)

in their drinking water from 3 until 9 weeks of age (i.e. 6 weeks of treatment). Mice were sacrificed at 9 weeks of age, which was the age that all investigations were performed.

2.11 Statistical analysis

Data are expressed as mean $\pm$ SEM; n = number of cells, N = number of animals.

Differences between means were analysed using one-way analysis of variance (ANOVA)

or with the Students t-test (SigmaStat).

CHAPTER THREE

Defective Ca^{2+} Handling in *Mdx* Hearts

3.1 Introduction

It is widely recognized that failing hearts have defects in Ca^{2+} handling and EC coupling (Lehnart *et al.*, 1998). It is believed that these defects are due to changes in the expression levels and phosphorylation status of Ca^{2+} handling proteins (Lehnart *et al.*, 1998; Schwinger *et al.*, 1999a), in addition to alterations in the pathways that regulate these proteins (Bristow *et al.*, 1982; Brodde, 1991). *Mdx* hearts have been shown to have defects in Ca^{2+} handling, such as decreased levels of SR luminal Ca^{2+} -binding proteins (Lohan & Ohlendieck, 2004), decreased SERCA2 mRNA (Rohman *et al.*, 2003), and an increase in resting $[\text{Ca}^{2+}]_i$ (Alloatti *et al.*, 1995). However, no studies have investigated Ca^{2+} transients in *mdx* mouse hearts.

In DMD, heart failure develops with age; the number of patients exhibiting DCMs increases from one third at 14 years to all by 18 years (Nigro *et al.*, 1990). In *mdx* mice the cardiac phenotype also progresses with age, with 9 month old animals displaying cardiac dilation, reduced fractional shortening (Quinlan *et al.*, 2004), conduction defects (Bia *et al.*, 1999) and fibrosis (Wehling-Henricks *et al.*, 2005). The aim of the current study was to investigate Ca^{2+} handling in *mdx* mice hearts, with a focus on age-related changes.

3.2 Methods

3.2.1 Body and heart weights

Following excision (see Chapter 3), mouse hearts of either 2-3 month or 9-12 months of age (young and old respectively) were washed in ice-cold PSS and palpated gently to remove blood from the ventricles. Hearts were trimmed of adipose tissue, blotted dry and weighed. Both atria and the right ventricle (RV) were removed, leaving the left ventricle (LV) and septum. The LV and RV were dried and weighed. Ventricular tissue was stored at -80°C for subsequent use.

3.2.2 Isolation of cardiomyocytes and measurement of $[Ca^{2+}]_i$

Isolation of single ventricular myocytes and measurement and calibration of $[Ca^{2+}]_i$ were performed as described in Chapter 2.

3.2.3 Immunoblotting

The relative levels of SERCA2, RyR2, PLN, Serine-16 phosphorylated PLN (Ser¹⁶ p-PLN) and GAPDH were determined by quantitative immunoblotting (Chapter 2). Cardiac tissue homogenates were prepared and separated via SDS-PAGE (Bio-Rad, Hercules, CA, USA) and transferred to a nitrocellulose membrane in a Mini Trans- Blot Transfer Cell (Bio-Rad), as described in Chapter 2. Membranes were incubated with primary antibodies against SERCA2, RyR2, PLN and Ser¹⁶ p-PLN before being incubated with HRP-conjugated secondary antibodies (see Table 3.1 for conditions) and developed using an enhanced chemiluminescence detection system (Amersham BioSciences, Uppsala, Sweden). All antibodies were diluted in

PBST-block. Total protein loading was normalised using a Bradford assay (Bio-Rad). All bands were normalised to the protein GAPDH.

	SERCA2	RyR2	PLN	Ser 16 p-PLN
Lysis/Immunoblotting				
Lysis temp. (°C)	37	37	37	37
Lysis time (mins)	10	10	10	10
Separating gel (%)	10	6	15	15
Stacking gel (%)	4	4	4	4
Protein per well (µg)	4	20	15	15

Primary antibody				
Type	Mouse anti-dog cardiac SERCA2 IgG (Affinity BioReagents)	Rabbit anti-human skeletal and cardiac RyR IgG (SantaCruz)	Mouse anti-dog cardiac PLN IgG (Affinity BioReagents)	Rabbit anti-human Ser ¹⁶ phosphorylated PLN IgG (Badrilla)
Dilution	1:20,000	1:1,000	1:10,000	1:5,000
Incubation time	1 hr	Overnight	1 hr	1 hr
Incubation temperature	Room temp.	4°C	Room temp.	Room temp.
Reference	(He <i>et al.</i> , 1997)	(Zhang <i>et al.</i> , 2003)	(Radzyukevich <i>et al.</i> , 2004)	(Sanchez <i>et al.</i> , 2005)

Secondary antibody				
Type	Anti-mouse IgG (Amersham)	Anti-rabbit IgG (SantaCruz)	Anti-mouse IgG (SantaCruz)	Anti-rabbit IgG (SantaCruz)
Dilution	1:2,500	1:5,000	1:7,500	1:1,000
Incubation time	1 hr	1 hr	1 hr	1 hr
Incubation temperature	Room temp.	Room temp.	Room temp.	Room temp.

Table 3.1 Immunoblotting conditions for analysis of SERCA2, RyR2, PLN and Serine-16 phosphorylated PLN in mice hearts.

Conditions used for immunoblotting. Abbreviations: hr, hour; IgG, immunoglobulin G; PLN, phospholamban; Ser 16 p-PLN, Serine-16 phosphorylated PLN; SERCA, sarcoplasmic reticulum Ca²⁺ ATPase; temp., temperature. Company addresses: Affinity Biosciences, Golden, CO, USA; Amersham, Uppsala, Sweden, USA; Badrilla, Leeds, UK; Santa Cruz, Santa Cruz, CA, USA.

3.3 Results

3.3.1 Body and heart weights

Young *mdx* mice had increased body weights, but decreased left and right ventricle and heart weights (Table 3.2). Young *mdx* mice also had decreased LV/body and RV/body weight ratios. Old *mdx* mice were heavier than WT mice. All other parameters were unchanged in old *mdx* mice compared to WT mice.

Parameters		Wild-Type	<i>Mdx</i>
Young	BW (g)	25.1 ± 0.3 (80)	26.1 ± 0.2 (98) ***
	HW (mg)	120 ± 11 (12)	109 ± 8.8 (13) *
	RV (mg)	26.3 ± 0.9 (12)	19.1 ± 2.5 (16) **
	RV/BW (mg/g)	1.1 ± 0.02 (12)	0.78 ± 0.09 (12) **
	LV + S (mg)	88.0 ± 2.4 (19)	79.1 ± 1.2 (22) **
	LV + S/BW (mg/g)	3.5 ± 0.06 (12)	3.1 ± 0.03 (22) ***
Old	BW (g)	33.6 ± 0.5 (48)	35.0 ± 0.3 (88) ***
	HW (mg)	162 ± 3.8 (6)	159 ± 5.5 (6)
	RV (mg)	30.2 ± 1.1 (6)	30.2 ± 0.6 (6)
	RV/BW (mg/g)	0.91 ± 0.01 (6)	0.90 ± 0.01 (6)
	LV + S (mg)	120.5 ± 2.8 (6)	117 ± 5.5 (6)
	LV + S/BW (mg/g)	3.6 ± 0.10 (6)	3.48 ± 0.16 (6)

Table 3.2 Heart muscle and body weights from young and old wild-type and *mdx* mice

Abbreviations: BW, body weight; HW, heart weight; RV, right ventricle; LV+S, left ventricle + septum. * P < 0.05, ** P < 0.01, *** P < 0.001 between age-matched WT and *mdx* mice. Numbers in brackets indicate number of mice.

3.3.2 Resting $[Ca^{2+}]_i$ and Ca^{2+} transients in ventricular myocytes

It is well established that in heart failure changes occur in the Ca^{2+} handling properties of ventricular myocytes (Lehnart *et al.*, 1998). In order to determine whether similar changes occurred in ventricular myocytes from *mdx* mice, resting $[Ca^{2+}]_i$ and Ca^{2+} transients were recorded. Resting $[Ca^{2+}]_i$ was found to be higher in old *mdx* mice compared to age-matched WT (old *mdx*, 67 ± 6 nM; old WT 42 ± 3 nM; Fig. 3.1A). There was no difference in the resting $[Ca^{2+}]_i$ of young *mdx* and WT mice.

Ca^{2+} transients produced by electrical stimulation were measured in isolated ventricular myocytes from WT and *mdx* mice. Myocytes were allowed to rest for 5 minutes before a stimulation series. The first Ca^{2+} transient in a series had the largest amplitude, with subsequent transients becoming smaller until a steady-state was reached (Fig. 3.1B). We analysed both the first Ca^{2+} transient in a series and the steady-state Ca^{2+} transients. Here, results are only presented for the first Ca^{2+} transient, though similar changes were also observed in the steady-state transients. In both young and old *mdx* mice the amplitude of the first Ca^{2+} transient was greater than that of age-matched WT mice (young WT, 1003 ± 11 nM; young *mdx*, 1358 ± 18 nM; old WT, 1167 ± 6 nM; old *mdx*, 1700 ± 18 nM; Figs. 3.1C and 3.1D). The time to peak was shorter in *mdx* compared to WT mice of both ages (young WT, 77 ± 4 ms; young *mdx*, 62 ± 3 ms; old WT, 71 ± 5 ms; old *mdx*, 55 ± 3 ms; Fig. 3.1E). The time constant of Ca^{2+} transients was greater in *mdx* mice compared to age-matched WT (young WT, 134 ± 6 ms; young *mdx*, 183 ± 10 ms; old WT, 142 ± 7 ms, old *mdx* 188 ± 13 ms; Fig. 3.1F), indicating that *mdx* cardiomyocytes took longer to lower $[Ca^{2+}]_i$ following a Ca^{2+} transient. These results demonstrate that even in young *mdx* cardiomyocytes significant changes have already occurred in Ca^{2+} handling.

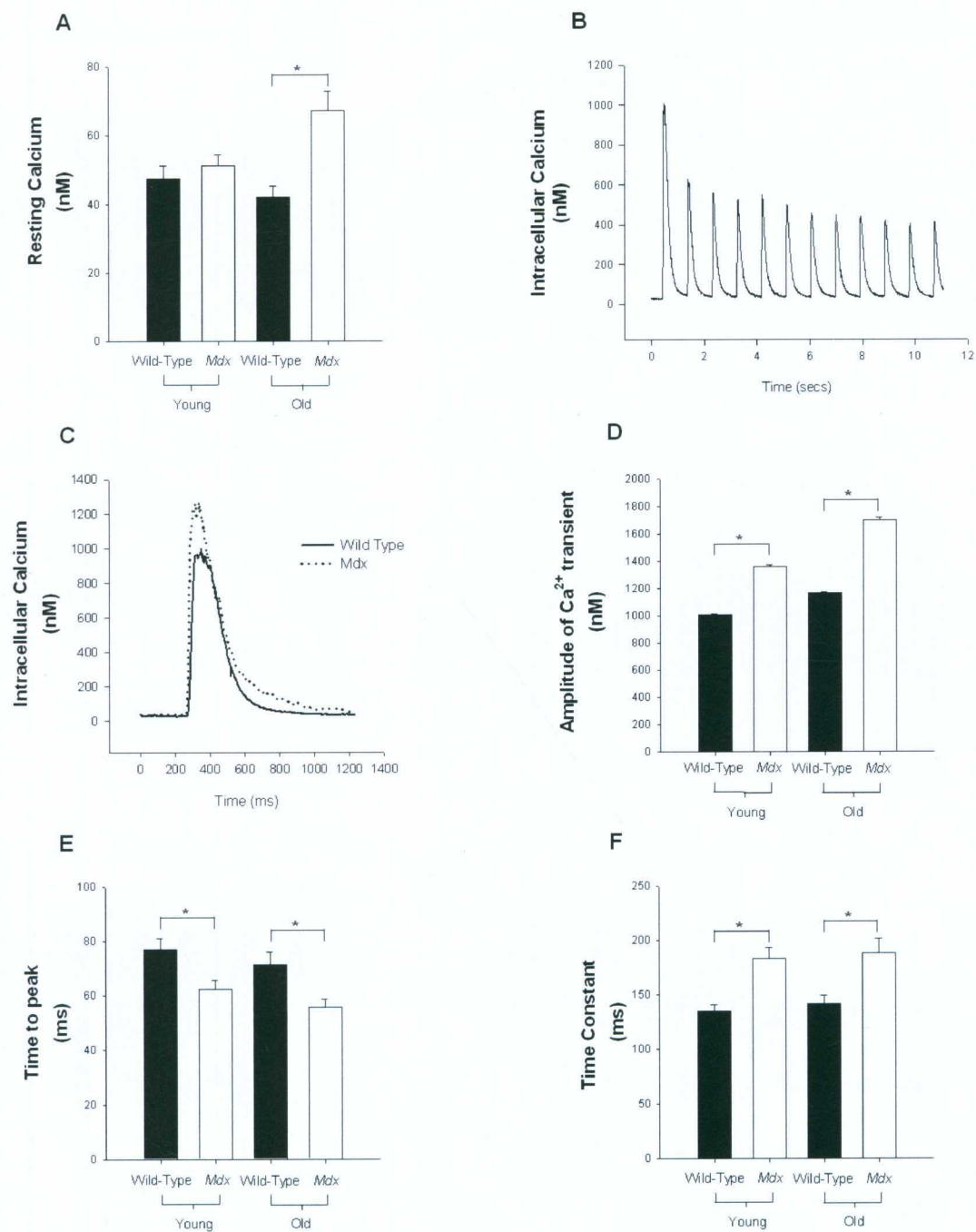


Figure 3.1 Resting $[Ca^{2+}]_i$ and Ca^{2+} transients in isolated cardiomyocytes

A: Resting $[Ca^{2+}]_i$ in young and old WT and *mdx* mice. **B:** Sample trace showing a Ca^{2+} transient series (1 Hz) in a young WT cardiomyocyte. **C:** Sample trace showing first Ca^{2+} transient from a Ca^{2+} transient series in a young WT (solid line) and *mdx* (dashed line) cardiomyocyte. Group data showing amplitude (**D**), time to peak (**E**) and time constant (**F**) of first Ca^{2+} transient in a series for young and old, WT and *mdx* mice. $N \geq 4$; $n \geq 11$. * $P < 0.05$ between indicated groups.

3.3.3 Alterations in Ca^{2+} handling protein expression levels

Immunoblotting experiments were performed on left ventricular tissue to investigate whether the observed changes in the Ca^{2+} transients of *mdx* mice (Fig. 3.1) were accompanied by alterations in the protein expression levels of Ca^{2+} handling proteins. RyR2 protein expression levels in young *mdx* mice showed a 2-fold increase compared to young WT mice (Fig. 3.2A and 3.2B), while old *mdx* hearts showed a 3-fold increase over old WT hearts. These results suggest that the increased amplitude and the shorter time to peak of the Ca^{2+} transients in *mdx* mice could be due to increased RyR2 expression which would allow for more rapid release of Ca^{2+} from the SR store.

SERCA2 and the NCX are the two primary mechanisms for returning $[\text{Ca}^{2+}]_i$ to resting levels following a Ca^{2+} transient. Thus, a decrease in either the expression and/or function of SERCA2 and/or NCX could be responsible for the slower decay (i.e. increased time constant) of Ca^{2+} transients in *mdx* mice. SERCA2 can be reversibly inhibited by PLN. In the dephosphorylated state, PLN inhibits SERCA2 function (Simmernan & Jones, 1998), while phosphorylation of PLN at Ser¹⁶ and Threonine¹⁷ following β -adrenergic stimulation reverses this inhibition. It is believed that a dynamic equilibrium exists between PLN monomers and pentamers (Cornea *et al.*, 1997), and that the PLN monomer is responsible for inhibiting SERCA2 (Autry & Jones, 1997; Kimura *et al.*, 1997).

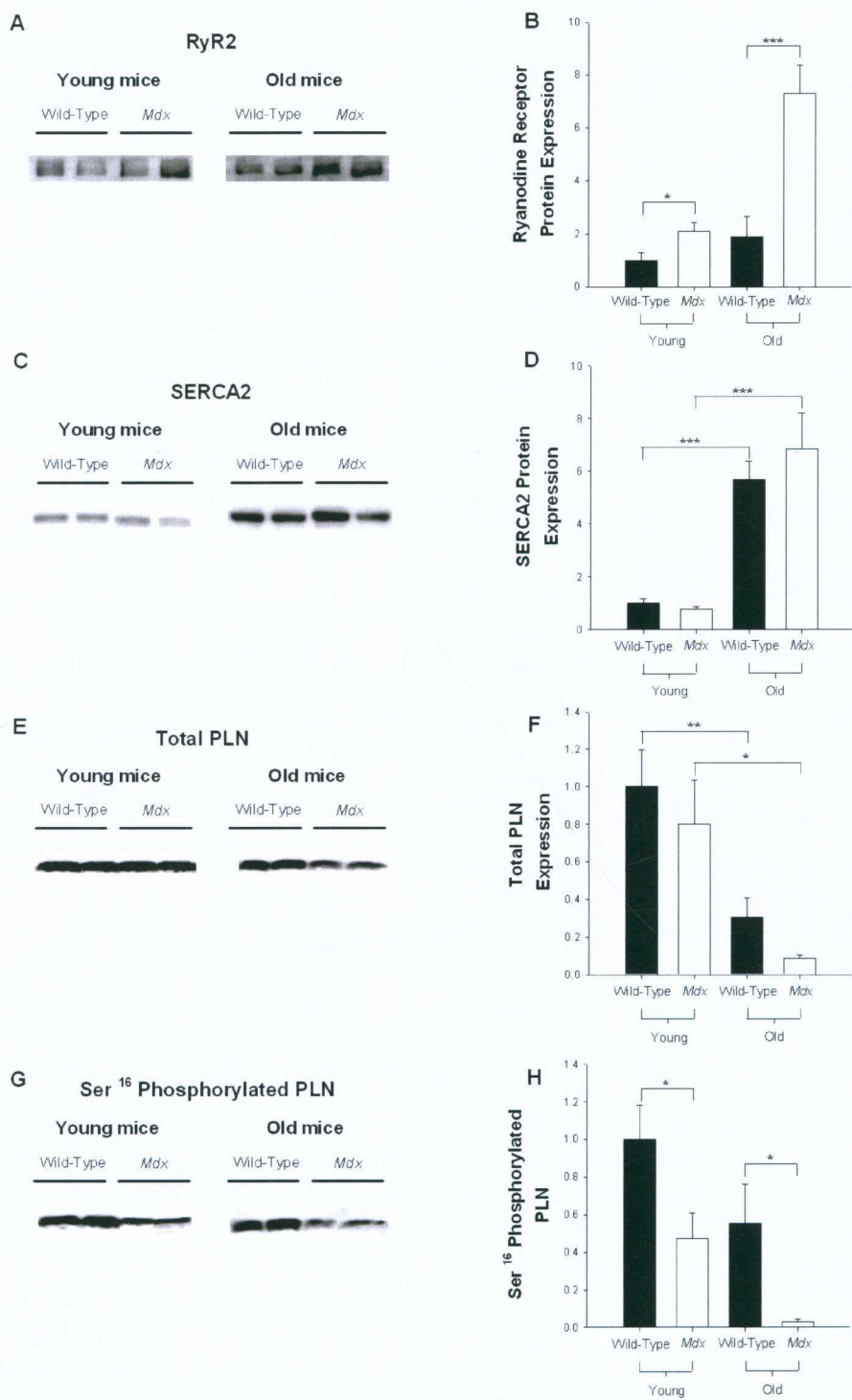


Figure 3.2 Protein expression levels of Ca²⁺ handling-related proteins

Sample immunoblots showing expression levels of **A**: RyR2 (564 kDa); **C**: SERCA2 (110 kDa); **E**: PLN (5 kDa) and **G**: Ser¹⁶ phosphorylated PLN (5 kDa). **B**, **D**, **F** and **H** are group data for figures **A**, **C**, **E**, and **G** respectively. N ≥ 5. * P < 0.05, *** P < 0.001 between indicated groups. Note: In Fig. 3.2B, an ANOVA test found no difference in RyR expression between young *mdx* and young WT mice. However, an unpaired t-test found P < 0.05.

In order to test whether decreased levels of SERCA2, and/or altered PLN or Ser¹⁶ p-PLN protein expression were responsible for the increased time constants of Ca²⁺ transients in *mdx* mice, additional immunoblotting experiments were performed. In young and old *mdx* hearts SERCA2 protein expression was not significantly different from age-matched WT hearts (Figs. 3.2C and 3.2D). Thus, a change in expression levels of SERCA2 cannot explain the increased time constants of Ca²⁺ transients in *mdx* mice. However, expression levels of Ser¹⁶ p-PLN monomer were found to be lower in *mdx* hearts compared to age-matched WT hearts (Figs. 3.2G and 3.2H). This suggests that the increased time constants of Ca²⁺ transients in *mdx* mice is due, at least in part, to increased inhibition of SERCA2 by dephosphorylated PLN monomer. In relation to protein expression of total PLN monomer (Figs. 3.2E and 3.2F), and total and Ser¹⁶ p-PLN pentamer (data not shown), no difference was found between *mdx* and age-matched WT mice.

With regard to changes in expression of EC coupling proteins with ageing, SERCA2 expression was 7-fold greater in old *mdx* hearts versus young *mdx* hearts (Fig. 3.2C and 3.2D). SERCA2 expression was 5-fold greater in old WT hearts compared to young WT hearts. In both *mdx* and WT hearts, total PLN monomer expression decreased in old mice by 3-fold and 9-fold, respectively (Figs. 3.2E and 3.2F). Ser¹⁶ p-PLN monomer was not significantly altered in young versus old mice (Figs. 3.2G and 3.2H). Although not statistically significant, a trend in Ser¹⁶ p-PLN expression with age was observed, with old WT and old *mdx* hearts decreasing by 2-fold ($P = 0.065$) and 15-fold ($P = 0.053$) respectively, versus strain-matched young mice.

3.3.4 Caffeine-induced Ca^{2+} transients

To evaluate whether the increase in time constant of Ca^{2+} transients in *mdx* mice was due to decreased removal of cytosolic Ca^{2+} via the NCX, caffeine-induced Ca^{2+} transients were measured. The peak of the Ca^{2+} transient produced by rapid application of caffeine (10 mM) provides an estimate of SR Ca^{2+} content (Bers, 2001), while the time constant is indicative of NCX function (Callewaert *et al.*, 1989). In order to achieve steady-state SR Ca^{2+} -loading, cells were stimulated at 1 Hz for 15 seconds, allowed to rest for 15 seconds and then exposed to caffeine.

In young and old *mdx* mice, the amplitude of caffeine-induced Ca^{2+} transients was increased compared to age-matched WT mice (young WT, 1006 ± 9 nM; young *mdx*, 1319 ± 9 nM; old WT, 1123 ± 8 nM; old *mdx*, 1712 ± 19 nM; Figs. 3.3A, 3.3B and 3.3C) suggesting that SR Ca^{2+} content is greater in *mdx* cardiomyocytes of both ages. In old *mdx* mice, the caffeine-induced Ca^{2+} transient time constant was decreased compared to age-matched WT mice (old WT, 3158 ± 251 ms, old *mdx*, 2053 ± 319 ms; Fig. 3.3D) suggesting that NCX function is increased. As decreased NCX function does not appear to be involved in the increased time constant of Ca^{2+} transients in *mdx* mice, attenuated SERCA2 function is probably responsible for the increased time constant.

