An Investigation of NADPH Oxidase in Normal and Diseased Skeletal Muscle

Thesis submitted for the assessment of

Doctor of Philosophy

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Victoria University, Faculty of Health Engineering and Science

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2009

Overview

The recent discovery of functional NADPH oxidase enzymes in a variety of cells and tissues proposes various roles for the superoxide generating enzyme systems. The controlled release of NADPH oxidase-dependent superoxide production has recognised the enzyme system as an important component in various redox-sensitive signaling pathways. Inappropriate activation of the superoxide generating system has the potential to alter normal cellular function and cause considerable harm to healthy tissue. In order to avoid the harmful effects of these reactive molecules, antioxidant enzymes function to convert reactive oxidants into less reactive oxygen species and are therefore an important regulator of cellular oxidative stress. Oxidative stress has been implicated in various progressive degenerative conditions, such as skeletal muscle wasting and therefore this study sought to determine a role for the superoxide generating NADPH oxidase and antioxidant enzyme systems in conditions of skeletal muscle wasting. The results of these studies indicated changes in the gene expression of important components of NADPH oxidase in animal models of age-associated sarcopenia, cancer-induced cachexia and a model of antioxidant superoxide dismutase overexpression. In addition, we observed changes in superoxide dismutase that appeared to contribute significantly to alterations in cellular reactive oxygen species and contribute to skeletal muscle wasting in these conditions. While these oxidative and antioxidative systems demonstrated complex changes in these models, NADPH oxidase is indeed altered in response to aging, cancer and superoxide dismutase overexpression, which appear to be involved in complex redox-sensitive signaling that essentially regulates skeletal muscle atrophy and hypertrophy pathways.

Doctor of Philosophy Declaration

"I, Melanie Sullivan-Gunn, declare that the PhD thesis entitled An Investigation of NADPH Oxidase in Normal and Diseased Skeletal Muscle is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

Signature ..... Date .....

Acknowledgements

I would like to firstly express my sincere gratitude to my supervisors Dr Siun O'Sullivan & Dr Paul Lewandowski for sharing their expertise and guidance in this field of research and also for their encouragement and ongoing support throughout my candidature.

I would like to thank all the staff at Victoria University for their support, with special thanks to Dr Alan Hayes and Dr Chris Stathis for the use of the EMU Lab, where I was able to carry out experimental analyses. I would like to also thank Dr Emma Rybalka for her demonstrations in animal tissue collection and technical assistance. I would like to thank Nikola Popovik and the technical staff at Victoria University for their lab support, and Kim Pastore and the staff at the Victoria University animal house for their assistance with the animal models. I would like to also thank Victoria University for their financial support, which made it possible for me to continue this research. I would also like to acknowledge and thank Dr Patrick Mclaughlin for his ongoing support and assisting me with the organization of the International NOX conference (2008). A special thankyou also to my fellow PhD students who helped to create a friendly and supportive research environment.

I would like to express my gratitude to Professor Michael J. Tisdale for his generous donation of the cancer-induced cachectic skeletal muscle tissue and cancer cell lines, which made it possible to carry out the cachectic studies. I would also like to express my gratitude to Dr Michael Mathai for his generous donation of the SOD1^{G93A} mouse model tissue. I would like to thank Amy Larsen for her assistance with cell culture techniques and use of the cell culture lab facilities at Deakin University. I would also

like to thank Dr Louise Judd and Professor Andy Giraud and their team at the Murdoch Children's Research Institute for allowing me access to their PCR equipment for gene analyses.

I would like to personally thank Holy Cannoli, Tre Biccheri, Auction Rooms and Le Chien and to the staff at Fandango for their especially warm hospitality. I would like to express my sincere thanks to my friends for all their support, encouragement and understanding throughout my candidature with special thanks to Ella for always being there for me. A very special thankyou to Mile for all his assistance and advice over the past three years, but most importantly for being a great support and loving friend.

This achievement would not have been possible without the amazing love and support of my family, who have always encouraged and supported me, but who have also been very patient and understanding, particularly during this time. A very special thankyou to Jona for being extremely supportive, encouraging and ever so patient and understanding.