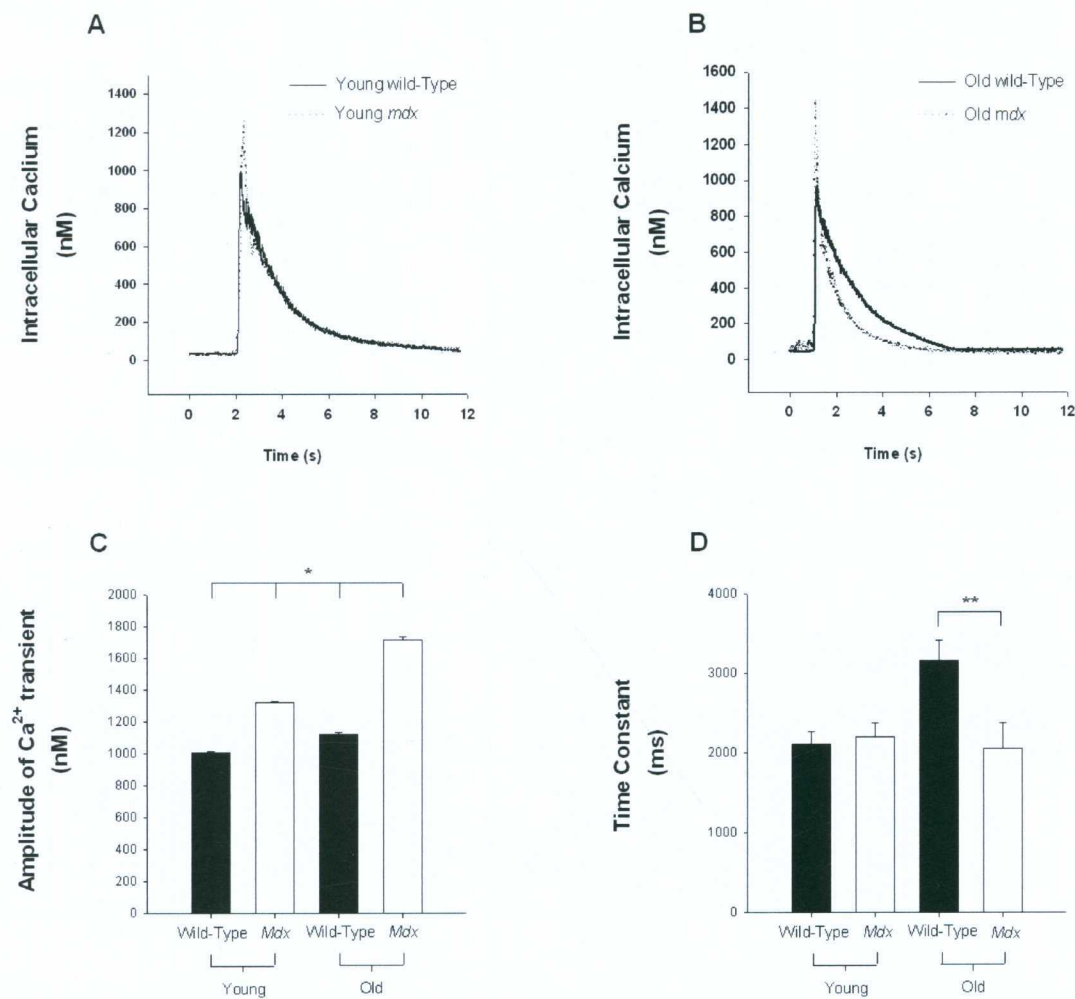


Figure 3.3 Caffeine-induced Ca^{2+} transients

Sample traces showing the effect of rapid application of 10 mM caffeine on isolated ventricular myocytes from young and old (A and B respectively) WT (solid line) and *mdx* (dashed line) cardiomyocytes. C: Group data showing amplitude (C) and time constant (D) of caffeine-induced Ca^{2+} transient in young and old, WT and *mdx* mice. $N \geq 4$; $n \geq 6$. * $P < 0.05$, ** $P < 0.01$ between indicated groups.

3.4 Discussion

It is unknown why absence of the protein dystrophin leads to heart failure in DMD. In skeletal muscle it is generally accepted that changes in Ca^{2+} handling are involved in the pathogenic process (Gillis, 1999). The aim of the current chapter was to explore *mdx* cardiomyocyte Ca^{2+} handling. The results presented are the first to demonstrate that Ca^{2+} transients and Ca^{2+} handling in *mdx* cardiomyocytes are abnormal.

3.4.1 Body and heart weights

The changes in young *mdx* body and heart weights show a similar trend to others (Lu & Hoey, 2000a). The decreased LV/body weight and RV/body weight ratios in *mdx* mice suggest heart growth is not matching body weight gains, resulting in increased cardiovascular strain.

Heart weights in old mice correlate well with findings from others who found no difference in total heart weights, LV, LV/body weight ratio, RV or RV/body weight ratio in old *mdx* mice (Bia *et al.*, 1999; Lu & Hoey, 2000a). It is unclear why *mdx* mice overcome earlier differences in heart weight. Old *mdx* body weights remained heavier than WT, which is in contrast to results presented by Lu & Hoey (2000a), and Bia *et al.* (1999). It is unclear why these opposing findings occur. Possible explanations include variations in animal housing conditions and mouse access to food.

As a point of interest, the absolute heart weights described in the present study mirror those of Lu & Hoey (2000a). For instance, old WT mice had an average heart weight of 117 mg

in the present study compared to the 116 mg presented by Lu & Hoey (2000a). In contrast, Bia et al. (1999) found the hearts of old *mdx* and WT mice were 290 mg, as opposed to the 160 mg found in the current study. It is unclear why disparity exists, but may be due to differences in weighing and drying techniques.

3.4.2 Changed resting $[Ca^{2+}]_i$ and SR Ca^{2+} content

The finding that resting $[Ca^{2+}]_i$ is raised in old *mdx* mice hearts corresponds well with previous findings from cardiac muscle (Alloatti *et al.*, 1995; Dunn & Radda, 1991). Raised resting $[Ca^{2+}]_i$ would be expected to lead to an increase in SR Ca^{2+} content due to augmented availability of Ca^{2+} for SERCA. With regard to *mdx* skeletal muscle it is disputed as to whether SR Ca^{2+} content is higher (Robert *et al.*, 2001) or lower (Plant & Lynch, 2003) than WT.

We were surprised that young *mdx* hearts had raised SR Ca^{2+} content in the absence of increased resting $[Ca^{2+}]_i$; several mechanisms may be responsible for this finding: 1) resting $[Ca^{2+}]_i$ was raised in *mdx* cardiomyocytes, but any difference was not sufficient to reach statistical significance; 2) Resting $[Ca^{2+}]_i$ was raised in small microdomains (Davidson & Duchen, 2006) within *mdx* cardiomyocytes which were not detected due to the limited spatial resolution of the current imaging technique. Thus, in the present study, although global resting $[Ca^{2+}]_i$ was not increased in young *mdx* hearts, small microdomains of increased $[Ca^{2+}]_i$ may result in increased SR Ca^{2+} uptake. As the *mdx* mouse ages, localised increases in $[Ca^{2+}]_i$ in microdomains may summate, leading to the global increase in resting $[Ca^{2+}]_i$ found in old

mdx hearts. In support of the Ca^{2+} microdomain concept, Mallock et al. (2000) have described elevated levels of subsarcolemmal Ca^{2+} in *mdx* skeletal muscle.

Interestingly, previous work has shown that luminal SR Ca^{2+} binding proteins are reduced in *mdx* mice hearts (Lohan & Ohlendieck, 2004), suggesting that the increased SR Ca^{2+} content found in the current study may be influenced in a complex manner by the resting $[\text{Ca}^{2+}]_i$, the rate of Ca^{2+} uptake by the SR, and a reduction in bound SR Ca^{2+} due to changes in SR Ca^{2+} binding proteins.

3.4.3 Increased amplitude and time to peak of *mdx* cardiomyocyte Ca^{2+} transients

The increase in the amplitude of Ca^{2+} transients in *mdx* cardiomyocytes was unexpected, as other models of heart failure typically have a decreased amplitude (O'Rourke *et al.*, 1999). However, an increase in the amplitude of Ca^{2+} transients has been reported in trabeculae from failing human and rat hearts (Vahl *et al.*, 1994; Ward *et al.*, 2003). The time to the peak of the Ca^{2+} transient was shorter in *mdx* mice, which again is not usually found in established DCM (Sen *et al.*, 2000). However, the raised SR Ca^{2+} levels in *mdx* cardiomyocytes would be expected to lead to augmentation of the amplitude of Ca^{2+} transients, as found in the present study (Bers, 2001; Lindegger & Niggli, 2005). Alternatively, the augmented amplitude of *mdx* Ca^{2+} transients and the shorter time to peak of Ca^{2+} transients might be the result of increased RyR2 protein expression. This argument is based on the idea that increased RyR2 protein expression would, assuming all other parameters were equal, allow more Ca^{2+} to leave the SR (i.e. greater amplitude) and at a

greater rate (i.e. shorter time peak). Interestingly, Rohman et al. (2003) have reported a decrease in *mdx* heart RyR2 mRNA expression, so that in order to explain the increased RyR2 protein expression it seems possible that RyR2 degradation is decreased in *mdx* hearts.

Studies have suggested that human DMD skeletal myotubes have altered activity of the RyR (Deval *et al.*, 2002), with increased SR Ca^{2+} leakage possibly due to changes in RyR function (Takagi *et al.*, 1992). However, others have found no difference in SR Ca^{2+} leak in *mdx* skeletal muscle (Plant & Lynch, 2003). Although the mechanism of SR Ca^{2+} release in cardiac and skeletal muscle differ, changes in *mdx* skeletal muscle RyR function indicates that RyR and SR Ca^{2+} release are altered in the absence of dystrophin.

3.4.4 Increased time constants of *mdx* cardiomyocyte Ca^{2+} transients

This study is the first to demonstrate that young and old *mdx* mice have decreased Ca^{2+} transient time constant. This slowing of the rate of removal of cytosolic Ca^{2+} following a Ca^{2+} transient occurs without any change in SERCA2 protein expression, which is in accord with findings from DCM patients (Schwinger *et al.*, 1995).

The rate at which intracellular Ca^{2+} declines following a Ca^{2+} transient, is dependent on Ca^{2+} being removed from the intracellular milieu; the two main pathways through which this occur are active pumping of Ca^{2+} back into the SR via SERCA, or Ca^{2+} efflux across

the sarcolemma via the NCX. Thus, we explored these two mechanisms to elucidate whether they were dysfunctional in *mdx* cardiomyocytes.

In *mdx* mice of both ages, reduced SERCA2 function appears to be responsible for the increased time constant of Ca^{2+} transients, as NCX function is not decreased. In fact, NCX function was increased in old *mdx* mice, as found in other models of heart failure (Bers, 2001). As SERCA2 expression was unchanged in *mdx* mice, inhibition of SERCA2 by PLN monomer appears to be responsible for the increased time constant of Ca^{2+} transients in *mdx* mice. Total PLN monomer was unchanged between age-matched *mdx* and WT hearts. However, Ser¹⁶ p-PLN monomer was decreased in *mdx* compared to age-matched WT mice, implying that PLN was present in its dephosphorylated inhibitory state. Thus, reduced SERCA2 function is, at least partly, the result of increased inhibition via dephosphorylated PLN monomer. This finding correlates well with findings from patients with DCM (Schwinger *et al.*, 1999a) and from rat models of myocardial infarction (Huang *et al.*, 1999; Sande *et al.*, 2002). A reduction in p-PLN monomer may result from β -adrenoceptor down-regulation which is thought to occur in human heart failure (Bristow *et al.*, 1982) and *mdx* mice (Lu & Hoey, 2000a).

3.4.5 Ageing and Ca^{2+} handling

The current study found that old WT and *mdx* hearts had increased SERCA2 expression compared to young mice, which was surprising as others have reported no change (Isenberg *et al.*, 2003; Kaplan *et al.*, 2007) or a decrease in protein levels (Janczewski *et al.*, 2002). It is unclear why this disparity occurs, but may be due to differences in the age and species of

animals used by different research groups. The present study found that total PLN was decreased in old WT and *mdx* mice compared to young mice, which is in accord with trends presented by others (Slack *et al.*, 2001).

It is disputed whether ageing of adult hearts affects the time constant of Ca^{2+} transients (Howlett *et al.*, 2006; Isenberg *et al.*, 2003; Lim *et al.*, 2000); in the current study no change was found in *mdx* or WT hearts. On first inspection, this observation is surprising, as one might predict the time constant to be reduced due to the finding that SERCA2 expression was increased, and total PLN was decreased in *mdx* and WT old hearts. This conflict can be reconciled by the knowledge that SERCA activity is decreased in isolated SR vesicles extracted from aged animals (Froehlich *et al.*, 1978; Knyushko *et al.*, 2005). In addition, although not statistically significant, PLN phosphorylation was decreased in old hearts, implying that more PLN was present in its dephosphorylated inhibitory state. Importantly, the ability of PLN to be phosphorylated, and SERCA's responsiveness to PLN phosphorylation is not affected by age (Jiang & Narayanan, 1990). Thus, in the current study, the increase in SERCA2 expression may be offset by a decrease in SERCA2 activity; the net result being no change in the time constant of Ca^{2+} transients in young versus old cardiomyocytes.

3.4.6 Are changes in Ca^{2+} handling an early pathological event?

A key question that arises from the findings in the current chapter is whether changes in *mdx* myocardial Ca^{2+} handling are an early pathological event or whether they represent a compensatory mechanism that occurs later in disease progression? Furthermore, is there a

direct connection between *mdx* myocardial Ca^{2+} handling defects and absence of dystrophin?

With regard to *mdx* hearts, data presented in the later chapters of this thesis suggest that changes in Ca^{2+} handling may, in part, occur secondary to alterations in intracellular ROS levels. However, in relation to other forms of DCM, the importance of Ca^{2+} handling defects in disease progression is unclear. In some cases, mutations in Ca^{2+} handling proteins appear to be the primary defect. However, in other forms of DCM it is uncertain if Ca^{2+} handling changes are the cause of myocardial dysfunction or secondary consequences of abnormal cardiac remodelling during the development of heart failure.

With regard to the RyR2, the general consensus is that altered RyR2 function occurs secondary to other changes, such as β -adrenergic signalling (Lehnart *et al.*, 2004). No RyR2 mutations have been found to be associated with DCM, although mutations appear to cause arrhythmogenic right ventricular dysplasia type 2 (Tiso *et al.*, 2001). To the best of my knowledge, no DCM-inducing mutations have been reported in the SERCA gene. However, overexpression of SERCA confers some protection in animal models of heart failure (Miyamoto *et al.*, 2000). A mutation in the human PLN gene results in a lethal DCM (Haghighi *et al.*, 2003; Schmitt *et al.*, 2003). In this situation, changed Ca^{2+} handling appears to be the primary pathology. In support of this finding is data showing that in animal models of DCM, procedures that reduce the inhibitory action of PLN improve cardiac function (Hoshijima *et al.*, 2002; Minamisawa *et al.*, 1999).

In light of the above findings, it appears that two paradigms may exist with regard to the importance of Ca^{2+} handling defects in the pathogenesis of DCMs. In the first paradigm, defective Ca^{2+} handling is the primary cause of heart failure. However, in the second paradigm, defective Ca^{2+} handling is most likely a final common pathway, as the diverse aetiology and plethora of genes associated with the disease suggest that several different pathological mechanisms lead to heart failure (Fatkin & Graham, 2002). This hypothesis is supported by the finding that β -adrenergic blockade restores defective Ca^{2+} handling in an animal model of DCM (Plank *et al.*, 2003), implying that aberrant Ca^{2+} handling occurs secondary to alterations in β -adrenergic signalling. However, regardless of whether changes in Ca^{2+} handling are the primary cause, or a secondary consequence of cardiac dysfunction, it is clear that changes in Ca^{2+} handling are a hallmark of heart failure. Thus, they provide an attractive therapeutic target in the treatment of DCM.

3.5 Conclusion

In summary, Ca^{2+} handling is altered in *mdx* cardiomyocytes. The changes in Ca^{2+} handling may, in part, be due to changes in SR Ca^{2+} content and differential expression of Ca^{2+} handling proteins. It is interesting to note that although young *mdx* mice display abnormal Ca^{2+} homeostasis and Ca^{2+} dynamics, they do not show signs of heart failure (Quinlan *et al.*, 2004). This observation suggests that abnormalities in myocardial Ca^{2+} handling could play a role in the development of the cardiac damage which precedes heart failure.

CHAPTER FOUR

The Role of Stretch-Activated Channels in *Mdx* Hearts

4.1 Introduction

The previous chapter presented data showing that the Ca^{2+} handling properties of *mdx* cardiomyocytes are defective. This raises the question, how does absence of dystrophin lead to Ca^{2+} handling defects? One hypothesis to explain the increase in resting $[\text{Ca}^{2+}]_i$ seen in *mdx* mice hearts is that dystrophin is involved in regulating sarcolemmal channels and, in its absence, some channels are abnormally regulated, as established for the L-type Ca^{2+} channel and aquaporin (Frigeri *et al.*, 2001; Woolf *et al.*, 2006). One channel of particular interest is the SAC. It is believed that the canonical transient receptor potential channel 1 (TRPC1) gene encodes the mammalian SAC (Maroto *et al.*, 2005). In both the *mdx* mouse and DMD, the lack of dystrophin results in increased activity of SACs in skeletal muscle (Franco-Obregon, Jr. & Lansman, 1994; Vandebrouck *et al.*, 2001). The resulting increase in resting $[\text{Ca}^{2+}]_i$ is thought to activate proteases and has been implicated in the pathogenesis of skeletal muscle damage in DMD (Yeung *et al.*, 2005). SACs have been reported in ventricular cells (Craelius *et al.*, 1988) and are proposed to have a role in tachycardia-induced chronic heart failure (Clemo *et al.*, 1998). However, the role of SACs in the DCM associated with DMD is unknown. Thus, the aim of the current chapter was to investigate the role of SACs and Ca^{2+} handling in *mdx* mice hearts, with a focus on age-related changes.

4.2 Methods

4.2.1 Animals

Experiments were performed on male WT and *mdx* mice, of either 2-3 months or 9-12 months of age (young and old, respectively).

4.2.2 Isolation of cardiomyocytes and measurement of $[Ca^{2+}]_i$

Isolation of single ventricular myocytes, and measurement and calibration of $[Ca^{2+}]_i$ were performed as described in Chapter 2.

4.2.3 Effect of stretch-activated channel inhibitors on resting $[Ca^{2+}]_i$

The effect of two SAC inhibitors on resting $[Ca^{2+}]_i$ levels in quiescent, un-stretched cardiomyocytes was investigated. The two SAC inhibitors used were streptomycin (100 μ M) (Hamill & McBride, Jr., 1996) and the spider venom toxin GsMTx-4 (10 μ M) (Suchyna *et al.*, 2000).

4.2.4 Effect of streptomycin on Ca^{2+} transients

Isolated cardiomyocytes were stimulated at 1 Hz and at 10% suprathreshold. The viability of cells was confirmed by performing three Ca^{2+} transient series in control physiological salt solution (PSS) over a period of 5 minutes; cardiomyocytes were discarded if the properties of the Ca^{2+} transients deteriorated during this period. Healthy cells were then

perfused with a PSS solution containing 100 μM streptomycin or 10 μM GsMTx-4 for 5 minutes, before cells were stimulated and Ca^{2+} transients recorded.

4.2.5 TRPC1 protein expression

The relative level of TRPC1 was determined by quantitative immunoblotting. Cardiac tissue homogenates were prepared and separated via SDS-PAGE (see Chapter 2). In brief, tissue homogenate was solubilized in Laemmli sample buffer and heated at 95°C for 5 minutes. 15 μg of protein/well was loaded onto a 4% stacking gel and separated via SDS-PAGE on a 10% separating gel. Following transfer, membranes were blocked before being incubated overnight at 4°C with rabbit anti-human TRPC1 IgG primary antibody at a dilution of 1:800 (Alomone, Jerusalem, Israel). Membranes were washed, before being incubated for 1 hour at room temperature with anti-rabbit IgG HRP-conjugated secondary antibody at a dilution of 1:1000 (SantaCruz, Santa Cruz, CA, USA) and developed using an enhanced chemiluminescence detection system. All antibodies were diluted in PBST-block. Total protein loading was normalised using a Bradford assay (Bio-Rad, Hercules, CA, USA). All bands were normalised to the protein GAPDH.

4.3 Results

4.3.1 Effect of stretch-activated channel inhibitors on resting $[Ca^{2+}]_i$

In order to ascertain if the raised resting $[Ca^{2+}]_i$ found in old *mdx* mice (Chapter 3, Fig. 3.1A) was due to increased Ca^{2+} influx through SACs, the effect of the SAC channel inhibitor streptomycin on resting $[Ca^{2+}]_i$ was assessed.

Streptomycin (100 μ M) reduced resting $[Ca^{2+}]_i$ in old *mdx* cardiomyocytes by $15 \pm 4\%$, but had no effect on old WT mice (Fig. 4.1B). In order to confirm that the action of streptomycin was through blocking SACs, the more potent and specific SAC inhibitor GsMTx-4 (Suchyna *et al.*, 2000) was also used. GsMTx-4 (10 μ M) reduced resting $[Ca^{2+}]_i$ in old *mdx* mice by $35 \pm 4\%$ from $159 \pm 13\%$ to $107 \pm 8\%$ of the old WT value (Fig. 4.1C). GsMTx-4 had no effect on old WT mice.

Streptomycin had no effect on resting $[Ca^{2+}]_i$ in young *mdx* or WT cardiomyocytes (Fig. 4.1A). This result was not surprising, as resting $[Ca^{2+}]_i$ was not significantly raised in young *mdx* hearts. However, any increase in Ca^{2+} influx through SACs may have been too small to be detected in young *mdx* hearts (see Chapter 3, Discussion, *Changed resting $[Ca^{2+}]_i$ and SR Ca^{2+} content*). To circumvent this issue, resting $[Ca^{2+}]_i$ was raised in the hope of amplifying any previously undetectable Ca^{2+} influx through SACs.