Abbreviations

Aracodonic Acid	AA
Beta-Actin	ACTB
Activation Domain	AD
Animal Experimental Ethics Committee	AEEC
Animal Experimentation Ethics	AEETH
Auto Immune Deficiency Syndrome	AIDS
Autoinhibitory Region	AIR
Amyotrophic Lateral Sclerosis	ALS
Angiotensin II	Ang II
Analysis Of Variance	ANOVA
Activating Protein-1	AP-1
Animal Resources Centre	ARC
Atrogin-1	At-1
1-Bromo-3-Chloropropane	BCP
Bovine Serum Albumen	BSA
Catalase	Cat
Chronic Granulomatous Disease	CGD
Chronic Obstructive Pulmonary Disease	COPD
Cyclicoxygenase	COX
Critical Threshold	C_T
Cardiovascular Disease	CVD
Delta	Δ
Dihydroethidium	DHE
Dimethyl Sulfoxide	DMSO

Ethylene Diamine Tetraacetic Acid	EDTA
Ethylene Glycol Tetraacetic Acid	EGTA
Electron Transport Chain	ETC
Flavin Adenine Dinucleotide	FAD
Familial ALS	FALS
Fetal Bovine Serum	FBS
Forkhead Box O	FOXO
Glyceraldehyde 3-phosphate Dehydrogenase	GAPDH
Guanosine Diphosphate	GDP
Glutathione Peroxidase	GPx
Glutathione (reduced)	GSH
Glutathione (oxidised)	GSSG
Guanosine Triphosphate	GTP
Heme-iron	Heme(Fe^{3+})
4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic acid	HEPES
Insulin-like Growth Factor-1	IGF-1
I-kB kinase complex	IKK
Interleukin-6	IL-6
Linearized Rate	LR
Murine Adenocarcinoma	MAC
Mitogen-Activated Protein Kinase	MAPK
Minor Groove Binder	MGB
Mammalian Target Of Rapamycin	mTOR
Muscle Ring Finger-1	MuRF-1
Nicotinamide Adenine Dinucleotide (reduced)	NADH

Nicotinamide Adenine Dinucleotide Phosphate (oxidised)	$NADP^+$
Nicotinamide Adenine Dinucleotide Phosphate (reduced)	NADPH
Nuclear Factor-Kappa Beta	NF-κB
Nuclease Free Water	NFW
NADPH Oxidase	NOX
NOX Activator 1	NOXA1
NOX Organiser 1	NOXO1
Optimal Cutting Temperature	O.C.T
Oxidative Stress	OS
70-kDa ribosomal protein S6 kinase	p70S6k
Phox and Bem 1	PB1
Phosphate Buffered Saline	PBS
Phagocyte NADPH Oxidase	PHOX
Phosphatidylinositol-3-kinase	PI3K
Proteolysis Inducing Factor	PIF
Protein Kinase B	PKB/Akt
Protein Kinase C	PKC
Phospholipase A	PLA
Phorbol 12-Myristate 13-Acetate	PMA
Proline Rich Region	PRR
Phox	PX
Reactive Oxygen Species	ROS
Reverse Transcription -Polymerase Chain Reaction	RT-PCR
Rho GDP-dissociation inhibitor	RhoGDI
Somatic ALS	SALS

Standard Error of the Mean	SEM
Src Homologue 3	SH3
Superoxide Dismutase	SOD
Copper Zinc Superoxide Dismutase	SOD1/CuZn-SOD
Manganese Superoxide Dismutase	SOD2/Mn-SOD
Extracellular Superoxide Dismutase	SOD3/Ec-SOD
Total Dilution Factor	TDF
Transmembrane	TM
Tumour Necrosis Factor-alpha	TNF-α
Tetratricopeptide Repeat	TPR
Ubiquitin	Ub
Wild Type	WT
Xanthine Oxidase	XO
Hydrogen	H^+
Hydrogen Peroxide	H_2O_2
Hydrochloric acid	HCl
Dipotassium Phosphate	K_2HPO_4
Potassium Dihydrogen Phosphate	Kh_2PO_4
Potassium Hydroxide	KOH
Sodium Bicarbonate	$NaHCO_3$
Oxygen	O_2
Superoxide	O_2^-
Hydroxyl Radical	$OH^\bullet$

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Chapter 1 Literature Review

1.1 Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidase

Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidase (NOX) was firstly considered as a multi-component enzyme system that functions to generate the Reactive Oxygen Species (ROS), superoxide anion (O_2^-) in phagocytes (Bedard & Krause 2007; El-Benna et al. 2005) (Reaction 1).

Reaction (1) $NADPH + 2O_2 \xrightarrow{\text{(NADPH oxidase)}} NADP^+ + H^+ + 2O_2^-$

Upon stimulation NOX is activated and utilises electrons derived from intracellular NADPH to generate O_2^- (Bokoch & Diebold 2002). First discovered in phagocytes, O_2^- generated by phagocyte NOX is toxic to a variety of microorganisms and its functionality is therefore a major component in immune defence (Li et al. 2002; Shiose et al. 2001; Sukhanov et al. 2007; Vazquez-Torres et al. 2000). The phagocyte NOX enzyme is a five-subunit complex that requires the process of subunit assembly for oxidase activation. The subunit components of phagocyte NOX consist of two membrane bound subunits, $gp91^{phox}$ and $p22^{phox}$, and three cytosolic subunits, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ (Babior 2002; Kitiyakara et al. 2003). This localisation of the membrane and cytosolic subunit complexes resembles the resting state of the oxidase (Groemping et al. 2003) and the subunits must assemble to complete the enzyme complex for activation and generation of O_2^- (Takeya & Sumimoto 2003). The protein

components of NOX along with the GTP binding protein Rac1 assemble via the translocation of the cytosolic subunits to the membrane to form the active oxidase (El-Benna et al. 2005; Groemping & Rittinger 2005).