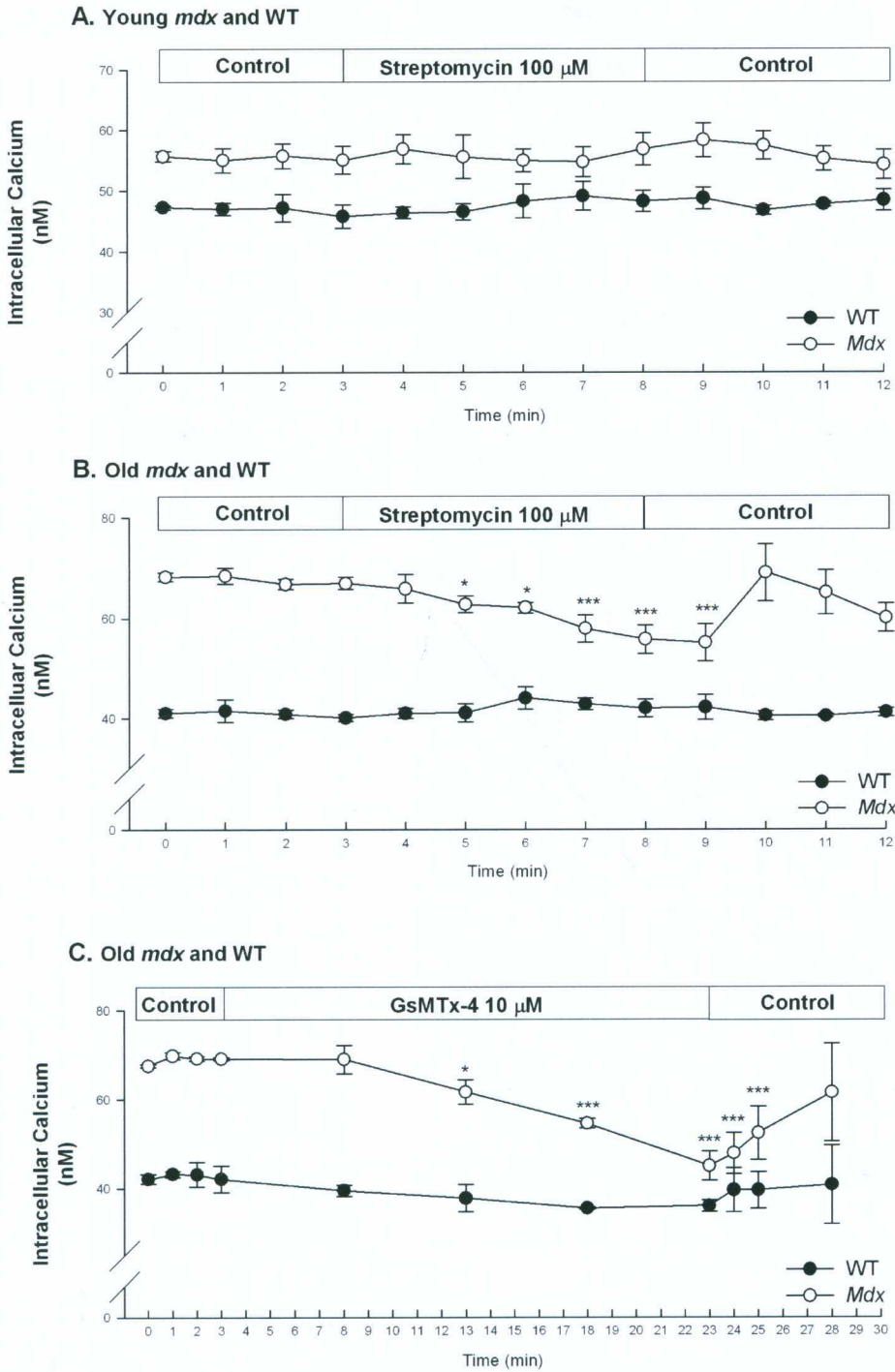


Figure 4.1. Effect of streptomycin and GsMTx-4 on resting $[Ca^{2+}]_i$ in young and old, WT and *mdx* cardiomyocytes

A: Effect of 100 μ M streptomycin on resting $[Ca^{2+}]_i$ levels in young WT and *mdx* cardiomyocytes ($\bullet$ WT, $\circ$ *mdx*). **B:** Effect of 100 μ M streptomycin on resting $[Ca^{2+}]_i$ levels in old WT and *mdx* cardiomyocytes. $N \geq 4$; $n \geq 4$. **C:** Effect of spider toxin GsMTx-4 (10 μ M) on resting $[Ca^{2+}]_i$ in old WT and *mdx* cardiomyocytes. $N = 3$ for both groups. * $P < 0.05$, *** $P < 0.001$ between control and treatment in old *mdx* cardiomyocytes.

A correction factor was applied to each individual experiment presented in Figure 4.1. It is important to note that prior to application of the correction factor, analysis of individual experiments suggested that streptomycin and GsMTx-4 reduced resting $[Ca^{2+}]_i$ in old *mdx* mice, but had no effect on WT resting $[Ca^{2+}]_i$. However, due to variability in the resting $[Ca^{2+}]_i$ within experimental groups the effect of the SAC inhibitors on resting $[Ca^{2+}]_i$ did not reach statistical significance when analysing pooled data. In order to reduce the number of mice required to overcome the variability within groups a correction factor was applied to data. The correction factor was calculated for each individual experiment. The correction factor was calculated by comparing the average resting $[Ca^{2+}]_i$ obtained from a particular experiment with the average resting $[Ca^{2+}]_i$ calculated from earlier calibration experiments (Figure 3.1A). The ratio of these two values was used to correct all data points within that particular experiment. It is important to note that trends present in raw data were not altered by applying the correction factor.

In order to increase resting $[Ca^{2+}]_i$ in cells, cardiomyocyte were perfused with a PSS containing 4mM Ca^{2+} ; this induced a rapid increase in resting $[Ca^{2+}]_i$, which reached a plateau (Fig. 4.2A). In preliminary investigations, application of streptomycin (100 μ M) during the plateau phase had no effect on resting $[Ca^{2+}]_i$ in either young *mdx* (Fig. 4.2A) or young WT mice (data not shown); consequently these experiments were discontinued. However, increasing extracellular Ca^{2+} to 4 mM caused almost a 2-fold greater increases in *mdx* resting $[Ca^{2+}]_i$ compared to WT (WT, 11 ± 0.9 nM; *mdx* 20 ± 2.3 nM; Fig. 4.2B and 4.2C). This finding suggests that *mdx* cardiomyocytes are not able to regulate $[Ca^{2+}]_i$ following increased Ca^{2+} influx as well as WT cardiomyocytes.

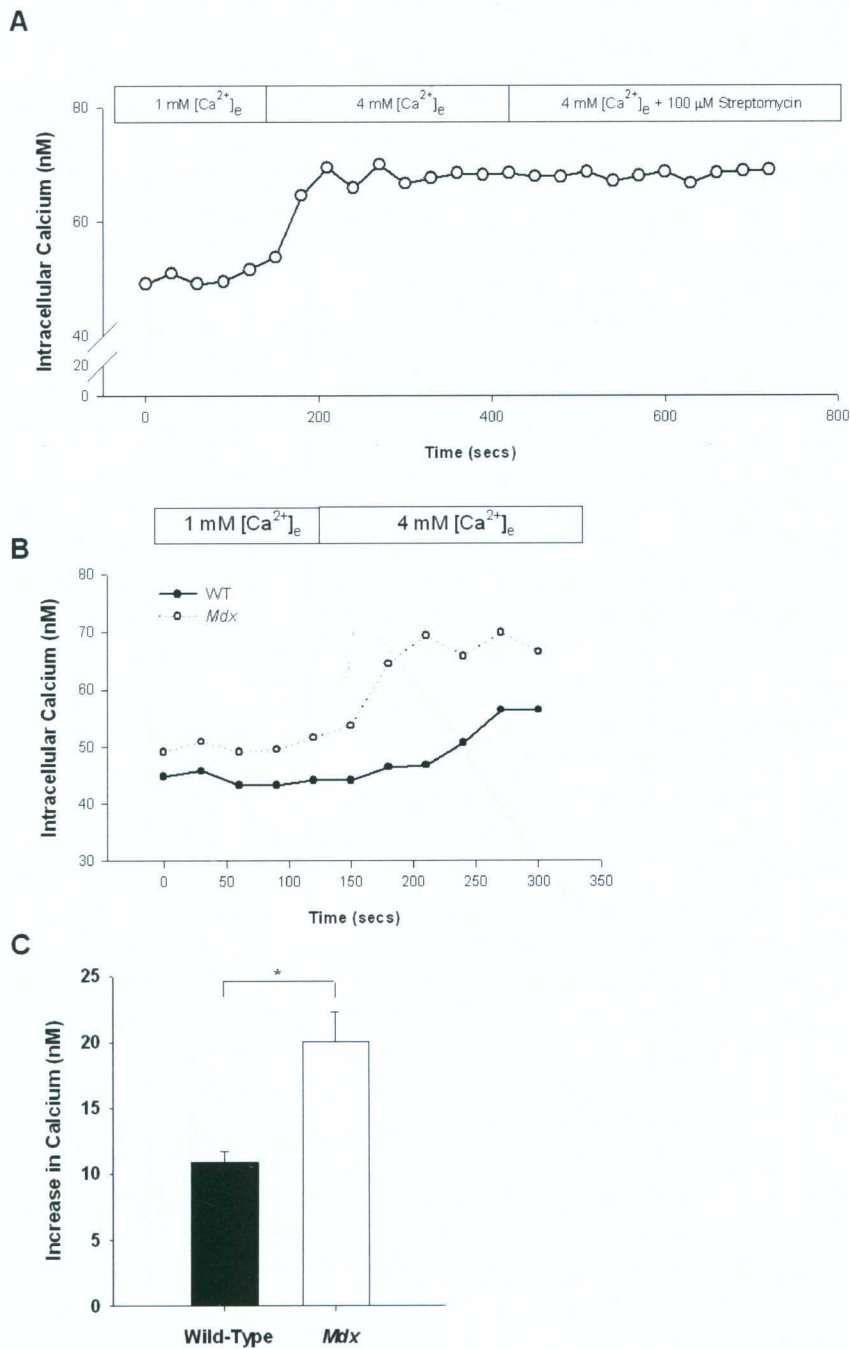


Figure 4.2 Effect of streptomycin on resting $[Ca^{2+}]_i$ in the presence of increased extracellular $[Ca^{2+}]$

A: Sample trace showing effect of 100 μ M streptomycin on resting $[Ca^{2+}]_i$ following exposure of cardiomyocytes to 4 mM extracellular Ca^{2+} ($[Ca^{2+}]_e$). **B:** Sample trace showing rise in resting $[Ca^{2+}]_i$ following exposure to 4 mM $[Ca^{2+}]_e$ in young WT and *mdx* cardiomyocytes ($\bullet$ WT, $\circ$ *mdx*). **C:** Group data showing absolute increase in resting $[Ca^{2+}]_i$ in *mdx* and WT cardiomyocytes following exposure to 4 mM $[Ca^{2+}]_e$. $N \geq 4$; * $P < 0.05$ between indicated groups.

4.3.2 Effect of streptomycin on $[Ca^{2+}]_i$ transients

As discussed in Chapter 3, increased SR Ca^{2+} content may account for the increased amplitude of *mdx* cardiomyocyte Ca^{2+} transients. As raised resting $[Ca^{2+}]_i$ may lead to increased SR Ca^{2+} content, it was predicted that if streptomycin reduced resting $[Ca^{2+}]_i$ in old *mdx* mice (Fig. 4.1B), it may also be able to reduce the amplitude of Ca^{2+} transients. As shown in Figure 4.3 streptomycin (100 μ M) reduced the amplitude of Ca^{2+} transients in old *mdx* mice (1746 ± 10 nM and 1638 ± 35 nM before and after streptomycin application, respectively), but had no significant effect on any other Ca^{2+} transient parameters. Streptomycin had no effect on young WT, old WT or young *mdx* cardiomyocyte Ca^{2+} transients.

4.3.3 TRPC1 protein expression

To further explore the finding that streptomycin and GsMTx-4 reduced resting $[Ca^{2+}]_i$ in old *mdx* cardiomyocytes, immunoblotting experiments were performed to evaluate TRPC1 protein expression levels. TRPC1 was found to be present in all groups studied. Control experiments using the TRPC1 blocking peptide (antigen) prevented TRPC1 expression detection (data not shown). There was no difference in the expression levels of TRPC1 between young *mdx* and WT mice. However, old *mdx* mice displayed a 3-fold increase in TRPC1 protein expression compared to old WT mice (Fig. 4.4A & 4.4B). The above findings suggest that increased protein expression of TRPC1 could play a role in the observed increase in resting $[Ca^{2+}]_i$ in old *mdx* mice.

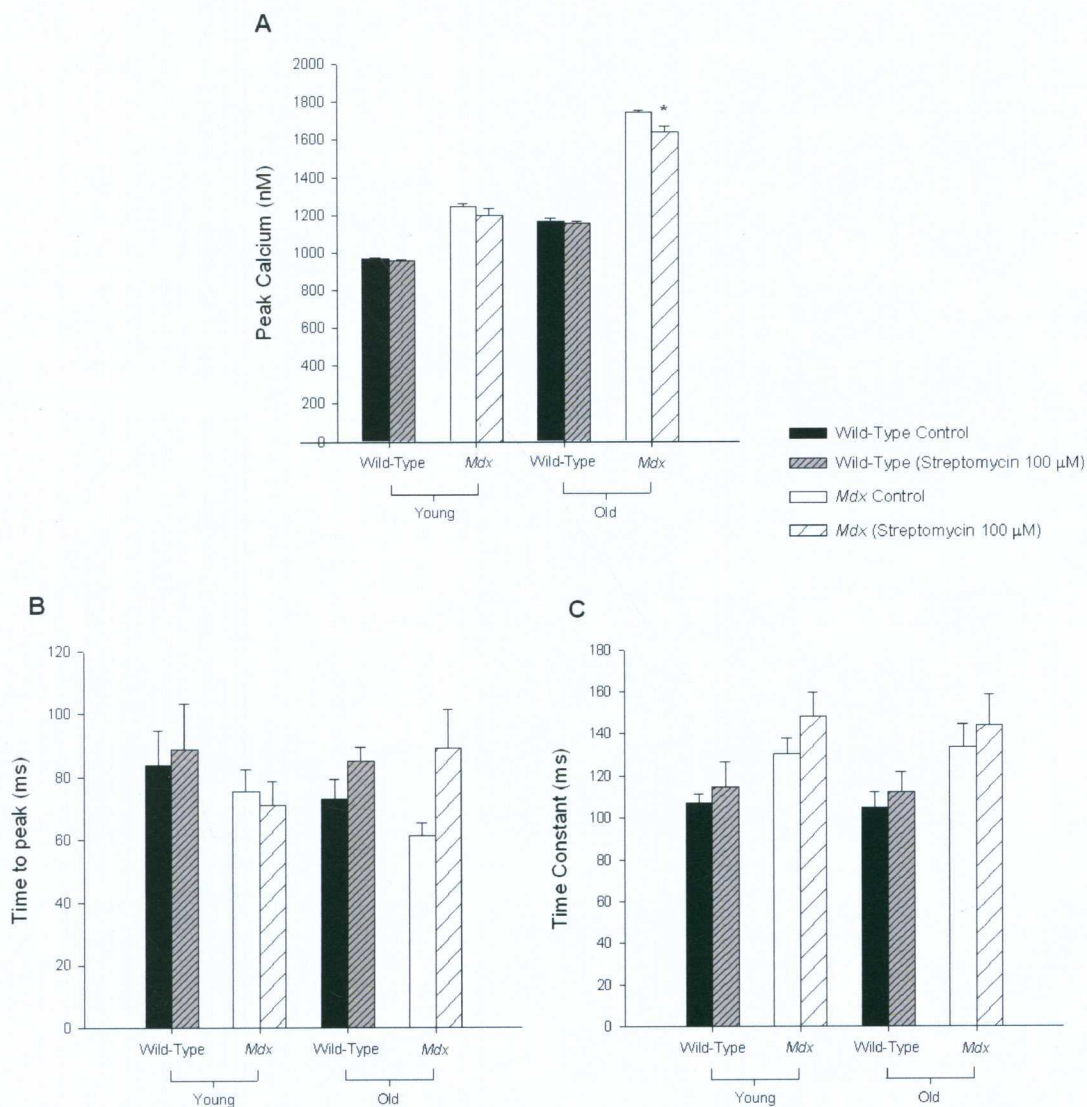


Figure 4.3 Effect of streptomycin on Ca^{2+} transients in isolated cardiomyocytes

Effect of acute exposure of streptomycin (100 μ M) on the amplitude (A), time to peak (B) and time constant (C) of Ca^{2+} transients in young and old, *mdx* and WT cardiomyocytes. $N \geq 3$, $n \geq 4$. * $P < 0.05$ paired t-test between old *mdx* control and old *mdx* streptomycin-treated group.

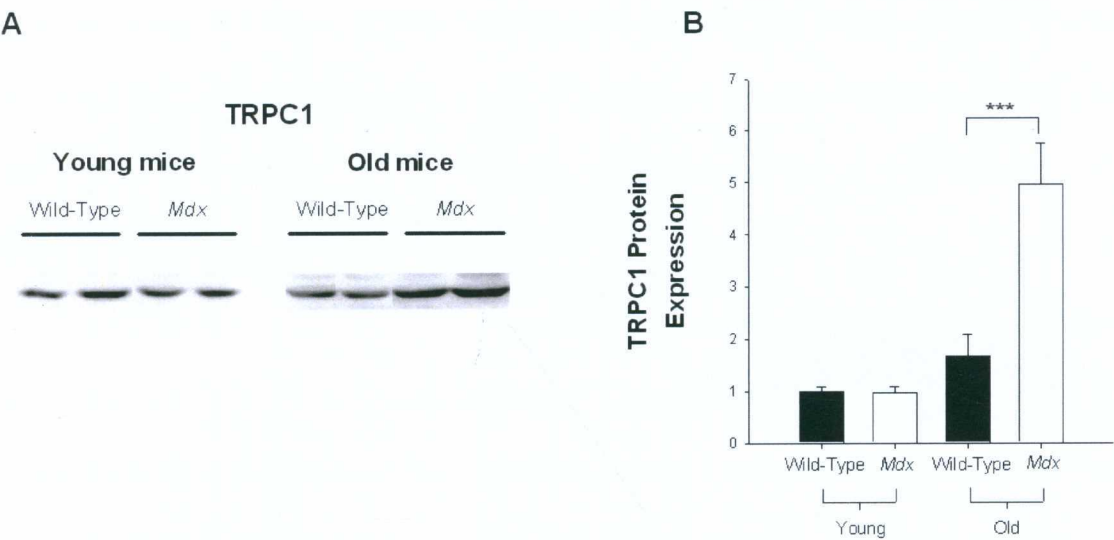


Figure 4.4 TRPC1 protein expression

A: Sample immunoblots showing expression levels of TRPC1 in young and old, WT and *mdx* mice. Bands represent proteins with a molecular weight of 85 kDa. **B:** Group data from A. $N \geq 6$. $^{+++} p < 0.001$ between indicated groups.

4.3.4 Effect of hydrogen peroxide on resting $[Ca^{2+}]_i$: role of SACs

Oxidative damage and SACs have been implicated in the pathology of DMD skeletal muscle. It has previously been shown in cardiomyocytes that a sarcolemmal non-selective cation current is activated by ROS (Jabr & Cole, 1995). We hypothesized that in *mdx* cardiomyocytes ROS may be able to increase the opening probability of SACs, resulting in increased Ca^{2+} influx and raised resting $[Ca^{2+}]_i$. The rise would be expected to be greatest in old *mdx* cardiomyocytes due to their increased expression of TRPC1. To test this hypothesis, hydrogen peroxide (10 μ M) was applied to fluo-4 loaded resting cardiomyocytes and resting $[Ca^{2+}]_i$ recorded. Hydrogen peroxide induced a similar increase in resting $[Ca^{2+}]_i$ in both young *mdx* and WT cardiomyocytes (data not shown). Thus, experiments involving young mice were discontinued. Hydrogen peroxide increased resting $[Ca^{2+}]_i$ in old *mdx* cardiomyocytes over 2-fold more than in WT cardiomyocytes (Fig. 4.5A and 4.5B). In order to test if the augmented hydrogen peroxide induced rise in resting $[Ca^{2+}]_i$ in old *mdx* cardiomyocytes was due to ROS-induced opening of SACs, streptomycin was applied to cells. In preliminary experiments, streptomycin (100 μ M) returned the hydrogen peroxide induced rise in resting $[Ca^{2+}]_i$ close to starting levels (Fig. 4.5C). To confirm that streptomycin's action was not the consequence of fluo-4 leaking out of cells or loss of cell viability, streptomycin was removed from the perfusate when resting $[Ca^{2+}]_i$ had returned close to baseline. Once again, hydrogen peroxide induced a rise in resting $[Ca^{2+}]_i$ confirming the earlier action of streptomycin (Fig. 4.5C). Due to the limited number of experiments performed regarding the effects of hydrogen peroxide, statistical analysis of the data was not possible. However, these preliminary findings suggest that ROS increase SAC opening, resulting in increased Ca^{2+} influx into cardiomyocytes.

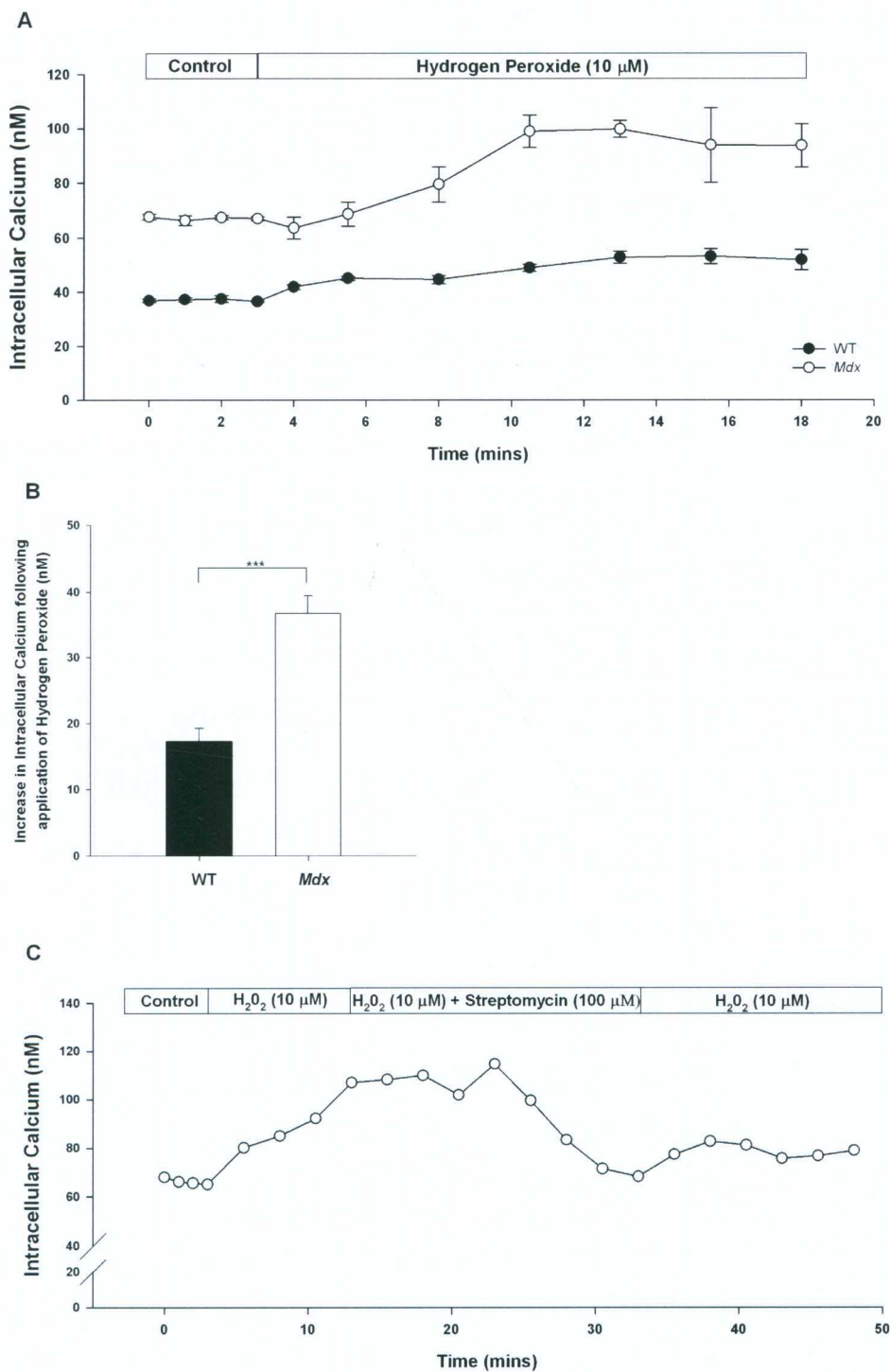


Figure 4.5 Effect of hydrogen peroxide on resting $[Ca^{2+}]_i$ in old *mdx* and WT cardiomyocytes

Average effect of hydrogen peroxide (10 μ M) on resting $[Ca^{2+}]_i$ (A) and maximum increase in resting $[Ca^{2+}]_i$ (B) in old *mdx* and WT cardiomyocytes. C: Sample trace showing effect of streptomycin (100 μ M) on the hydrogen peroxide (10 μ M) induced rise in resting $[Ca^{2+}]_i$. $N \geq 4$, $n \geq 4$. *** $p < 0.001$ between indicated groups.