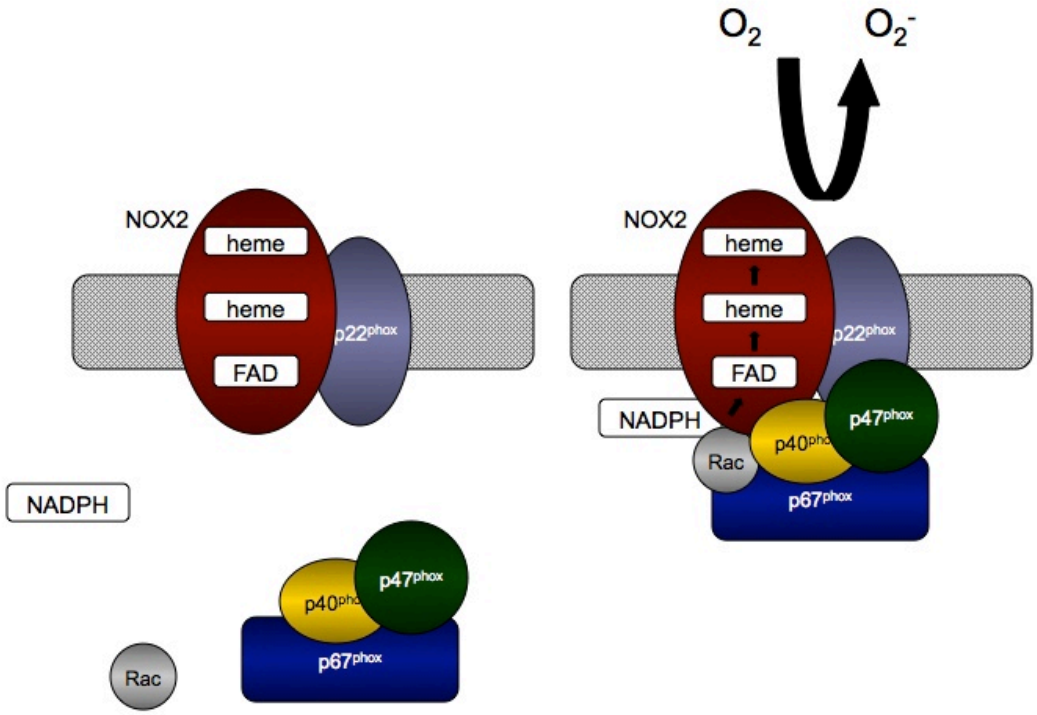
In addition to the well established phagocyte NOX, the enzyme system is present and active in non-phagocytes, indicating that the NOX enzyme is not just a mechanism of host defence, rather this ROS producing enzyme system functions in various roles in numerous cells and tissues (Banfi et al. 2003; Davis et al. 2004; Griendling, Sorescu & Ushio-Fukai 2000; Javesghani et al. 2002). With this finding came the discovery of a number of homologues for the catalytic subunit $gp91^{phox}$ that has since been renamed NOX2. NOX2 contains the transmembrane machinery necessary for NOX activation and generation of O_2^- (Babior 2004; Lassegue & Clempus 2003). Currently, there are five known homologues for the well-established phagocyte NOX2, which include NOX1, NOX3, NOX4 and NOX5, found to be expressed in specific cell types and tissues (Bedard & Krause 2007; Geiszt 2006). Subsequently, NOX enzymes have been found to generate O_2^- in tissues including smooth muscle (Salles et al. 2005), endothelial (Gorlach et al. 2000), neuronal (Kudin et al. 2004), and skeletal muscle (Javesghani et al. 2002) tissues. While the structure and function of the NOX enzyme system in phagocytes is relatively understood, the non-phagocyte NOX enzymes in other tissues are yet to be elucidated.