4.4 Discussion

The aim of the current chapter was to explore the possibility that absence of dystrophin leads to increased activity of SACs and contributes towards dysfunctional cardiomyocyte Ca^{2+} handling. This study is the first to explore the role of SACs and TRPC1 in hearts from *mdx* mice. The data suggests that SACs, and in particular TRPC1, may play a role in the pathogenesis of the cardiomyopathy associated with DMD.

4.4.1 What causes changes in intracellular Ca^{2+} handling

Two main hypotheses are proposed to explain the raised $[\text{Ca}^{2+}]_i$ in *mdx* muscle. The first proposes that lack of dystrophin renders the sarcolemma more susceptible to damage and, as a result, Ca^{2+} leaks into cells via membrane ruptures (Head *et al.*, 1992; Petrof *et al.*, 1993). However, there is little direct evidence that increased membrane fragility causes the increased membrane permeability to Ca^{2+} .

The second hypothesis suggests that dystrophin, directly or indirectly, regulates channels involved in $[\text{Ca}^{2+}]_i$ homeostasis. In the absence of dystrophin, the activity of these channels becomes greater and causes increased Ca^{2+} entry into *mdx* myocytes. One group of channels that may be regulated via such a mechanism are SACs.

SACs are more active in skeletal muscle of *mdx* mice (Franco-Obregon, Jr. & Lansman, 1994) and in humans with DMD (Vandebrouck *et al.*, 2001). Moreover, increased influx of Ca^{2+} through these channels has been demonstrated in *mdx* skeletal muscle (Yeung *et al.*,

2005). The results from the present study in dystrophic cardiac muscle suggest that SACs might be involved in the DCM found in DMD.

In quiescent, un-stretched young *mdx* cardiomyocytes streptomycin had no effect on Ca^{2+} handling or resting $[\text{Ca}^{2+}]_i$. Furthermore, TRPC1 expression was unchanged. These findings suggest that SACs are not involved in the pathology of young *mdx* mice. However, in old *mdx* cardiomyocytes streptomycin and GsMTx-4 both reduced resting $[\text{Ca}^{2+}]_i$ towards that seen in old WT mice; these findings correlate well with the effect of streptomycin on un-stretched *mdx* skeletal muscle (Yeung *et al.*, 2005). Furthermore, streptomycin also returned the amplitude of old *mdx* Ca^{2+} transients close to WT. These results suggest increased influx of Ca^{2+} through SACs is involved in the *mdx* pathology. Increased influx of Ca^{2+} through SACs in old *mdx* muscle could arise from increased expression and/or increased activity of SACs. The present study suggests that in old *mdx* mice, increased TRPC1 expression accounts for the observed changes in Ca^{2+} homeostasis and dynamics. However, increased activity of SACs cannot be excluded. With regard to the effect of streptomycin on old *mdx* cardiomyocytes Ca^{2+} transients, it is possible streptomycin's ability to decrease resting $[\text{Ca}^{2+}]_i$, resulted in decreased SR Ca^{2+} content and consequently a fall in the amplitude of Ca^{2+} transients. However, the data in this chapter does not conclusively prove this hypothesis. It is also possible that streptomycin reduced the amplitude of old *mdx* cardiomyocyte Ca^{2+} transients by blocking the DHPR, although this is unlikely in light of the low dose of streptomycin used (Belus & White, 2002). In order to further support the hypothesis that changes in Ca^{2+} transients were due to inhibition of SACs, it would be preferable to test the effect of the more specific SAC inhibitor GsMTx-4.

Interestingly, the above observations relating to streptomycin imply that in the absence of stretch, SAC activity is increased in old *mdx* cardiomyocytes, which is in accord with findings from *mdx* skeletal muscle (Franco-Obregon, Jr. & Lansman, 1994; Suchyna & Sachs, 2007). It is hypothesized that in healthy animals, when cardiomyocytes become stretched during ventricular filling, SACs open appropriately due to regulation by dystrophin. However, in dystrophin-deficient *mdx* mice, myocardial stretch results in inappropriate opening of SACs and an increase in resting $[Ca^{2+}]_i$.

Increasing extracellular Ca^{2+} concentration had a greater effect on resting $[Ca^{2+}]_i$ in young *mdx* cardiomyocytes compared to WT. One interpretation of this finding is that the augmented increase in resting $[Ca^{2+}]_i$ in *mdx* cardiomyocytes was due to greater Ca^{2+} influx through SACs due to increased activity/expression of the channel. However, a number of alternative mechanisms could explain this observation, including increased Ca^{2+} influx through other sarcolemmal channel/s and/or altered SR Ca^{2+} handling. Nevertheless, this finding is interesting as it provides further evidence that even in young *mdx* cardiomyocytes Ca^{2+} handling is already abnormal.

4.4.2 Effect of hydrogen peroxide on resting $[Ca^{2+}]_i$: role of SACs

Preliminary experiments investigating ROS and SACs suggested that the rise in resting $[Ca^{2+}]_i$ following hydrogen peroxide application was due to Ca^{2+} influx through SACs. Increased sample numbers and the use of more specific SAC inhibitors are required to confirm these preliminary findings. It is also important to note that hydrogen peroxide has also been shown to increase the opening of the DHPR which could also account for the

increase in resting $[Ca^{2+}]_i$ observed in the current study (Viola *et al.*, 2007). However, the contribution of the DHPR to the increase in resting $[Ca^{2+}]_i$ in response to hydrogen peroxide is likely to be very small, as the DHPR is closed at the resting membrane potential (Bers, 2001). If it is confirmed that ROS are able to activate SACs it raises the question, are ROS involved in the *mdx* cardiac pathology? Furthermore, if increased oxidative stress is involved in *mdx* cardiac damage, do changes in ROS occur before changes in SAC activity? In order to address these questions the role of ROS in the *mdx* myocardium was investigated; these studies will be the focus of the next three chapters.

4.5 Conclusions

This chapter has shown that the *mdx* myocardium has altered SAC activity and increased TRPC1 expression. It is possible that early in the disease process, and before the onset of heart failure, increased SAC activity could explain the observed alterations in Ca^{2+} handling and the increased SR Ca^{2+} content in *mdx* mice.

CHAPTER FIVE

Increased ROS in *Mdx* Hearts: NADPH Oxidase as a Possible Source

5.1 Introduction

Increasing evidence suggests that reactive oxygen species (ROS) may play a critical role in the pathogenesis of DCMs and DMD. In DCMs an imbalance exists between ROS production (Heymes *et al.*, 2003) and myocardial antioxidative stress defence mechanisms (Baumer *et al.*, 2000; Sam *et al.*, 2005) leading to increased ROS induced myocardial injury with disease progression (Keith *et al.*, 1998).

Various enzymatic sources within the myocardium are capable of producing excess ROS, including the mitochondria, nitric oxide synthase (NOS), xanthine oxidase (XO) and NADPH oxidase. NADPH oxidases are multimeric enzymes that contain a membrane-bound core, comprising a catalytic gp91^{phox} and p22^{phox} subunit, and four regulatory subunits p40^{phox}, p47^{phox}, p67^{phox} and rac1, (Murdoch *et al.*, 2006). In DCMs NADPH oxidase activity and subunit protein expression are increased (Maack *et al.*, 2003). While it is not known if NADPH oxidase activity is increased in the myocardium in DMD, it appears to be a good candidate for augmented ROS production. This is because caveolae, which are known to affect NADPH oxidase activity, are altered in DMD (Repetto *et al.*, 1999) and are required for NADPH oxidase activation (Kawamura *et al.*, 2003). Disruption of caveolae prevents activation of the cytosolic protein rac1 (Kawamura *et al.*, 2003) and results in a shift in NADPH oxidase enzyme subunits out of caveolae and into other membranous and intracellular compartments (Yang & Rizzo, 2007).

It is also possible that the increased elasticity (Pasternak *et al.*, 1995) and susceptibility of *mdx* myocytes to stretch (Yasuda *et al.*, 2005) leads to increased NADPH oxidase activity (Hwang *et al.*, 2003b).

ROS have been implicated in the pathogenesis of the skeletal muscle damage that occurs in DMD (Rando, 2002). However, no studies have investigated the role of ROS in the DCM associated with DMD. The aim of the current chapter was to determine whether oxidative stress is increased in *mdx* hearts, and if so, whether NADPH oxidase is a source of raised ROS production.

5.2 Methods

5.2.1 *In vivo treatment of mice with the antioxidant N-acetylcysteine*

NAC-treatment followed protocol 2, as described in Chapter 2. Mice were divided into 4 groups; WT and *mdx* control mice that received tap water, and WT and *mdx* mice that received 1% NAC (WT-NAC, *mdx*-NAC) in their drinking water from 3 until 9 weeks of age (i.e. 6 weeks of treatment). Mice were sacrificed at 9 weeks of age, which was the age that all investigations were performed.

5.2.2 *Measurement of reactive oxygen species*

Oxidative stress was measured using di-hydroethidine (DHE: van Empel *et al.*, 2006). DHE sensitivity to ROS has the following potencies: peroxynitrite > hydroxyl radical > superoxide > hydrogen peroxide (Al Mehdi *et al.*, 1997). DHE is cell permeable and in the presence of ROS is converted to the fluorescent product, ethidium bromide (EtBR), which is trapped by binding with DNA (Rothe & Valet, 1990). 10 μm heart sections were prepared as previously discussed (Chapter 2). 5 μM DHE was applied to heart cross-sections and incubated in a light-protected and humidified chamber at 37°C for 30 minutes. Sections were then rinsed with PBS in order to remove excess DHE. Sections were mounted (Chapter 2) and EtBr *in situ* fluorescence assessed using fluorescence microscopy.

5.2.3 NADPH oxidase assay

NADPH oxidase activity was measured in total left ventricular homogenate using a bis-N-methylacridinium (lucigenin)-enhanced chemiluminescence assay (Heymes *et al.*, 2003). In the presence of ROS, lucigenin releases a photon which can be detected by a luminometer. In the current study, heart tissue homogenization produced small membranous vesicles. As NADPH oxidase is membrane bound it presumably associates with the small vesicles. NADPH (the substrate for NADPH oxidase) is not membrane permeable. Thus, NADPH oxidase would only be able to access NADPH and produce ROS in situations where the enzyme had associated with membranous vesicles in the inside-out configuration.

Left ventricular tissue was mechanically homogenized (5 secs) in ice-cold buffer containing in mM: 150 NaCl, 0.05 Tris-HCl, 0.025 EGTA, 0.025 EDTA, and the following inhibitors to prevent protein degradation: 1% protease inhibitor cocktail; a 0.5% phosphatase inhibitor and 0.35% calpain inhibitor 1 (all Sigma, St. Louis, MO, USA). Following homogenization, samples were stored on ice for 1 hour to allow froth formed during homogenization to settle to produce a uniform solution. A Bradford assay was performed to assess protein content. Homogenate was stored at -80°C for subsequent use. Samples were used within 2 weeks of homogenization.

On experimental days samples were allowed to thaw on ice. The activity of NADPH oxidase was measured in 600 μ L aliquots using a photomultiplier-based luminometer. Aliquots contained: 2 mg/ml total protein, NADPH (200 μ M) and a low concentration of lucigenin (10 μ M). It is important to use a low dose of lucigenin as higher doses (>20 μ M)

are thought to lead to superoxide production via lucigenin-based redox cycling (Munzel *et al.*, 2002). Experiments were performed at 37°C. NADPH oxidase activity was also measured in the presence of the antioxidants 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron, 5 and 10 mM; Ide *et al.*, 2000) or NAC (10 mM). The following agents were used to assess potential sources of superoxide production: 10 µM diphenyleneiodonium (DPI), a flavoprotein inhibitor, to block NADPH oxidase activity (Sano *et al.*, 2001); 100 µM Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), a NOS inhibitor (Vasquez-Vivar *et al.*, 1998); 50 µM rotenone, a mitochondrial site I electron transport inhibitor (Becker, 2004); or 100 µM oxypurinol, a XO inhibitor (Kogler *et al.*, 2003).

5.2.4 Immunoblotting

The relative levels of caveolin 3, rac1, and gp91^{phox} were determined by quantitative immunoblotting (Chapter 2). Membranes were incubated with primary antibodies against caveolin 3, rac1, and gp91^{phox}, washed in PBST and incubated with HRP-conjugated secondary antibodies (see Table 5.1 for conditions) and developed using an enhanced chemiluminescence detection system (Amersham BioSciences, Uppsala, Sweden). All antibodies were diluted in PBST-block. Total protein loading was normalised using a Bradford assay (Bio-Rad Ltd). All bands were normalised to the protein GAPDH.

	Caveolin 3	Rac1	gp91 ^{phox}
Lysis/Immunoblotting			
Lysis temperature (°C)	95	95	95
Lysis time (mins)	5	5	5
Separating gel (%)	12	12	12
Stacking gel (%)	4	4	4
Protein per well (µg)	5	80	200

Primary antibody

Type	Mouse anti-rat IgG (BD Biosciences)	Mouse anti-human IgG (BD Biosciences)	Mouse IgG (BD Biosciences)
Dilution	1:8,000	1:1,000	1:500
Incubation time	1 hr	1 hr	Overnight
Incubation temperature	Room temperature	Room temperature	4°C
Reference	(Zhao <i>et al.</i> , 2002)	(Kawamura <i>et al.</i> , 2003)	(Sanchez <i>et al.</i> , 2005)

Secondary antibody

Type	Anti-mouse IgG (SantaCruz)	Anti-mouse IgG (SantaCruz)	Anti-mouse IgG (Amersham)
Dilution	1:1,00	1:1,000	1:2,500
Incubation time	1 hr	1 hr	1 hr
Incubation temperature	Room temperature	Room temperature	Room temperature

Table 5.1 Immunoblotting conditions for analysis of caveolin 3, rac1 and gp91^{phox} protein expression in WT and *mdx* hearts.

Conditions used for immunoblotting. Abbreviations: hr, hour; IgG, immunoglobulin G. Company addresses: Amersham, Uppsala, Sweden, USA; BD Biosciences, San Jose, CA, USA; Santa Cruz, Santa Cruz, CA, USA.

5.3 Results

5.3.1 Effect of NAC-treatment on body weight and behaviour

NAC-treatments were well-tolerated, and no overt changes were seen in the behaviour of mice. The body weight of mice was not affected by NAC-treatment (Table 5.2). However, mice receiving NAC in their water drank less than mice receiving water alone. These observations correlate well with others (Farid *et al.*, 2005).

	Treatment	
	Control	NAC-treated
Wild Type	24.7 ± 0.7 g	23.8 ± 1.0 g
<i>Mdx</i>	24.2 ± 0.4 g	24.2 ± 0.2 g

Table 5.2. Body weights of mice receiving control or NAC in their drinking water.

Animal weights of 9 week old WT, WT-NAC, *mdx* and *mdx*-NAC mice. Treatment followed protocol 2. $n \geq 7$.

5.3.2 ROS are increased in *mdx* hearts

In order to ascertain if ROS levels are increased in *mdx* hearts we measured the levels of EtBr fluorescence in *mdx* and WT heart sections. Compared to WT, *mdx* hearts displayed an increase ($19 \pm 4 \%$) in EtBr fluorescence, reflecting an increase in ROS levels (Fig. 5.1). We hypothesized that, as ROS levels are augmented in *mdx* mice, treatment with the antioxidant NAC (1% w/v) may prevent or reduce some of the abnormalities observed in these animals. The use of NAC and its dosage were based on findings from previous trials (Farid *et al.*, 2005). In *mdx* hearts, NAC-treatment returned EtBr fluorescence to WT levels (Fig. 5.1). NAC-treatment had no effect on WT hearts.

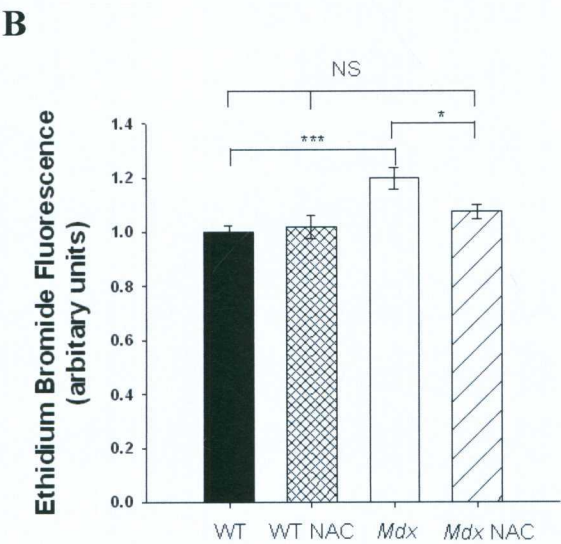
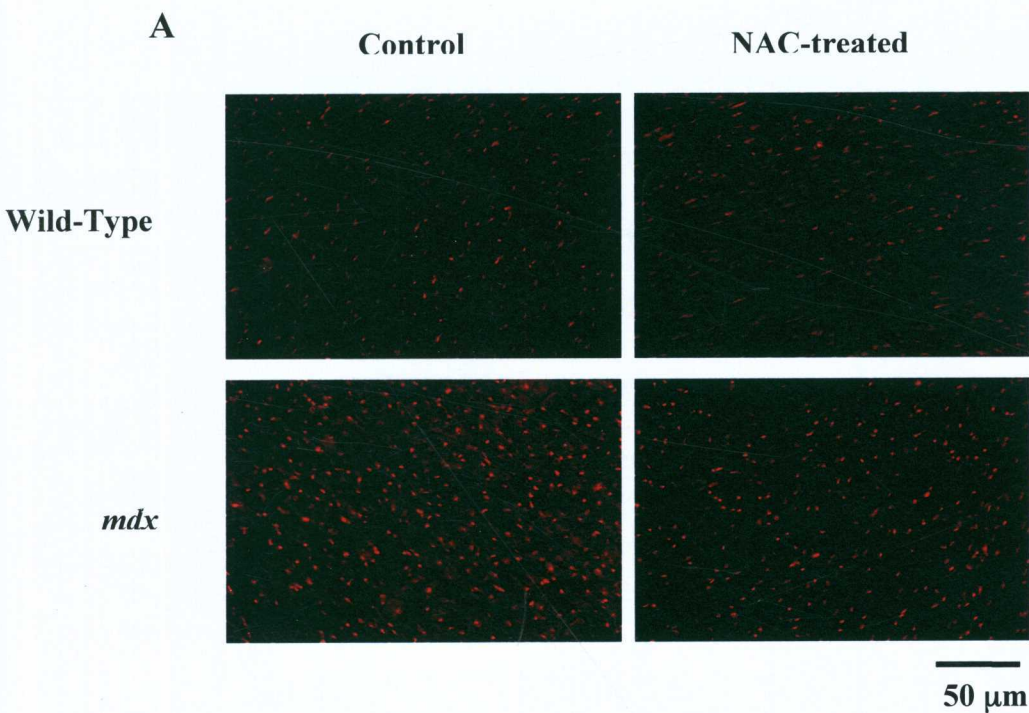


Figure 5.1 Effect of NAC-treatment on ROS levels in *mdx* and WT hearts.

A: Representative images showing EtBr fluorescence (a measure of ROS levels) in WT, WT-NAC, *mdx* and *mdx*-NAC heart cross sections. B: Group data from A. N $\geq$ 5; NS, not significant; * P < 0.05, *** P < 0.001 between indicated groups. Bar = 50 μ m.

5.3.3 Potential sources of ROS in *mdx* hearts

NADPH oxidase activity was increased 1.4 fold in *mdx* hearts compared to WT (Fig. 5.2A). In all tissues, NADPH-dependent chemiluminescence was virtually abolished by the ROS scavengers Tiron (5 and 10 mM) and NAC (10 mM; Fig. 5.2B). DPI (10 μ M), an inhibitor of NADPH oxidase, blocked ROS production, confirming that the assay was measuring NADPH oxidase-dependent ROS production. ROS production was unaffected by oxypurinol (100 μ M; XO inhibitor), rotenone (50 μ M; mitochondrial site I electron transport inhibitor) or L-NAME (100 μ M; NOS inhibitor). NAC-treatment did not affect NADPH oxidase activity in either WT or *mdx* hearts.

In order to explore the underlying mechanism for increased NADPH oxidase activity in *mdx* hearts, we measured the protein expression of the membrane bound NADPH oxidase subunit, gp91^{phox}, and the regulatory cytosolic subunit rac1. These two subunits were chosen as they have been shown to be up-regulated (Hwang *et al.*, 2003a) or activated by stretch (Kawamura *et al.*, 2003). Furthermore, as caveolae are known to play an important role in the regulation of NADPH oxidase, we measured the protein level of caveolin 3, a key component of caveolae in cardiac muscle. The expression of rac1 was unchanged in *mdx* hearts (Fig. 5.3A and 5.3B). However, expression of gp91^{phox} and caveolin 3 were both increased in *mdx* hearts. NAC-treatment had no effect on the protein expression of gp91^{phox} or caveolin 3 (Fig. 5.3C and 5.3D).

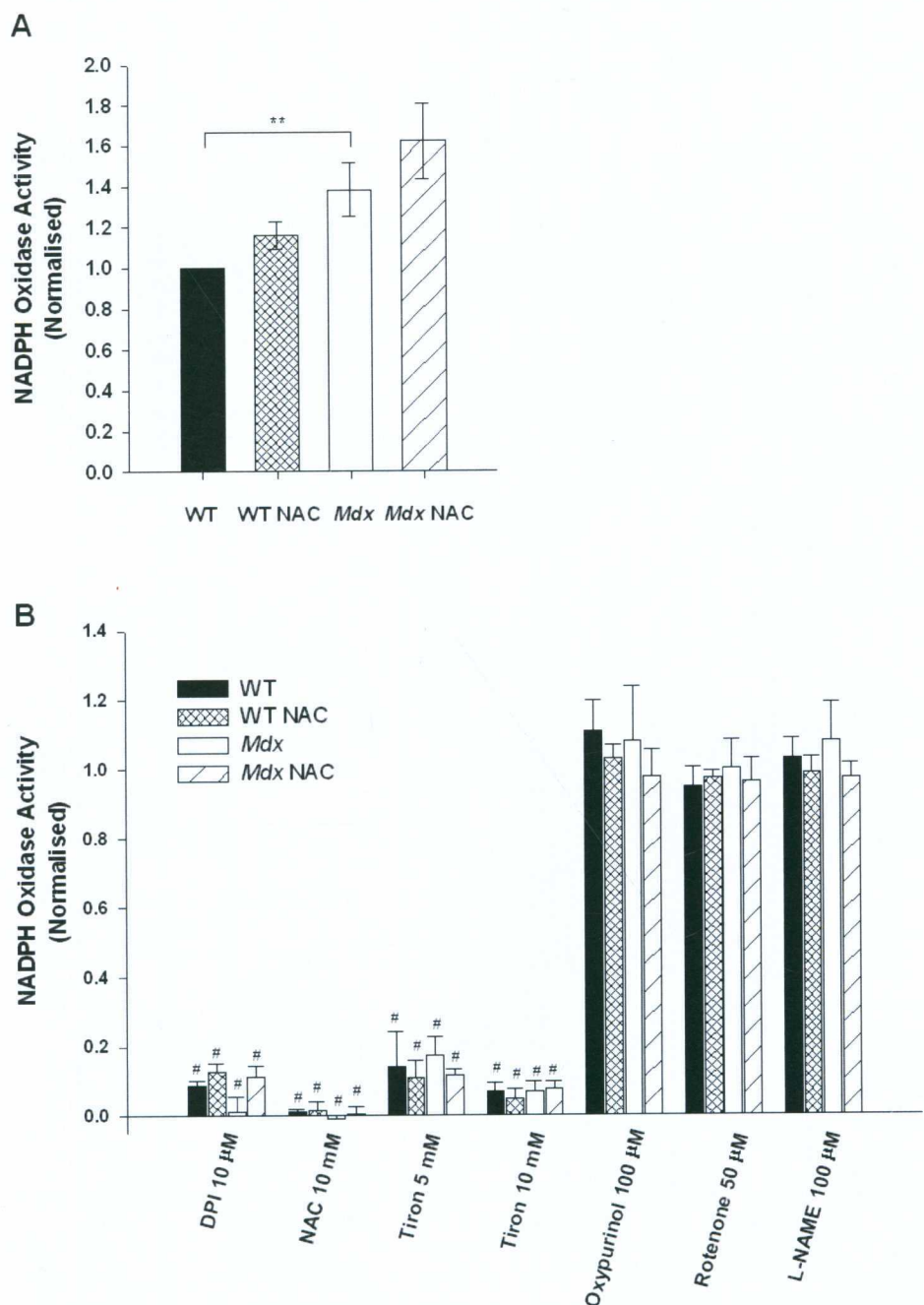


Figure 5.2 NADPH oxidase activity

A: NADPH-dependent ROS production in WT, WT-NAC, *mdx*, and *mdx*-NAC hearts. ** $P < 0.01$ between groups. Data are expressed normalised to WT control. $N \geq 5$; $n \geq 16$. **B:** Effect of various agents on NADPH-dependent ROS production in WT, WT-NAC, *mdx*, and *mdx*-NAC hearts. Data are expressed relative to baseline before application of inhibitor. $N = 5$; # $P < 0.001$ before and after application of inhibitor.

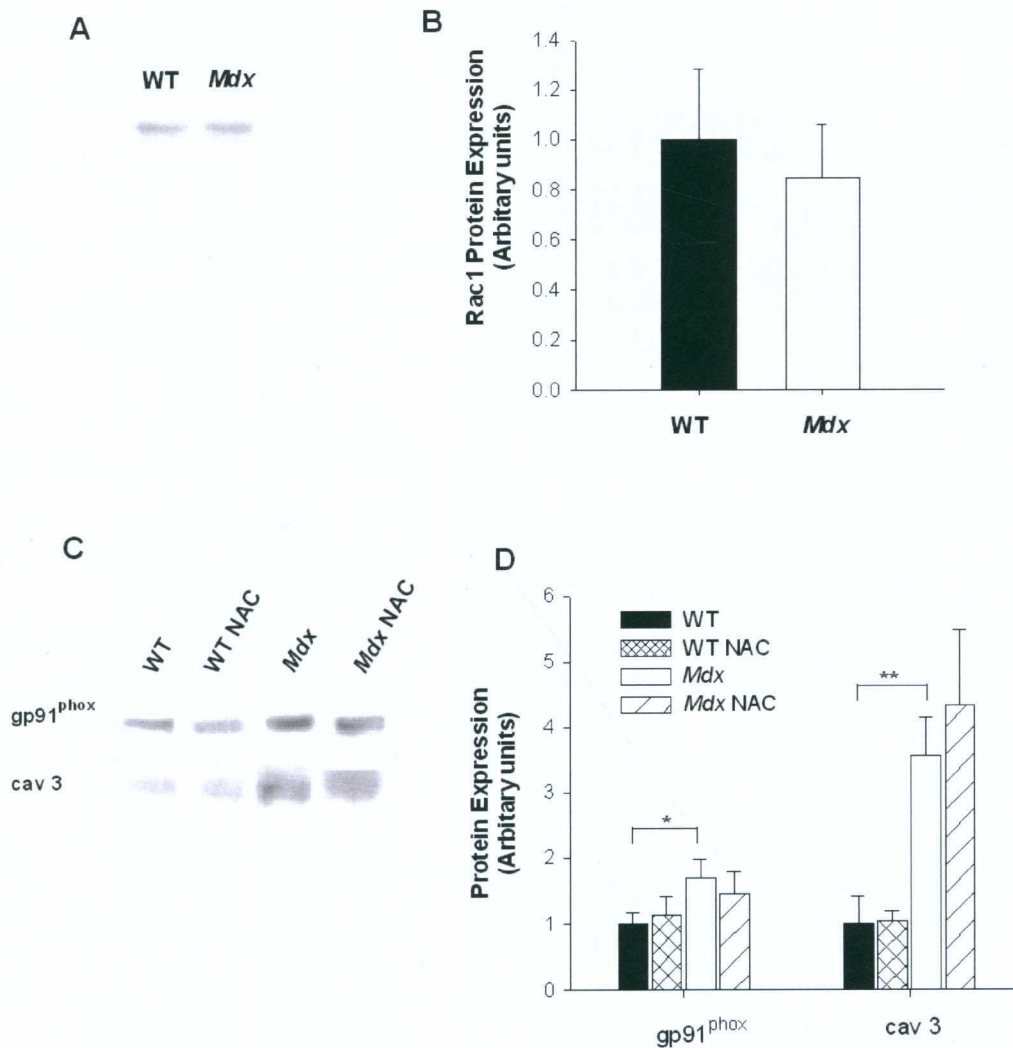


Figure 5.3 Gp91^{phox}, rac1 and caveolin 3 protein expression

A: Sample blots showing protein expression levels of rac1 (21 kDa). **B:** Group data from **A**. **C:** Sample blots showing protein expression levels of gp91^{phox} (58 kDa) and caveolin 3 (cav 3; 18 kDa) in WT, WT-NAC, *mdx*, and *mdx*-NAC hearts. **D:** Group data from **C**. Data are expressed normalised to WT control. N ≥ 5. * P < 0.05, ** P < 0.01 between indicated groups.

5.4 Discussion

In skeletal muscle there is good evidence that abnormalities in ROS levels are involved in the pathology of DMD (Rando, 2002). However, no studies have explored the involvement of ROS in the DCM that occurs in DMD. The present chapter found that increased ROS levels in *mdx* cardiomyocytes, which could be reversed by the antioxidant NAC, were associated with increased NADPH oxidase-dependent ROS production.

5.4.1 Increased ROS in *mdx* hearts

The present study found that ROS levels were increased in *mdx* hearts compared to WT; this finding correlates well with observations from *mdx* and DMD skeletal muscle (Rando, 2002), and cardiac muscle from DCM patients (Sam *et al.*, 2005).

NAC-treatment returned ROS levels in *mdx* hearts close to WT values, suggesting that NAC-treatment was acting as an antioxidant; this finding is in accord with others who found that NAC-treatment reduced ROS levels in animal models of hypertrophic (Nakagami *et al.*, 2003) and diabetic (Fiordaliso *et al.*, 2006) cardiomyopathy. It is likely that NAC reduced *mdx* oxidative stress via its ability to; 1) directly scavenge ROS such as the hydroxyl radical and peroxynitrite (Aruoma *et al.*, 1989; Navarro-Antolin *et al.*, 2007); 2) increase superoxide dismutase activity (Barreiro *et al.*, 2005; Hicdonmez *et al.*, 2006) and; 3) stimulate GSH synthesis and activity (Gillissen *et al.*, 1997; Hicdonmez *et al.*, 2006).

NAC-treatment had no effect on any of the variables measured in WT hearts in this thesis, supporting previous findings that NAC only supports GSH synthesis under conditions of increased oxidative stress (Kelly, 1998).

5.4.2 Sources of raised ROS

The finding that ROS are increased in *mdx* hearts raises the question, what is the source of increased ROS? Furthermore, what mechanism links absence of dystrophin to increased ROS production? In DCMs various sources of ROS production have been proposed, including NADPH oxidase, XO, NOS and the mitochondria (Murdoch *et al.*, 2006). NADPH oxidase activity was studied as it has been shown to have increased activity in DCMs (Heymes *et al.*, 2003; Maack *et al.*, 2003). NADPH oxidase activation is altered by stretch (Hwang *et al.*, 2003b) and requires intact caveolae (Kawamura *et al.*, 2003); both caveolae and the response to stretch are altered in DMD (Pasternak *et al.*, 1995; Repetto *et al.*, 1999).

NADPH oxidase activity was increased 1.4 fold in *mdx* compared to WT hearts. NADPH oxidase, rather than dysfunctional NOS, XO, or mitochondria production was the source of NADPH-dependent ROS production, as ROS production was blocked by DPI (an NADPH oxidase inhibitor), but not by oxypurinol (XO inhibitor), rotenone (mitochondrial inhibitor) or L-NAME (NOS inhibitor). Although DPI can affect mitochondria ROS production (Li & Trush, 1998), it is unlikely that DPI was acting to prevent mitochondrial ROS production in the current study, as the mitochondrial site I electron transport inhibitor rotenone had no effect on NADPH-dependent ROS production. The current study does not rule out other

sources of ROS production; however, it does demonstrate that NADPH oxidase could be one source of increased ROS in *mdx* hearts.

NAC-treatment did not reduce *mdx* NADPH oxidase activity or gp91^{phox} expression, suggesting that the beneficial effects of NAC on *mdx* myocardial function are not due to its ability to reduce NADPH oxidase ROS production. Instead, it appears that NAC acts by enhancing endogenous ROS scavengers or directly scavenging excess ROS produced by NADPH oxidase. The increased superoxide produced by NADPH oxidase during the assay in *mdx* mice would not be expected to be reduced by any NAC present in the tissue homogenate of *mdx*-NAC mice, as NAC does not directly scavenge superoxide (Aruoma *et al.*, 1989; Winterbourn & Metodiewa, 1999).

If raised activity of NADPH oxidase is not secondary to increased ROS, what is the primary mechanism augmenting its activity? One possible mechanism relates to the finding that caveolae are increased in DMD (Repetto *et al.*, 1999). Dystrophin associates with caveolin 3 (Song *et al.*, 1996), and in DMD, where dystrophin is absent, caveolin 3 levels are augmented and leading to an increase in the number of caveolae (Galbiati *et al.*, 2000). It is unclear why absence of dystrophin results in up-regulation of caveolin 3. One possibility relates to the finding that caveolin 3 and dystrophin both compete for a similar binding site on β -dystroglycan (Sotgia *et al.*, 2000). In the absence of dystrophin, the stoichiometry of the dystroglycan complex is changed, resulting in a decrease in caveolin 3 degradation and consequently an increase in caveolin 3 expression. NADPH oxidase subunits have been shown to co-localize with caveolin 1 in vascular smooth muscle

(Hilenski *et al.*, 2004). Furthermore, caveolae have been shown to be important in the spatial regulation and agonist stimulation of NADPH oxidase (Kawamura *et al.*, 2003; Yang & Rizzo, 2007). Our finding that caveolin 3 expression is greater in *mdx* hearts may allow increased localization of gp91^{phox} to caveolae, resulting in increased NADPH oxidase-dependent ROS production.

A second mechanism to account for increased NADPH oxidase activity relates to the possibility that the increased elasticity (Pasternak *et al.*, 1995) and susceptibility of *mdx* myocytes to stretch (Yasuda *et al.*, 2005) leads to increased activation of the enzyme. Stretch leads to activation of both NADPH oxidase dependent ROS production (Hwang *et al.*, 2003b) and activation of the enzyme's regulatory subunit rac1 (Kawamura *et al.*, 2003), in addition to increasing the expression of gp91^{phox} (Hwang *et al.*, 2003a). Thus, increased stretch may account for the raised NADPH oxidase activity and augmented expression of gp91^{phox} found in the current chapter.

Another possible mechanism linking increased caveolin 3 expression to augmented NADPH oxidase activity relates to NOS. In addition to being associated with NADPH oxidase, caveolin 3 is also able to reduce NOS activity (Feron & Balligand, 2006; Garcia-Cardena *et al.*, 1997). As NADPH oxidase is inhibited by NO (Fujii *et al.*, 1997) it is possible that the increased levels of caveolin 3 found in *mdx* hearts decreases NO levels resulting in augmented NADPH oxidase ROS production. In addition to caveolin-dependent inhibition of NO production, *mdx* hearts also have decreased nNOS protein

levels (Bia *et al.*, 1999). Thus, NADPH oxidase activity may also be increased as a consequence of decreased nNOS-dependent NO production.

Interestingly, in all treatment groups, acute exposure of 10 mM NAC abolished the signal from the NADPH oxidase assay. The signal measured in the NADPH oxidase assay results from superoxide reacting with lucigenin. Thus, the finding that acute exposure of NAC abolishes NADPH oxidase activity implies that NAC reduced superoxide levels. This observation is interesting, as NAC *per se* is a poor scavenger of superoxide (Aruoma *et al.*, 1989; Winterbourn & Metodiewa, 1999). One possible explanation for this observation is that acute exposure of NAC stimulated endogenous SOD activity, resulting in decreased superoxide levels (Barreiro *et al.*, 2005). It is also possible that the relatively high dose of NAC (10 mM) was great enough to directly scavenge superoxide. Finally, the current findings can not exclude the possibility that acute NAC exposure reduced superoxide production by inhibiting NADPH oxidase-dependent superoxide production.

5.5 Conclusion

Our data support the hypothesis that *mdx* mice hearts have increased intracellular ROS levels. An increase in NADPH oxidase-dependent ROS production may, in part, account for the increased oxidative stress in the *mdx* myocardium. Raised NADPH oxidase activity might be due to increased expression of the catalytic subunit, gp91^{phox}, and greater activation of the enzyme due to increased association with caveolin 3. Alternatively, increased NADPH oxidase activity might be due to the increased susceptibility of *mdx* myocytes to stretch.

CHAPTER SIX

Role of ROS in Ca^{2+} Handling and Myofilament Dysfunction in *Mdx* Mice Hearts

6.1 Introduction

The previous chapter (Chapter 5) demonstrated that *mdx* hearts have increased levels of ROS. It has been shown that raised ROS levels leads to oxidation of various proteins resulting in myocardial dysfunction. Myocardial proteins affected by ROS include sarcolemmal (Viola *et al.*, 2007), EC coupling (Zima & Blatter, 2006), contractile (Gao *et al.*, 1996) and stress sensitive signalling proteins (Aikawa *et al.*, 1997). Abnormalities in the function of all the above classes of proteins have been reported in DCM. Thus, raised ROS levels may contribute to the dysfunction that occurs in the DCM associated with DMD. The aim of the current chapter was to determine whether the EC coupling defects observed in *mdx* cardiomyocytes were due to increased levels of oxidative stress in *mdx* hearts. Furthermore, the effect of raised ROS on *mdx* cardiomyocyte myofilament Ca^{2+} -sensitivity and fractional shortening was explored.

6.2 Methods

6.2.1 In vivo treatment of mice with the antioxidant N-acetylcysteine

NAC-treatment followed protocol 1 and 2, as described in Chapter 2. In protocol 1 mice were divided into 3 groups, WT and *mdx* control mice that received tap water, and *mdx* mice that received 1% NAC in their drinking water from 6 until 9 weeks of age (i.e. 3 weeks of treatment). In protocol 2, mice were divided into 4 groups; WT and *mdx* control mice that received tap water, and WT and *mdx* mice that received 1% NAC (WT-NAC, *mdx*-NAC) in their drinking water from 3 until 9 weeks of age (i.e. 6 weeks of treatment). All mice were sacrificed at 9 weeks of age, which was the age that all investigations were performed.

6.2.2 Isolation of cardiomyocytes and measurement of $[Ca^{2+}]_i$

Isolation of single ventricular myocytes, and measurement and calibration of $[Ca^{2+}]_i$ were performed as described in Chapter 2.

6.2.3 Measurement of cardiomyocyte Ca^{2+} -sensitivity and fractional shortening

Measurement of cardiomyocyte Ca^{2+} -sensitivity and fractional shortening were performed as described in Chapter 2.

6.2.4 Immunoblotting

The relative levels of full length native troponin I (TnI) and of TnI degradation products were determined by quantitative immunoblotting (Chapter 2). Membranes were incubated with primary antibodies against TnI, before being incubated with HRP-conjugated secondary antibodies (see Table 1 for conditions) and developed using an enhanced chemiluminescence detection system (Amersham BioSciences, Uppsala, Sweden). All antibodies were diluted in PBST-block. Total protein loading was normalised using a Bradford assay (Bio-Rad Ltd). All bands were normalised to the protein GAPDH.

	TnI degrad. band	TnI native band
Lysis/Immunoblotting		
Lysis temperature (°C)	95	95
Lysis time (mins)	5	5
Separating gel (%)	12	12
Stacking gel (%)	4	4
Protein per well (µg)	200	40
Primary antibody		
Type	Mouse anti- bovine (Chemicon)	Mouse anti- bovine (Chemicon)
Dilution	1:1,000	1:8,000
Incubation time	1 hour	1 hour
Incubation temperature	Room temperature	Room temperature
Reference	(Kehat <i>et al.</i> , 2001)	Kehat <i>et al.</i> , 2001)
Secondary antibody		
Type	Anti-mouse IgG (SantaCruz)	Anti-mouse IgG (SantaCruz)
Dilution	1:1,000	1:1,000
Incubation time	1 hour	1 hour
Incubation temperature	Room temp.	Room temp.

Table 6.1 Immunoblotting conditions for analysis of troponin I protein expression

Conditions used for immunoblotting. Abbreviations: TnI, troponin I; degrad., degradation.
Company addresses: Chemicon, Temecula, CA, USA; SantaCruz, Santa Cruz, CA, USA.

6.3 Results

6.3.1 Effect of NAC on intracellular Ca^{2+} handling

As presented in Chapter 3, Ca^{2+} handling is abnormal in *mdx* cardiomyocytes. In order to determine if ROS are involved in the Ca^{2+} handling defects in young *mdx* cardiomyocytes, Ca^{2+} transients were recorded from control and NAC-treated *mdx* and WT mice.

As discussed in Chapter 2, initially a pilot study was performed to assess the effects of 3 weeks of NAC-treatment on Ca^{2+} handling. NAC protocol 1 was used. Calibration of $[\text{Ca}^{2+}]_i$ was not performed in the pilot study. Instead, the ratio of the peak fluo-4 signal (F_p) and the baseline fluo-4 signal (F_o) was used as an estimate of the peak of the Ca^{2+} transient.

NAC-treatment returned F_p/F_o of *mdx* myocytes to WT values (WT, 7.4 ± 0.2 ; *mdx*, 8.4 ± 0.3 ; NAC-treated *mdx*, 7.4 ± 0.2 ; Fig. 6.1A). NAC-treatment had no effect on the time to peak or time constant of *mdx* Ca^{2+} transients (Fig 6.1B, and 6.1C). As NAC-treatment returned F_p/F_o to WT, but had no effect on the time to peak or time constant, it was decided to increase the duration of NAC-treatment. Hence, as discussed in Chapter 2 NAC-treatment was given from 3 week until 9 weeks of age (i.e. 6 weeks of treatment; protocol 2).

6 weeks of NAC-treatment had no effect on *mdx* or WT resting $[\text{Ca}^{2+}]_i$ (Fig. 6.2A). NAC-treatment corrected the time to peak, and returned the amplitude of the Ca^{2+}

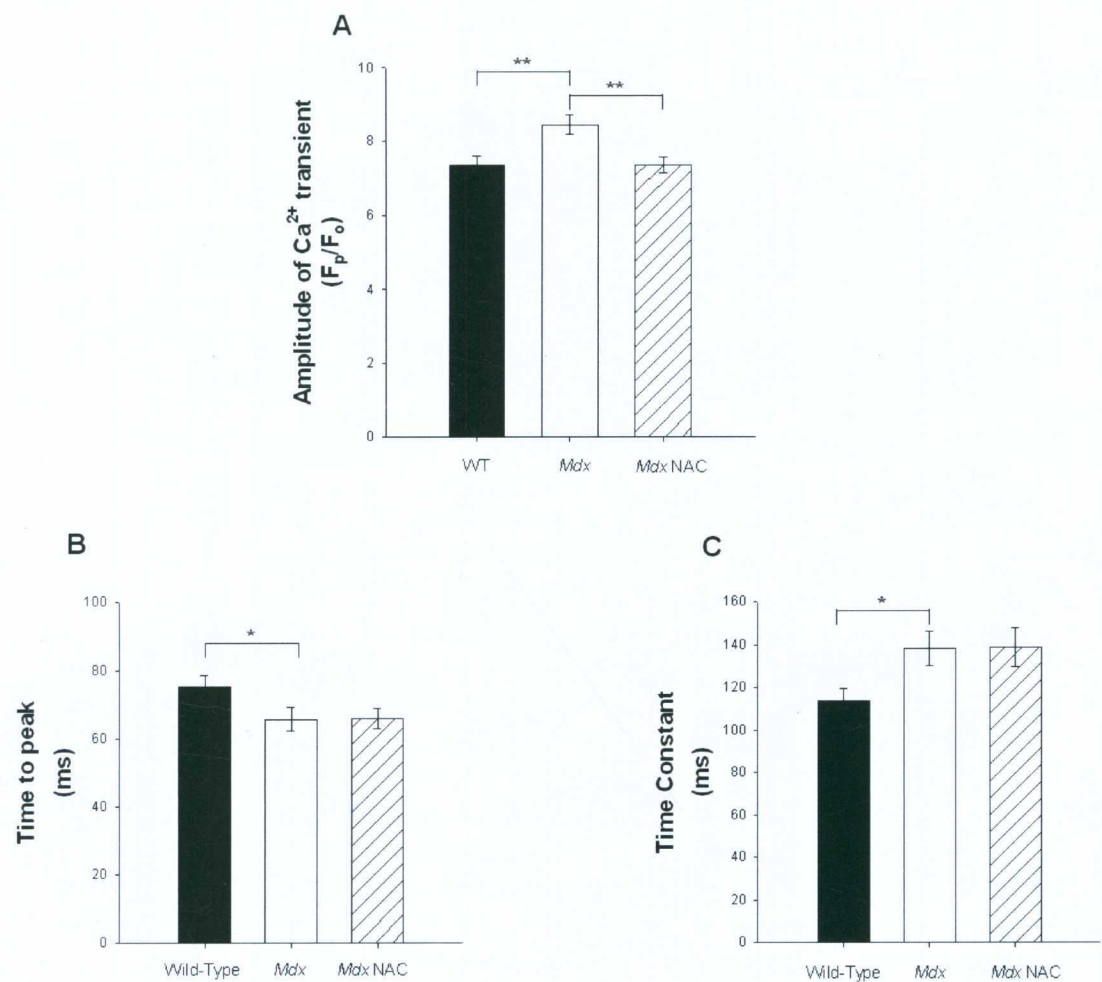


Figure 6.1 Effect of 3 weeks of NAC-treatment on Ca^{2+} transients in *mdx* and WT cardiomyocytes

Effect of 3 weeks (6 weeks until 9 weeks of age) of NAC-treatment (1%) on *mdx* and WT cardiomyocyte Ca^{2+} transients. Effect of NAC-treatment on the amplitude (A), time to peak (B), and time constant of Ca^{2+} transients (C). $N \geq 4$, $n \geq 20$; NS, not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ between indicated groups.

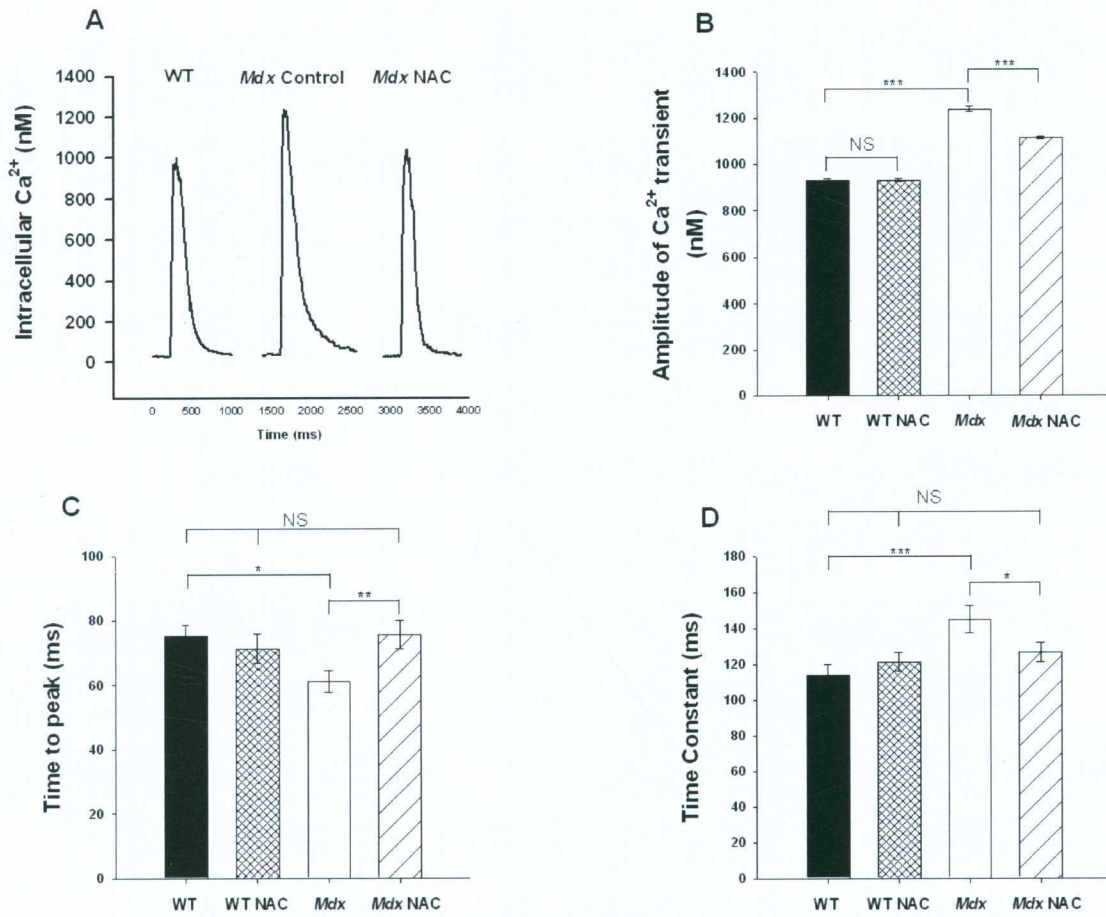


Figure 6.2 Effect of 6 weeks of NAC-treatment on Ca^{2+} transients in *mdx* and WT cardiomyocytes

Effect of 6 weeks (3 weeks until 9 weeks of age) of NAC-treatment (1%) on *mdx* and WT cardiomyocyte Ca^{2+} transients (**A**), the amplitude of Ca^{2+} transients (**B**), the time to peak of Ca^{2+} transients (**C**), and time constant of Ca^{2+} transients (**D**). $N \geq 5$, $n \geq 20$; NS, not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ between indicated groups.

transient and the time constant close to WT values (Fig. 6.2B, 6.2C and 6.2D). NAC-treatment had no effect on WT Ca^{2+} transients.

6.3.2 Effect of NAC on cardiomyocyte myofilament Ca^{2+} -sensitivity and fractional shortening

As ROS have been shown to affect cardiomyocyte myofilament Ca^{2+} -sensitivity (Gao *et al.*, 1996) and whole heart fractional shortening (Grieve *et al.*, 2006) we investigated whether the raised ROS in *mdx* hearts affected these parameters in *mdx* cardiomyocytes. Figure 6.3A shows a cell shortening versus $[\text{Ca}^{2+}]_i$ trajectory for one contraction. The arrow shows the relaxation phase during which $[\text{Ca}^{2+}]_i$ and cell shortening are thought to be close to their steady-state equilibrium (Spurgeon *et al.*, 1992). Figure 6.3B shows the $[\text{Ca}^{2+}]_i$ - length trajectory during the relaxation phase in a WT, *mdx*, and *mdx*-NAC cardiomyocyte. The $[\text{Ca}^{2+}]_i$ at which 50% shortening occurred is greater in *mdx* than WT indicating a decrease in Ca^{2+} -sensitivity (Fig. 6.3C). *Mdx* cardiomyocytes also have decreased fractional shortening (Fig. 6.3D). NAC-treatment returned *mdx* cardiomyocyte fractional shortening values back to WT values, suggesting that increased oxidative stress underlies the decreased fractional shortening (WT, $19.5 \pm 1.0\%$; WT-NAC $20.2 \pm 1.6\%$; *mdx* 16.6 ± 0.8 , *mdx*-NAC 19.3 ± 0.9). NAC-treatment had no significant effect on the Ca^{2+} -sensitivity of *mdx* cardiomyocytes implying that an NAC-insensitive mechanism is responsible for the changed Ca^{2+} -sensitivity. NAC-treatment had no effect on WT fractional shortening or Ca^{2+} -sensitivity (WT 262 ± 22 nM; WT-NAC 252 ± 21 nM; *mdx* 364 ± 20 nM; *mdx*-NAC 316 ± 19 nM).

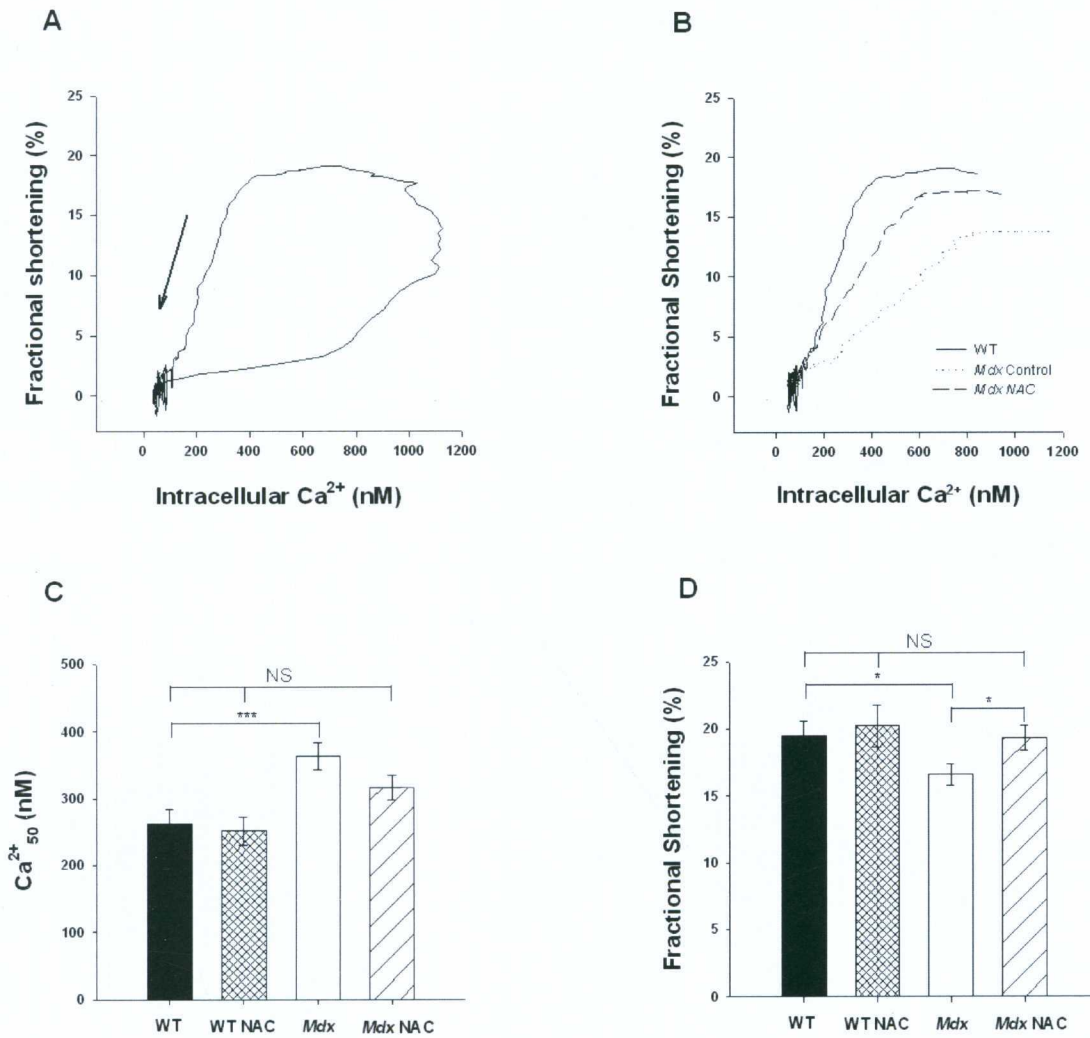


Figure 6.3 Effect of NAC-treatment on Ca^{2+} -sensitivity and fractional shortening in *mdx* and WT cardiomyocytes.

A: Trace showing $[\text{Ca}^{2+}]_i$ against cell length during a contraction. Arrow shows the relaxation phase. **B:** $[\text{Ca}^{2+}]_i$ - length trajectory during the relaxation phase of a Ca^{2+} transient. The $[\text{Ca}^{2+}]_i$ at 50% cell shortening was termed Ca^{2+}_{50} and was used as an estimate of Ca^{2+} -sensitivity. **C:** Grouped fractional shortening data. **D:** Group Ca^{2+} -sensitivity data. WT, $n = 11$; WT NAC, $n = 5$; *Mdx*, $n = 20$; *Mdx* NAC, $n = 11$; NS, not significant; * $P < 0.05$, *** $P < 0.001$ between groups.

To further explore the changes in *mdx* cardiomyocyte fractional shortening and Ca^{2+} -sensitivity we measured TnI degradation, as degradation of this protein is thought to underlie changes in these parameters in myocardial stunning (Gao *et al.*, 1997). Immunoblotting experiments revealed that full-length native TnI (~26 kDa) was present in all heart samples. NAC-treatment had no effect on full-length TnI expression (data not shown). In both WT and *mdx* hearts, a weaker, lower molecular weight band was seen at ~20 kDa (Fig. 6.4A); this band was assumed to be a TnI degradation product. The expression of the 20 kDa TnI degradation band was over 5-fold greater in *mdx* hearts and was unaffected by NAC-treatment (Fig. 6.4B).

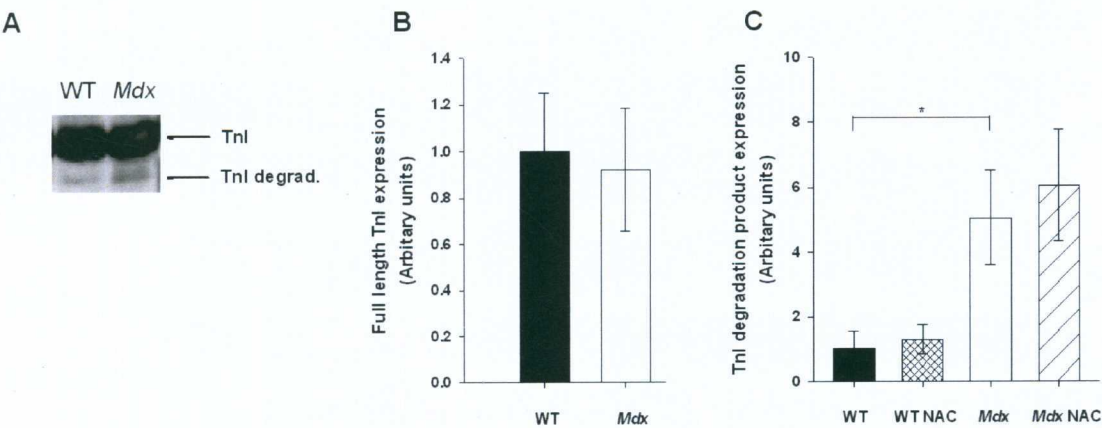


Figure 6.4 TnI degradation in *mdx* and WT hearts

A: Sample blots showing full-length native TnI (~26 kDa) and TnI degradation (~20 kDa) expression levels. **B:** Group data showing native TnI expression. **(C)** Group data showing TnI degradation product expression in WT, WT-NAC, *mdx* and *mdx*-NAC hearts. N ≥ 6; * P < 0.05 between indicated groups.

6.4 Discussion

The current chapter found that increased ROS levels in *mdx* cardiomyocytes were associated with irregular intracellular Ca^{2+} handling and myofilament dysfunction. The observation that NAC-treatment reduced ROS levels and returned the above parameters towards WT levels suggests a causal relationship.

6.4.1 Why do ROS affect Ca^{2+} transients?

It is well established that intracellular Ca^{2+} handling is defective in DCM (Lehnart *et al.*, 1998). Chapter 3 established that Ca^{2+} handling is abnormal in *mdx* hearts. The current chapter found that NAC-treatment corrected or improved all of the Ca^{2+} handling defects in *mdx* cardiomyocytes. ROS are known to modulate the function of many of the proteins involved in EC coupling (Zima & Blatter, 2006). Thus, it is probable that NAC-treatment corrected the Ca^{2+} handling defects in *mdx* hearts by preventing oxidation of EC coupling proteins, such as the RyR2, SERCA2 and PLN.

ROS have been shown to increase RyR2 activity and expression levels (Domenech *et al.*, 2003; Sanchez *et al.*, 2005) which would be predicted to decrease the time to peak and increase the amplitude of Ca^{2+} transients, as we found previously (Chapter 3); this may provide an explanation as to why NAC-treatment prevented the changes in the amplitude and time to peak of Ca^{2+} transients in *mdx* cardiomyocytes.

ROS are also known to inhibit SERCA2 function, through both a direct action on SERCA2 (Kaneko *et al.*, 1989a; Xu *et al.*, 1997) and through their ability to attenuate PLN

phosphorylation which preserves PLN's inhibitory action on SERCA2 (Sulakhe *et al.*, 1997). It is therefore likely that NAC-treatment increased the rate of recovery of Ca^{2+} transients through its ability to prevent oxidation of SERCA2 and PLN. This hypothesis is supported by the finding that NAC increases PLN phosphorylation (Cailleret *et al.*, 2004). Interestingly, in diabetic heart failure, increased NADPH oxidase activity and increased oxidative stress are associated with a decrease in SERCA activity (Li *et al.*, 2006). This finding correlates well with our finding that NAC-treatment increased the rate of recovery of *mdx* cardiomyocyte Ca^{2+} transients and that NADPH oxidase activity is increased in the *mdx* myocardium.

It is interesting to note that the beneficial effects of NAC-treatment occurred in viable cardiomyocytes *ex vivo*, and in the absence of NAC in the perfusate. Several interpretations of this finding are possible: (1) NAC-treatment altered cardiomyocyte protein expression. In the absence of NAC, the reversal of any changes in protein synthesis would not be expected to occur until *de novo* protein synthesis occurred, which may take longer than the 2-8 hours in which experiments were performed following heart excision. (2) NAC-treatment exerted post-translational effects on cardiomyocyte proteins; these effects were not reversed in the period between myocyte isolation and Ca^{2+} transient recordings. (3) NAC was still present in cardiomyocytes and was still able to exert its antioxidant effects. This idea is supported by the finding that in human skeletal muscle, NAC and cysteine (required for GSH synthesis) levels remained elevated 1 hour after NAC administration (Medved *et al.*, 2004). It is likely that a combination of all of the above three possibilities acted concurrently. Thus, in the hours immediately after cardiomyocyte isolation, residual NAC could still act as a ROS scavenger. Upon exhaustion of residual NAC, pre-cardiomyocyte isolation-induced changes in protein

synthesis, such as NAC-induced GSH synthesis, would prevent increases in ROS. Thus, it is likely that in the hours following isolation, the ROS levels of cardiomyocytes would have remained low. This would have ensured the continued presence of any beneficial effects of NAC on protein synthesis and post-translational modification of proteins. In addition, when ROS levels did begin to rise again, it may take several more hours before *de novo* protein synthesis, or for ROS to exert any post-translational changes on Ca^{2+} handling proteins. The above argument also relates to observations involving NACs effect on cardiomyocyte fractional shortening (see below).

6.4.2 Fractional shortening and Ca^{2+} -sensitivity in *mdx* cardiomyocytes

Mdx cardiomyocyte fractional shortening was found to be decreased. In support of previous findings from pressure overloaded whole hearts (Grieve *et al.*, 2006) and cocaine-induced cardiac dysfunction (Moritz *et al.*, 2003), antioxidant treatment corrected fractional shortening defects in *mdx* cardiomyocytes implicating ROS in the underlying pathology. The reduction in fractional shortening found in *mdx* cardiomyocytes is probably not related to TnI degradation as NAC-treatment corrected the fractional shortening defect, but did not prevent TnI degradation. The current study did not explore the mechanism/s by which oxidative stress reduced fractional shortening; however possible mechanisms include post-translation modification of the contractile proteins, either directly by ROS (Duncan *et al.*, 2005) or via ROS induced phosphorylation (He *et al.*, 2003).

Myofilament Ca^{2+} -sensitivity was decreased in *mdx* mice, as previously demonstrated in models of stunned (Gao *et al.*, 1995) and infarcted myocardium (Li *et al.*, 1997). However observations from DCM patients have variously shown Ca^{2+} -sensitivity to be unchanged (D'Agnolo *et al.*, 1992) or increased (Schwinger *et al.*, 1994; Wolff *et al.*, 1996). This disparity in the Ca^{2+} -sensitivity in the DCM that occurs in DMD and other forms of DCM probably relates to differences in the primary aetiology of the diseases.

Although ROS have previously been shown to be capable of reducing Ca^{2+} -sensitivity in ventricular muscle (Gao *et al.*, 1996), NAC-treatment did not significantly increase Ca^{2+} -sensitivity in *mdx* cardiomyocytes or prevent the occurrence of the 20 kDa TnI degradation band. One possibility is that NAC does have a real effect on Ca^{2+} -sensitivity but it failed to reach statistical significance in our experiments. This might have been due to issues relating to the optimization of dosage and timing of NAC administration.

Alternatively, if our findings are correct, the inability of NAC to correct Ca^{2+} -sensitivity defects suggest that increased ROS is not involved in the changes in Ca^{2+} -sensitivity. Thus, an alternative mechanism may underlie the observed changes in Ca^{2+} -sensitivity, such as altered myofilament gene expression, post-translational modification, such as phosphorylation (Gao *et al.*, 1996), or proteolysis of the myofilament proteins (Margossian *et al.*, 1999). In support of the proteolysis hypothesis, the current study found that the changes in Ca^{2+} -sensitivity in *mdx* mice was associated with an increase in TnI degradation. Although the current study did not demonstrate a causative link between Ca^{2+} -sensitivity and TnI degradation, previous studies have supported this connection (Gao *et al.*, 1995; Gao *et al.*, 1997). This raises the question,

why is TnI degradation increased in *mdx* hearts? One possibility is that increased resting $[Ca^{2+}]_i$ results in Ca^{2+} -dependent calpain degradation of TnI (see Chapter 1). Although resting $[Ca^{2+}]_i$ was not increased in young (9weeks) *mdx* hearts, it is possible that abnormal function of SACs allows increased Ca^{2+} entry resulting in small ‘microdomains’ of increased resting $[Ca^{2+}]_i$ (Davidson & Duchen, 2006). Localized increases in resting $[Ca^{2+}]_i$ may have been undetectable using global Ca^{2+} measurements, but could still result in a resting $[Ca^{2+}]_i$ great enough to activate Ca^{2+} -activated calpains.

As a point of interest, different research groups quote different values for the molecular weight of full length TnI. Based on the protein structure, Ensembl Gene Browser (www.ensembl.org) quotes TnI as having a molecular weight in mice, rats, dogs and humans of approximately 24 kDa. In accord with the predicted weight, both Colantonio et al. (2004) and Katrukha et al. (1998) present full length TnI immunoblots with a molecular weight of approximately 26 kDa in dogs and humans, respectively. However, both Gao et al. (1997) and Feng et al. (2001) report rat full length TnI immunoblots with a molecular weight of approximately 31 kDa. It is unclear as to why this discrepancy arises, but may result from inaccuracies relating to molecular weight markers. Importantly, all groups report TnI degradation products to be approximately 4 kDa lighter than the native TnI protein.

6.5 Conclusion

Our data support the hypothesis that oxidative stress may account for the changes in Ca^{2+} handling and cardiomyocyte fractional shortening that occurs in *mdx* hearts. However, raised ROS did not appear to be involved in changes to *mdx* cardiomyocyte Ca^{2+} -sensitivity. Although further studies are required to establish a causative link, the current data suggest changes in Ca^{2+} -sensitivity may relate to increased TnI degradation.

CHAPTER SEVEN

Role of ROS in the *Mdx* Myocardial Inflammatory Response

7.1 Introduction

The previous two chapters have provided evidence that raised ROS and increased activity of NADPH oxidase play a role in the pathogenesis of *mdx* myocardial damage. In addition to changes in intracellular Ca^{2+} handling, cardiac inflammation and fibrosis contribute towards the pathology of DCMs. DMD patients (Finsterer & Stollberger, 2003) and *mdx* mice (Quinlan *et al.*, 2004; Wehling-Henricks *et al.*, 2005) develop cardiac fibrosis. *Mdx* mouse hearts also develop inflammation (Bridges, 1986; Spencer *et al.*, 2001; Wehling-Henricks *et al.*, 2005). Cardiac inflammation could then lead to cardiac fibrosis via inflammatory cell secretion of pro-fibrotic proteins, such as TGF- β (Van Erp *et al.*, 2006). Increased fibrosis may result in reduced cardiac mechanical performance, in addition to ECG defects and arrhythmias due to changes in extracellular resistance.

Increased ROS can promote cardiac inflammation by inducing pro-inflammatory mediators, such as the transcription factor NF- κ B, which regulates a battery of inflammatory genes (Valen *et al.*, 2001). This results in increased expression of a host of proteins, including cytokines and adhesion molecules, leading to myocardial inflammatory cell aggregation (Valen *et al.*, 2001).

NADPH oxidase has been shown to be involved in both NF- κ B activation (Piao *et al.*, 2005) and in profibrotic pathways (Johar *et al.*, 2006). In addition to nuclear translocation

of NF- κ B, post-translation modification of NF- κ B, such as phosphorylation of the p65 (phospho-p65) NF- κ B subunit, has been shown to be important for agonist-induced stimulation of the NF- κ B pathway. Thus, the aim of the present chapter was to explore the role of ROS on the cardiac inflammation and fibrosis that occurs in *mdx* hearts. In addition, the role of phospho-p65 was explored.

7.2 Methods

7.2.1 In vivo treatment of mice with the antioxidant N-acetylcysteine

NAC-treatment followed protocol 2, as described in Chapter 2. Mice were divided into 4 groups; WT and *mdx* control mice that received tap water, and WT and *mdx* mice that received 1% NAC (WT-NAC, *mdx*-NAC) in their drinking water from 3 until 9 weeks of age (i.e. 6 weeks of treatment). Mice were sacrificed at 9 weeks of age, which was the age that all investigations were performed.

7.2.2 Immunohistochemistry

The relative levels of collagen type III and CD 68 (a macrophage specific protein) were assessed using immunohistochemistry. 10 μ m heart sections were placed on slides (Chapter 2) and blocked in PBS-BSA (2%) for 30 minutes to reduce non-specific antibody binding. Sections were incubated with either collagen type III primary antibody or CD68 primary antibody (see Table 7.1 for conditions). Sections were then washed in PBS for 10 minutes before being incubated with fluorescence-conjugated secondary antibodies. *In situ* fluorescence was assessed using fluorescence microscopy. All antibodies were diluted in PBS. CD 68 data is presented as the number of pixels exceeding a threshold divided by the total number of pixels.

Collagen Type III		CD 68
Primary antibody		
Type	Rabbit anti-rat IgG (Chemicon)	Rat anti-mouse IgG (AbD Serotec)
Dilution	1:80	1:200
Incubation time	2 hours	Overnight
Incubation temperature	Room temperature	4°C
Reference	(Wehling-Henricks <i>et al.</i> , 2005)	(Pugh <i>et al.</i> , 2007)
Secondary antibody		
Type	Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch)	FITC-conjugated goat anti-rat IgG (Jackson ImmunoResearch)
Dilution	1:400	1:100
Incubation time	1 hour	1.5 hours
Incubation temperature	Room temperature	Room temperature

Table 7.1 Immunohistochemistry conditions for analysis of collagen type III and CD 68 in mice hearts.

Conditions used for immunoblotting. Abbreviations: Cy3, cyanine 3; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G. Company addresses: AbD Serotec, Oxford, UK; Chemicon, Temecula, CA, USA; Jackson ImmunoResearch, West Grove, PA, USA.

7.2.3 Immunoblotting

The relative levels of phospho-p65 in the cytosol were determined by quantitative immunoblotting. Cardiac tissue homogenates was prepared as described in Chapter 2. In brief, tissue homogenate was solubilized in Laemmli sample buffer and heated at 95°C for 5 minutes. 40 µg of protein/well was loaded onto a 4% stacking gel and separated via SDS-PAGE on a 10% separating gel. Following transfer, membranes were blocked before being incubated overnight at 4°C with primary rabbit anti-mouse IgG antibodies against phospho-

p65 (1:300; Abcam, Cambridge, UK). Membranes were washed in PBST then incubated for 1 hour with HRP-conjugated secondary anti-rabbit IgG antibody (1:1,000, SantaCruz, Santa Cruz, CA, USA) and developed using an enhanced chemiluminescence detection system (Amersham BioSciences, Uppsala, Sweden). In some experiments the phospho-p65 blocking peptide (10 µg/ml; Abcam, Cambridge, UK) was used to confirm the specificity of the phospho-p65 antibody. The phospho-p65 blocking peptide was present during the primary incubation with phospho-p65 antibody. All antibodies were diluted in PBST-block. Total protein loading was normalised using a Bradford assay (Bio-Rad Ltd). All bands were normalised to the protein GAPDH.

7.3 Results

7.3.1 Cardiac inflammation and fibrosis

Mdx mouse hearts have increased inflammation and fibrosis (Wehling-Henricks *et al.*, 2005). Increased ROS levels may promote the inflammation and fibrosis found in *mdx* hearts via ROS-induced increases in pro-inflammatory proteins (Lee *et al.*, 2001; Valen *et al.*, 2001). In order to test this hypothesis we investigated whether NAC-treatment reduced signs of inflammation and fibrosis in *mdx* mouse heart sections.

Immunohistochemistry experiments revealed that *mdx* hearts had increased levels of a marker of inflammatory responses (the macrophage specific protein CD68), and of collagen type III (Fig. 7.1 and 7.2). NAC-treatment returned CD68 and collagen III expression close to WT values. NAC-treatment had no effect on WT hearts.

7.3.2 Phospho-p65 expression in *mdx* hearts

p65 is one subunit of the NF- κ B dimer. In addition to nuclear translocation of the p65/p50 dimer, phosphorylation of the p65 subunit is an important regulatory mechanism of NF- κ B. To investigate whether the ROS-induced inflammatory mediator NF- κ B was involved in the *mdx* inflammatory response, immunoblotting experiments were performed to determine the levels of S276 phospho-p65 in cytosolic homogenates from *mdx* and WT hearts. Phospho-p65 levels were over 3-fold greater in WT hearts compared to *mdx* (Fig. 7.3).

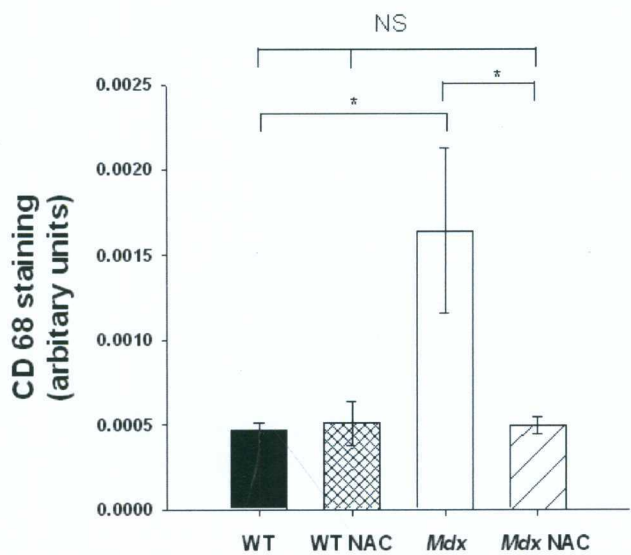


Figure 7.1 Effect of NAC-treatment on *mdx* and WT cardiac macrophage levels

Group data showing levels of the macrophage specific protein CD68 in WT, WT-NAC, *mdx* and *mdx*-NAC hearts. N ≥ 6; NS, not significant; * P < 0.05 between indicated groups.

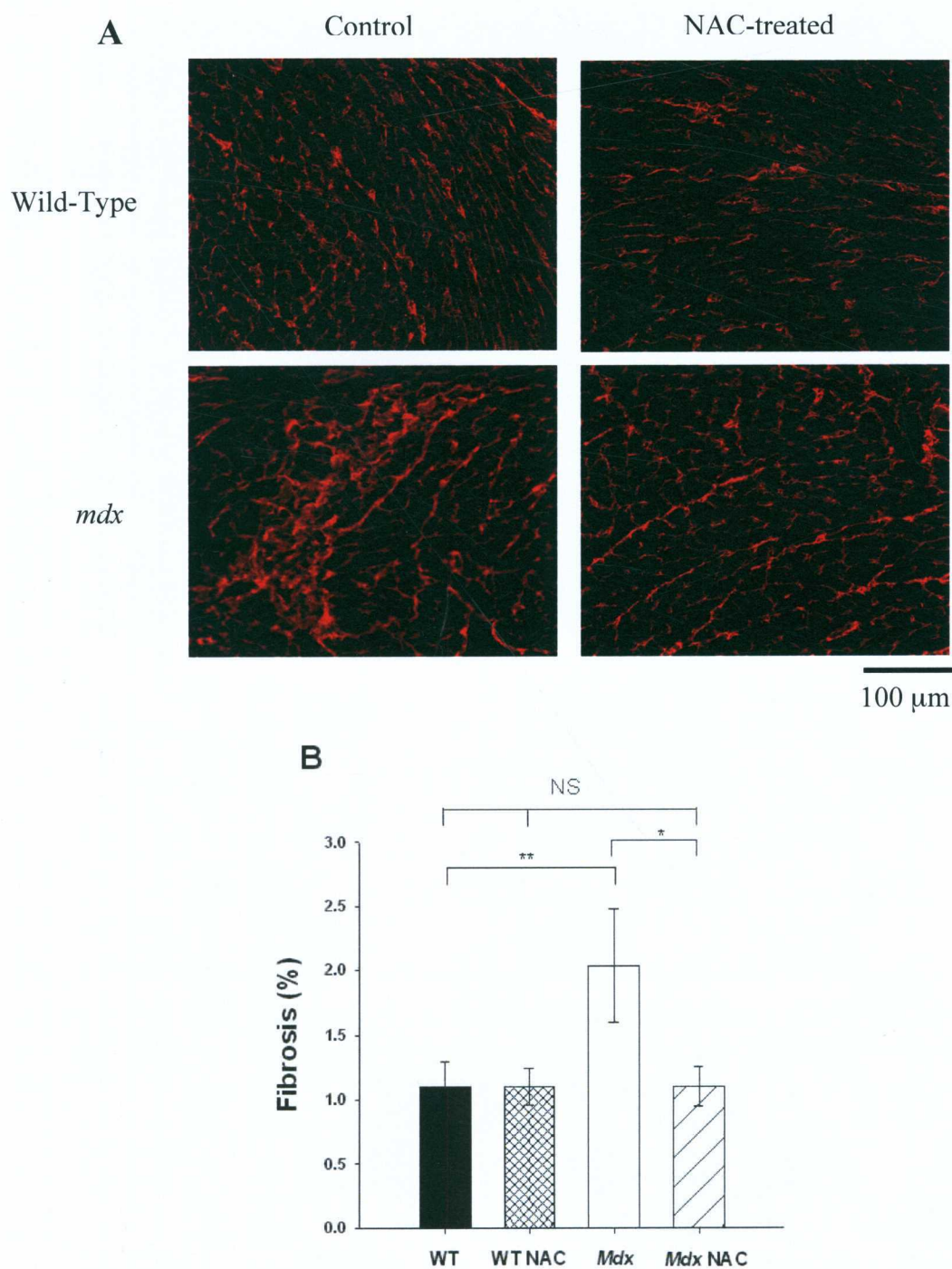


Figure 7.2 Effect of NAC-treatment on *mdx* and WT cardiac collagen type III levels

Effect of NAC-treatment on *mdx* and WT cardiac collagen type III levels. **A:** Representative images showing the distribution of collagen type III in WT, WT-NAC, *mdx* and *mdx*-NAC heart sections. **B:** Quantified collagen type III levels in the heart tissue. $N \geq 6$; NS, not significant; * $P < 0.05$, ** $P < 0.01$ between indicated groups. Bar = 100 μ m.

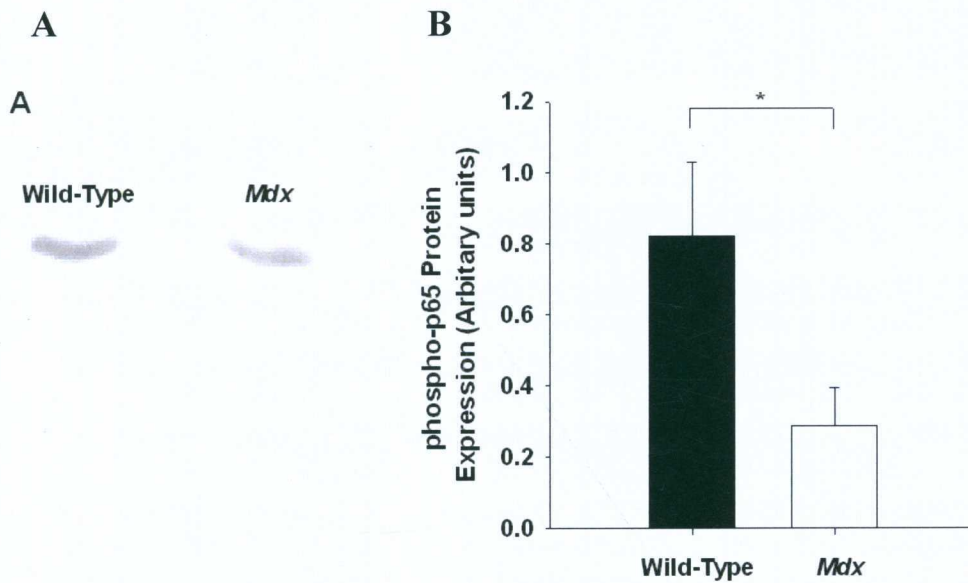


Figure 7.3 Phosphorylated p65 protein expression in WT and *mdx* hearts

A: Sample blots showing protein expression levels of phosphorylated p65 (phospho-p65; 65 kDa) in *mdx* and WT cytosolic tissue homogenate. **B:** Group data from A. $N \geq 6$. * $P < 0.05$ between *mdx* and WT.

7.4 Discussion

It is currently unknown why inflammation and fibrosis occurs in *mdx* hearts. The present study found that the increased inflammation and fibrosis in *mdx* hearts could be prevented by antioxidant treatment, suggesting that increased oxidative stress may be involved.

7.4.1 Cardiac inflammation and fibrosis

Antioxidant treatment prevented the increased myocardial inflammation and fibrosis in *mdx* mice, suggesting a role for ROS in this pathology. We hypothesised that increased ROS may lead to augmented inflammatory cell aggregation, which could then cause an increase in fibrosis. In cardiac muscle, ROS can promote inflammatory cell aggregation by activating pro-inflammatory mediators, such as NF- κ B (Eisner *et al.*, 2006; Valen *et al.*, 2001). ROS also increase expression of endothelial cell adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1; Lee *et al.*, 2001) and P-selectin (Patel *et al.*, 1991), as well as increasing expression of macrophage migration inhibitory factor, a cytokine that inhibits macrophage migration and concentrates them at the site of inflammation (Takahashi *et al.*, 2001). Cardiac inflammation could then lead to cardiac fibrosis via inflammatory cell secretion of pro-fibrotic proteins, such as TGF- β (Van Erp *et al.*, 2006).

With regard to the increase in collagen type III reported in the current study, it is important to note that we initially reported no increase in collagen type III expression in young *mdx* hearts compared to WT (Williams & Allen, 2007). However, with a greater sample size, a significant increase became apparent (Fig. 7.2). It is also important to be aware that cardiac fibrosis is made up of both collagen type I and III. Collagen type III was investigated here as previous

groups have presented data displaying impressive staining with the Chemicon antibody (Wehling-Henricks *et al.*, 2005; Chemicon, Temecula, CA, USA). In addition, as the collagen type I:III ratio is unchanged in fibrotic *mdx* hearts, levels of collagen type III give an estimate of the change in collagen type I levels.

The finding that a modest yet significant increase in cardiac fibrosis occurred in young *mdx* hearts correlates well with findings from others (Cohn *et al.*, 2007). The observation that myocardial inflammation and fibrosis occurs in young *mdx* mice is in accord with our data showing that Ca^{2+} handling and cardiomyocyte contractile function are also abnormal at this age (Chapters 3 to 6). In parallel with increased levels of fibrosis, macrophage levels were also increased in young *mdx* hearts, suggesting increased inflammatory cell aggregation. The 3-fold increase in macrophage levels reported presently is lower than the 5-fold increase reported by others, but is probably due to fact that 9 week old mice were used in the current investigation whereas Wehling-Henricks *et al.* (2005) used 12 month old mice. It is important to note that inflammation is probably not an initiating factor in the *mdx* pathology, since skeletal muscle membrane damage is observed before the appearance of macrophages (Cullen & Fulthorpe, 1975). However, inflammation and cardiac fibrosis are potentially damaging, as increased collagen can lead to conduction defects and increased myocardial stiffness, leading to reduced diastolic filling (Swynghedauw, 1999).

7.4.2 Phospho-p65 expression in *mdx* hearts

In order to ascertain if ROS-induced increases in cardiac inflammation and fibrosis was due to increased activation of NF- κ B, immunoblotting studies assessed the levels of cytosolic phospho-p65. Cytosolic phospho-p65 was decreased in *mdx* hearts, which was surprising, as an increase in expression was predicted. However, the result can be explained by the fact that a cytosolic homogenate rather than a nuclear homogenate was used to assess phospho-p65 levels (see Chapter 2). Thus, although phospho-p65 was decreased in cytosolic homogenate, it is possible that nuclear levels of phospho-p65 were raised due to augmented nuclear translocation of the activated mediator. This hypothesis is supported by Eisner *et al.* (2006) who showed that the levels of p65 in the cytosol decreased following activation, while nuclear levels increased. Furthermore, groups who have reported raised NF- κ B activation in either DCM patients (Wong *et al.*, 1998) or *mdx* skeletal muscle (Durham *et al.*, 2006; Messina *et al.*, 2006a) describe increases in nuclear staining. Interestingly, it has been reported that NF- κ B DNA-binding is not increased in *mdx* hearts (Durham *et al.*, 2006). However, in this study a trend was observed, and it is possible that as *mdx* mice age and the inflammatory process progresses, increased NF- κ B DNA binding in *mdx* hearts might occur.

Although the focus of the current chapter was to investigate the role of ROS in *mdx* cardiac fibrosis and inflammation, additional inflammatory pathways have been proposed that relate to stretch activated channels and $[Ca^{2+}]_i$. In lung (Kumar *et al.*, 2003) and coronary artery tissue (Hishikawa *et al.*, 1997), mechanical and pulsatile stretch increase NF- κ B activation. Furthermore, NF- κ B activation could be prevented by the SAC inhibitor

gadolinium (Kumar *et al.*, 2003). In skeletal muscle, stretch increases NF- κ B activation (Kumar & Boriek, 2003), although the presence of gadolinium during stretches did not affect activation. $[Ca^{2+}]_i$ overload also leads to NF- κ B activation (Zhang *et al.*, 2005). It is also interesting to note that in addition to its ability to activate NF- κ B, increased oxidative stress may also induce inflammation by reacting with NO, thereby reducing NO anti-inflammatory effects (Guzik *et al.*, 2003; Nakamura *et al.*, 2001).

7.5 Conclusion

Our data support the hypothesis that oxidative stress may be involved in the pathological inflammatory response that occurs in *mdx* hearts. Furthermore, limited data suggests that this ROS may act to increase NF- κ B activation, leading to increased nuclear translocation and decreased cytosolic levels. Thus, therapies designed to reduce oxidative damage or inhibit superfluous NF- κ B activation might be beneficial to DMD patients with heart failure.

CHAPTER EIGHT

General Discussion

8.1 Summary of findings

This thesis investigated possible causes of the DCM that occurs in DMD and *mdx* mice. It is currently unknown why absence of the protein dystrophin leads to heart failure in DMD and *mdx* mice. In *mdx* skeletal muscle it is generally accepted that changes in Ca^{2+} handling are involved in the pathogenic process, although the underlying mechanism leading to raised resting $[\text{Ca}^{2+}]_i$ is still disputed (Alderton & Steinhardt, 2000b; Carlson, 1998; Head *et al.*, 1992). One possibility is that absence of dystrophin leads to dysfunction of SACs and intracellular Ca^{2+} handling.

There is also good evidence that in *mdx* skeletal muscle abnormalities in levels may be involved in the DMD pathology (Rando, 2002). In other forms of DCM, an imbalance exists between ROS production (Heymes *et al.*, 2003) and myocardial antioxidative stress defence mechanisms (Baumer *et al.*, 2000; Sam *et al.*, 2005), leading to increased ROS. This may result in Ca^{2+} handling defects via increased oxidation of Ca^{2+} handling proteins (Zima & Blatter, 2006).

In addition to changes in intracellular Ca^{2+} handling, cardiac inflammation and fibrosis contribute towards the pathology of DCMs. DMD patients and *mdx* mice also develop cardiac fibrosis and inflammation (Finsterer & Stollberger, 2003; Wehling-Henricks *et al.*, 2005). Increased inflammation may lead to fibrosis which reduces cardiac

mechanical performance and promotes ECG defects and arrhythmias (Kass *et al.*, 2004; Weber *et al.*, 1994).

No studies have explored the involvement of either ROS or SACs in the DCM that occurs in *mdx* mice. Thus, this thesis explored the role of SACs and ROS on the Ca^{2+} handling properties of *mdx* cardiomyocytes. The potential involvement of ROS in the *mdx* cardiac inflammatory response was also explored. In summary, the novel and key findings of this thesis are: 1) Ca^{2+} handling is abnormal in *mdx* cardiomyocytes; 2) SAC activity and expression is altered in *mdx* cardiomyocytes; 3) ROS levels are raised in *mdx* hearts; 4) increased NADPH oxidase activity may, in part, account for augmented ROS production in *mdx* hearts; 5) antioxidant treatment reversed Ca^{2+} handling defects in *mdx* cardiomyocytes; 6) raised intracellular ROS may underlie the augmented inflammatory and fibrotic response in *mdx* hearts. Together, these findings suggest that SACs and ROS play a role in many of the damage pathways that lead to myocardial dysfunction in *mdx* mice. These results raise the general question; what is the sequence of pathological changes which follows the absence of dystrophin? More specifically, can we determine which of SAC activation or ROS production occur earlier in the sequence? The following section will discuss these two disease mechanisms and present evidence in support of each pathway.

8.2 Altered SAC activity as an early defect in the *mdx* pathology

8.2.1 Increased SAC activity and absence of dystrophin

The current study found that SAC expression and activity were altered in old *mdx* hearts. This raises the question, is altered SAC function an early defect in *mdx* hearts? In order for SACs to be considered an early pathology, there must be a link between absence of dystrophin and increased channel expression and activity. With regard to SAC expression, this link may occur through the intermediate protein caveolin 3 (Fig. 8.1). Caveolin 3 associates with dystrophin (Song *et al.*, 1996), and in the absence of dystrophin, caveolin 3 levels are augmented (Repetto *et al.*, 1999). Furthermore, experiments from our laboratory have demonstrated that TRPC1, the putative gene for SACs (Maroto *et al.*, 2005), and caveolin 3 directly interact with one another in skeletal muscle (unpublished observations, Gervasio, 2007). Increased binding of caveolin 3 to SACs may increase SAC activity by facilitating interaction between the channel and activators, such as *Src* kinase (unpublished observations, Gervasio, 2007). In addition, increased binding of SACs to caveolin 3 may help stabilize the channel and prevent its degradation. Thus, theoretically it is possible to link absence of dystrophin to increased SAC activity and expression.

8.2.2 Can increased SAC activity account for increased ROS?

The current study found that the *mdx* myocardium has raised intracellular ROS levels. If increased activity of SACs occurs before increased oxidative stress in *mdx* hearts, how does it lead to augmented ROS production? One possibility is that increased SAC activity results in increased Ca^{2+} influx, mitochondrial Ca^{2+} overload and consequently

augmented mitochondrial ROS production (Fig. 8.1; Brookes *et al.*, 2004). Although this hypothesis is attractive, little direct evidence exists in its support. A second possibility relates to the finding that intracellular Ca^{2+} can activate NADPH oxidase ROS production in human fibroblasts (Meier, 1996). However, this finding has not been replicated in the myocardium.

8.2.3 Are SACs altered before the onset of muscle damage?

One way to address the question of whether increased Ca^{2+} influx through SACs is an early defect in the pathology of *mdx* hearts is to investigate if alterations in SAC function occur at a sufficiently early stage in the disease process to be the cause of and not the consequence of other damage pathways. Although any changes detected at an earlier age do not necessarily confirm that a mechanism is an early defect, it at least identifies pathways that are closely linked to the primary pathology.

In *mdx* skeletal muscle, increased SAC activity has been observed before the onset of detectable muscle damage (Haws & Lansman, 1991). The current study found that the SAC inhibitor streptomycin had no effect on resting $[\text{Ca}^{2+}]_i$ in young *mdx* cardiomyocytes, suggesting that changes in SAC function were undetectable. In addition, TRPC1 expression was not significantly increased in young *mdx* hearts. No other studies have investigated SACs in *mdx* or DMD cardiac muscle. However, our observations do not exclude altered SAC activity as an early disease mechanism. Firstly, in the early stages of the *mdx* disease process, alterations in SAC function might be too small to detect. Secondly, the current study assessed SAC function by measuring streptomycin-induced changes in resting $[\text{Ca}^{2+}]_i$. Any small changes in resting $[\text{Ca}^{2+}]_i$ may have been beyond the limited spatial resolution of the current imaging technique.

Thirdly, SAC function was estimated in quiescent, un-stretched cardiomyocytes. Thus, it is conceivable that upon stretch, previously concealed differences in *mdx* and WT SAC function may have become apparent. Finally, changes in SAC function were not measured in very young mice. Thus, by 9 weeks of age (the age of the majority of the mice used in this thesis) changes had already occurred in *mdx* Ca^{2+} handling, inflammation and fibrosis. It could be argued that it would be more pertinent to investigate SAC function in very young mice, possibly before 3 weeks of age, which is the age when the first signs of *mdx* skeletal muscle damage occur (McGeachie *et al.*, 1993).

With regard to other forms of heart failure, a recent study explored the timing of SAC activation in a rat aortacaval fistula (ACF) model of heart failure (McNicholas-Bevensee *et al.*, 2006). Interestingly, 5 days after surgery and before the onset of overt heart failure, ACF myocytes, compared to sham animals, displayed a larger gadolinium and GsMTx-4 sensitive current. This finding supports the hypothesis that altered SAC activity may play a role in the pathogenesis of cardiac disease.

8.3 Increased ROS as an early defect in the *mdx* pathology

8.3.1 Increased NADPH oxidase dependent ROS production and absence of dystrophin

As discussed in Chapter 5, several potential mechanisms link lack of dystrophin to increased NADPH oxidase-dependent ROS production (Fig. 8.1). One possibility relates to the finding that caveolae are increased in DMD (Repetto *et al.*, 1999). Dystrophin associates with caveolin 3 (Song *et al.*, 1996), and in DMD, where dystrophin is absent, caveolin 3 levels are augmented leading to an increase in the number of caveolae (Galbiati *et al.*, 2000). NADPH oxidase subunits have been shown to co-localize with caveolin 1 in vascular smooth muscle (Hilenski *et al.*, 2004). Therefore, our finding that caveolin 3 expression is greater in *mdx* hearts may allow increased localization of gp91^{phox} to caveolae, resulting in a decrease in gp91^{phox} degradation, and consequently an increase in NADPH oxidase-dependent ROS production. Caveolae have also been shown to be important in the spatial regulation and agonist stimulation of NADPH oxidase (Kawamura *et al.*, 2003; Yang & Rizzo, 2007). Thus, in summary, it is possible that in *mdx* mice, absence of dystrophin leads to an increase in caveolin 3 and caveolae, resulting in increased NADPH oxidase activity.

A second mechanism to account for increased NADPH oxidase activity in the absence of dystrophin relates to the finding that enzymatic activity is increased by stretch (Hwang *et al.*, 2003b). In the absence of dystrophin, *mdx* myocytes have increased elasticity (Pasternak *et al.*, 1995) and are more susceptible to stretch (Yasuda *et al.*, 2005). Stretch leads to activation of both NADPH oxidase-dependent ROS production (Hwang *et al.*, 2003b) and activation of the enzyme's regulatory subunit rac1 (Kawamura *et al.*, 2003),

in addition to increasing the expression of gp91^{phox} (Hwang *et al.*, 2003a). Thus, it is possible that in *mdx* cardiomyocytes, stretch leads to the augmented NADPH oxidase activity and increased expression of gp91^{phox} found in *mdx* mice.

8.3.2 Can raised ROS account for changes in SAC activity?

SAC activity and expression were increased in old *mdx* mouse hearts. If increased ROS occurs before changes in SAC activity, a mechanism should exist that links raised ROS to augmented channel activity and expression.

Preliminary results presented in this thesis suggest that ROS are able to augment resting $[Ca^{2+}]_i$ in *mdx* cardiomyocytes, possibly by increasing Ca^{2+} influx through a SAC (Chapter 5). These preliminary observations are supported by the finding that hydrogen peroxide induced a rise in resting $[Ca^{2+}]_i$ in *mdx* skeletal muscle. The rise was reversed by the SAC inhibitor streptomycin, suggesting that hydrogen peroxide was increasing the opening of a SAC. Hydrogen peroxide had no effect on WT resting $[Ca^{2+}]_i$, (unpublished observations, Yeung, 2006). It is important to note that hydrogen peroxide has also been shown to increase the opening of the DHPR, which could account for the increase in resting $[Ca^{2+}]_i$ observed in the current study (Viola *et al.*, 2007). However, the contribution of the DHPR to the increase in resting $[Ca^{2+}]_i$ in response to hydrogen peroxide is likely to be very small, as the DHPR is closed at the resting membrane potential (Bers, 2001). Nonetheless, further studies employing more specific SAC inhibitors, such as GsMTx-4, would be required to confirm the role of SACs in the ROS-induced rise in resting $[Ca^{2+}]_i$.

Raised ROS levels may also underlie increased myocardial *mdx* SAC protein expression. ROS have been shown to activate the NF- κ B pathway (Kumar & Boriek, 2003), which has been shown to increase TRPC1 protein expression (Paria *et al.*, 2003). Thus, raised ROS may also account for the augmented expression of TRPC1 in old *mdx* myocardium (Fig. 8.1).

8.3.3 Are ROS increased before the onset of muscle damage?

The present study found that ROS were raised in young (9 week old) *mdx* mice that had not developed overt heart failure. As raised ROS levels precede the onset of muscle damage, it could be argued that they are an important early pathological mediator. However, at the same age, young (9 week old) *mdx* mice already had alterations in myocardial Ca^{2+} handling, myofilament function and inflammation. As with SACs (see above), a more definitive test would be to analyse ROS levels in very young animals. Although this was not conducted in the present study, evidence has been presented demonstrating that oxidative stress is increased in *mdx* skeletal muscle before the onset of damage, which lasted until 3 weeks of age (Disatnik *et al.*, 1998).

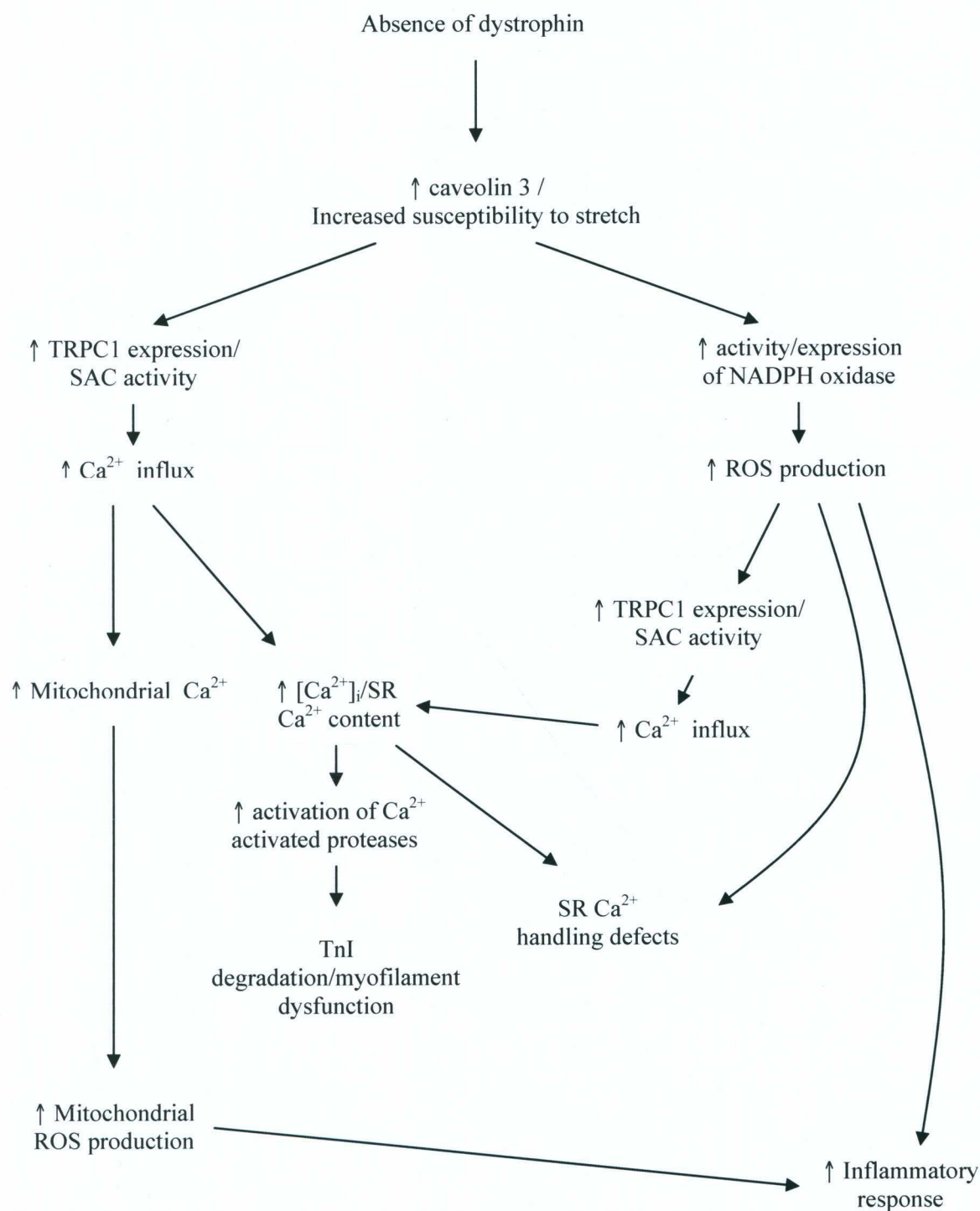


Figure 8.1 Flowchart showing SAC and ROS damage pathways in *mdx* cardiac muscle

Flowchart linking absence of dystrophin to increased SAC activity and augmented ROS production. A more detailed explanation of the pathways is found in the text.

8.4 ROS, SACs and additional pathways

The above section reviewed evidence in favour of altered SAC activity or increased ROS levels as early defects in the pathogenesis of *mdx* myocardial damage. Although one of these two mechanisms may be involved early in the DMD phenotype, based on the evidence presented above, it is possible that both pathways occur simultaneously. Furthermore, it is likely that each pathway will compound upon the other, leading to a positive feedback cycle that accelerates the damage process. In addition to alterations in SACs and ROS, a number of other intracellular pathways may also be involved in the pathogenesis of *mdx* cardiac and skeletal muscle damage. For example, in DMD many of the signalling proteins contained within the dystroglycan complex are down regulated (Rando, 2001). One such protein is nNOS, which in *mdx* cardiac muscle has decreased activity (Bia *et al.*, 1999) and is absent from the sarcolemma (Planitzer *et al.*, 2002). Over-expression of nNOS in *mdx* mice hearts reduces macrophage infiltration, cardiac fibrosis and ECG abnormalities (Wehling-Henricks *et al.*, 2005); these beneficial effects were attributed to the finding that NO produced by nNOS is anti-inflammatory (Guzik *et al.*, 2003). Thus, it is probable that the NOS pathway is also involved in the *mdx* pathology.

The finding that a variety of different proteins are altered in DMD suggests that effective pharmacological treatment will require a multi-drug approach, similar to the current combination therapy approach used to treat patients with heart failure (Adorisio *et al.*, 2006). Although combinatorial use of drugs may help to broaden therapeutic effects, it may also result in more side effects and complications.

8.5 Clinical trials

The results presented in this thesis support the hypothesis that altered SAC activity and increased ROS production may mediate many of the pathological features of the DCM that occurs in *mdx* mice. How do these findings relate to the treatment of DMD patients? This question is difficult to answer. One possibility would be to conduct clinical drug trials in DMD patients to investigate the effects of treatments that reduce oxidative stress or block SACs.

8.5.1 Antioxidant drug trials

In vivo antioxidant treatments have been shown to have beneficial effects in *mdx* skeletal muscle (Bornman *et al.*, 1998; Buetler *et al.*, 2002; Dorchies *et al.*, 2006). A recent trial using BN 82270, a membrane-permeable pro-drug of a chimeric compound, which acts as both a calpain inhibitor and antioxidant, exerted beneficial effects on *mdx* skeletal muscle (Burdi *et al.*, 2006). Unfortunately, clinical trials of antioxidants in DMD patients have shown no clear benefits (Fenichel *et al.*, 1988; Stern *et al.*, 1982). However, these trials have been limited in size and duration, and have failed to provide evidence that the ROS status of cells was altered. The studies were performed on patients who had already undergone severe muscle degeneration which may limit the effectiveness of antioxidant treatment. In support of this latter point, we found that *mdx* mice treated with NAC from 6 weeks of age to 9 weeks (i.e. for only 3 weeks) showed only marginal improvements in Ca^{2+} handling, suggesting that the starting time and/or duration of antioxidant treatment is of importance.

To the best of my knowledge no studies have investigated antioxidant treatment with regard to the DCM associated with DMD. However, in other forms of DCMs, inhibiting

xanthine oxidase dependent ROS production has been shown to improve myocardial function in both patients and animal models (Cappola *et al.*, 2001; Duncan *et al.*, 2005; Minhas *et al.*, 2006). It is interesting to note that in the above studies, although antioxidant treatments had beneficial effects, therapies were commenced after the onset of cardiac damage. Thus, in other forms of DCM the starting time of antioxidant treatment does not appear to be as critical as compared to DMD.

8.5.2 SAC inhibitor drug trials

An *in vivo* trial of the SAC inhibitor streptomycin reduced signs of muscle damage in *mdx* skeletal muscle (Yeung *et al.*, 2005). A recent clinical trial has provided circumstantial evidence supporting a role for SACs in DMD patients (Wagner *et al.*, 2001). In the trial the antibiotic gentamicin, which is also a SAC inhibitor, was administered to four DMD patients for two weeks. It was hoped that gentamicin would suppress nonsense mutations in the dystrophin gene, thus allowing for the translation of the full-length dystrophin protein. At the end of the trial, none of the patients had full-length dystrophin expression. However, all four patients had reduced serum creatine kinase levels. The authors proposed that the decreased kinase levels were due to bed rest during the trial. However, an alternative mechanism is that gentamicin blocked SACs, and thereby reduced $[Ca^{2+}]_i$ and muscle damage (Yeung *et al.*, 2005). *In vivo* assessment of SAC inhibitors with regard to DMD and *mdx* cardiac damage have not been performed.

No published studies have investigated the effects of SAC inhibitors in other forms of DCM. However, *in vivo* treatment with gentamicin has been shown to prevent left

ventricular hypertrophy in response to pressure overload induced by banding of the aortic arch in rats (personal communication, Michael Fenely, 2007).

8.5.3 Alternative treatment options

Alternative treatment options for DMD patients have explored interventions that reduce downstream effects of the disease process. One such downstream defect, although debated (Khurana & Davies, 2003), is the increased inflammatory response that occurs in *mdx* mice. Pharmacological approaches to reduce inflammation in *mdx* mice have shown some benefit. Antibody mediated depletion of CD4⁺ and CD8⁺ T cells (Spencer *et al.*, 2001) and treatment with anti-TNF-alpha etanercept (Pierno *et al.*, 2007) improved the *mdx* skeletal muscle pathology, while pirfenidone, a TGF- β antagonist, improved *mdx* cardiac muscle pathology (Van Erp *et al.*, 2006). Interestingly, in the pirfenidone trial, investigators suggested that drug's capacity to scavenge ROS may have been the basis for the improved cardiac contractility, lending support to findings from this thesis.

An additional therapeutic approach is to limit the effects of raised resting $[Ca^{2+}]_i$. Dantrolene, a drug that inhibits SR Ca²⁺ release, had some beneficial effects in a pilot clinical study (Bertorini *et al.*, 1991). *In vivo* pharmacological treatment with the calpain inhibitor, leupeptin, which is designed to prevent Ca²⁺-activated calpain damage, reduced *mdx* skeletal muscle degeneration (Badalamente & Stracher, 2000).

8.5.4 Non-pharmacological treatment options

In addition to pharmacological therapies, gene therapy (Duan, 2006) and cell therapy (Gussoni *et al.*, 1999; Partridge *et al.*, 1989) offer therapeutic promise in the treatment of DMD. Gene and cell therapy approaches have the advantage of addressing the primary pathology, circumventing the need to correct secondary defects. However, many technical challenges need to be overcome before these molecular approaches can be fully implemented into clinical trials for DMD. Major issues relating to gene therapy include producing sufficient expression of dystrophin in human muscles, while cell therapy requires a substantial increase in cell transplantation (reviewed in Wells, 2006). Unfortunately, studies focusing on gene therapy in DMD cardiac muscle lag behind skeletal muscle (Duan, 2006). Thus, although gene and cell therapy approaches are attractive, more work is required before they become viable treatment options.

8.6 Cellular source of ROS: Cardiomyocytes versus inflammatory cells

The observation that myocardial infiltration of macrophages is increased in *mdx* hearts (Chapter 7), and the previously published findings that macrophages produce ROS (Yagisawa *et al.*, 1996) raise the question, could macrophages be the source of increased ROS production in *mdx* hearts? In addition, are macrophages the source of the augmented gp91^{phox} expression found in *mdx* hearts? The answer to these questions was not directly addressed in this thesis. Thus, we can not exclude the possibility that macrophages were indeed the source of raised NADPH oxidase-dependent ROS production and increased gp91^{phox} expression. One argument in favour of cardiomyocytes being the main source of raised ROS/gp91^{phox} expression relates to the

distribution of EtBr fluorescence in the DHE stain. If inflammatory cells were the main source of ROS, one would expect to see foci of increased EtBr fluorescence in *mdx* heart sections in areas where macrophage infiltration had occurred. However, EtBr fluorescence was uniformly distributed across *mdx* heart sections, suggesting that increased ROS levels occurred in all cell types equally, including cardiomyocytes. Nonetheless, based on the current findings it is not possible to exclude either cell type as a possible source of the raised ROS/gp91^{phox} expression. In order to further explore this issue, future experiments could investigate ROS levels in isolated WT and *mdx* cardiomyocytes, using a ROS-sensitive dye, such as dichlorofluorescein (Sarvazyan, 1996).

8.7 Future directions

8.7.1 Long-term antioxidant trial

The antioxidant trial conducted in this study improved early signs of the *mdx* cardiac pathology. However, with regard to the DCM that occurs in DMD patients, the main challenge is to produce a treatment that prevents overt heart failure. Although *mdx* mice do develop heart failure, signs do not become apparent until 9 months of age (Quinlan *et al.*, 2004). Thus, it would be important to determine if antioxidant treatment could prevent *mdx* heart failure. The main drawback of such a trial is the length of time (9 months) required before it can be determined if the treatment has been successful. Thus, the development of a protocol which accelerates the onset of heart failure in *mdx* mice would be valuable. One such model is the aortic-banded *mdx* model (Danialou *et al.*, 2001; Kamogawa *et al.*, 2001). Banding of the abdominal aorta results in cardiac lesions within 2 days of surgery; thus this model would allow outcomes of drugs trials to be

determined quickly. Alternatively, exercising *mdx* mice has been shown to accelerate the disease process (Nakamura *et al.*, 2002). However, the main drawback of the exercise model is that it is labour intensive as mice have to be exercised regularly in order to accelerate damage.

8.7.2 Interactions between caveolin 3 and NADPH oxidase

The intracellular location of NADPH oxidase within cardiomyocytes was not studied in this thesis. However, it was hypothesized that NADPH oxidase associated with caveolin 3 and caveolae. In order to test this hypothesis, NADPH oxidase activity could be assessed in the presence and absence of agents that are known to specifically disrupt caveolae formation, such as methyl beta-cyclodextrin (Kawamura *et al.*, 2003). A decrease in NADPH oxidase activity in the presence of methyl beta-cyclodextrin would imply that caveolae are important for enzyme activation. To further explore potential interactions between caveolin 3 and NADPH oxidase, co-immunoprecipitation and fluorescence resonance energy transfer (FRET) studies could be performed.

8.7.3 ROS and inflammation

Chapter 7 of this thesis explored the involvement of ROS in the *mdx* cardiac inflammatory response. *In vivo* NAC-treatment reduced both macrophage infiltration and fibrosis; however, the mechanism by which ROS induced an increase in the inflammatory response was not explored. Preliminary experiments explored the potential involvement of the ROS-sensitive inflammatory mediator, NF- κ B. In order to fully explore the role of NF- κ B, it would be beneficial to develop an electrophoretic

mobility shift assay to study NF- κ B DNA binding. If NF- κ B DNA binding was found to be increased in *mdx* hearts, the effect of antioxidant on binding could be assessed.

8.7 Conclusions

In conclusion, increased activity of SACs and increased oxidative stress may account for the changes in Ca^{2+} handling, myofilament dysfunction and inflammation that occur in *mdx* hearts. NADPH oxidase activity and expression are also increased in *mdx* hearts, and may account, in part, for increased ROS production. Early therapeutic interventions that reduce SAC activity and attenuate oxidative damage might be beneficial to DMD patients with heart failure.

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