1.1.1 Phagocyte NADPH Oxidase (NOX)

The NOX2 enzyme mediates the respiratory burst in phagocytic leukocytes, a process that when appropriately stimulated initiates the consumption of oxygen to the production of O_2^- (Robinson 2008). This multicomponent enzyme system consists of five subunit proteins; NOX2, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$, all of which have distinct roles to initiate assembly and activation of the enzyme system (El-Benna et al. 2005). The enzyme is segregated with subunits bound to the plasma membrane, NOX2 and $p22^{phox}$, known as flavocytochrome b_{558}, for its spectral absorption at 558nm, and those localised in the cytosol, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ (El-Benna et al. 2005; Sumimoto et al. 2004). The localisation of the subunits resembles the resting state of the enzyme and interaction between these cytosolic and membrane complexes is required for enzyme activation (Groemping et al. 2003). In response to stimulation, the subunits rapidly assemble to form the active complex (Robinson 2008). In order to do so, the functional oxidase requires a three step process including, protein phosphorylation, GTPase activation and cytosolic translocation to the membrane (El-Benna, Dang & Gougerot-Pocidalo 2008). The cytosolic subunits are multi-domain proteins that essentially regulate the activation of the enzyme system (Groemping et al. 2003), as their inactivity stabilises the protein components and stimulation initiates their translocation to the membrane (Cheng, Ritsick & Lambeth 2004; Lambeth 2004). In response to regulated signaling events, one or more of the cytosolic subunits regulates specific protein-protein interactions by inducing conformational changes that reveal specific protein domains, required for oxidase assembly (Banfi et al. 2003; El-Benna et al. 2005; Groemping et al. 2003; Nauseef 2004). Once assembled, the oxidase undergoes respiratory burst, where the conversion of molecular oxygen to O_2^- occurs. Specifically, the reduced substrate NADPH binds to NOX2 of the assembled

complex, releasing electrons, which are passed in turn to Flavin Adenine Dinucleotide (FAD), then onto the first and second heme groups and accepted by molecular oxygen, to produce O_2^- (Reaction 2) (Figure 1.1). NADPH binds to NOX2 on the cytosolic side of the membrane and O_2^- is released on the opposite side of the membrane (Bokoch & Knaus 2003; Lassegue & Clempus 2003).

Reaction (2) $\quad NADPH \rightarrow FAD \rightarrow Heme\ (Fe^{3+}) \rightarrow 2O_2 \rightarrow 2O_2^-$



(A) (B)

Figure 1.1 The NOX2 enzyme in the resting and activation state. (A) The NOX2 enzyme in the resting state. The separation of the flavocytochrome b_{558} complex and the regulatory $p67^{phox}$/$p40^{phox}$/$p47^{phox}$ cytosolic complex renders the NOX2 enzyme inactive. (B) Formation of the assembled active NOX2 enzyme. Upon stimulation the cytosolic NOX complex translocates to the membrane where it assembles with the membrane complex. Rac also translocates to the membrane and associates with the oxidase. Once the oxidase is assembled, electrons are passed from NADPH to FAD, to the first heme and second heme redox centers and finally to molecular oxygen to produce O_2^- (Sumimoto, Miyano & Takeya 2005; Takeya & Sumimoto 2003).

1.1.1.1 The NOX Enzyme Subunit NOX2

The Flavocytochrome b_{558} membrane complex is described as the catalytic core of the NOX2 enzyme system (El-Benna et al. 2005; Sumimoto et al. 2004). NOX2 is the central redox component that binds NADPH substrate, essential for the transfer of electrons and generation of O_2^- (Lassegue & Clempus 2003; Samuelson et al. 2001; Shiose et al. 2001). Specifically, NOX2 contains all co-factors required for electrons transferred from NADPH onto FAD and to the heme group, to reduce oxygen to O_2^- (Groemping & Rittinger 2005) (Figure 1.2). The NOX2 component is therefore absolutely required for enzyme activation and O_2^- generation, however this key catalytic subunit also requires the assembly of the five subunits for activation (Li & Shah 2003; Takeya & Sumimoto 2003).

1.1.1.2 The NOX Enzyme Subunit p22^{phox}

The NOX subunit p22^{phox} colocalises with NOX2 at the membrane and provides a docking site for the cytosolic complex, p67^{phox}/p40^{phox}/p47^{phox} (Miyano et al. 2006). Structurally, p22^{phox} is composed of two Transmembrane (TM) segments, and a Proline Rich Region (PRR) at the C-terminus (Figure 1.4a) that binds p47^{phox} (Figure 1.5), securing the cytosolic complex at the membrane (Sumimoto, Miyano & Takeya 2005) (Figure 1.6). This is an important function for the oxidase as it essentially completes the complex, allowing the necessary interaction between NOX2 and p67^{phox} for catalytic activation. The importance of the membrane component p22^{phox} has been demonstrated, as a decrease in expression has been shown to significantly decrease NOX enzyme function (Bedard & Krause 2007).

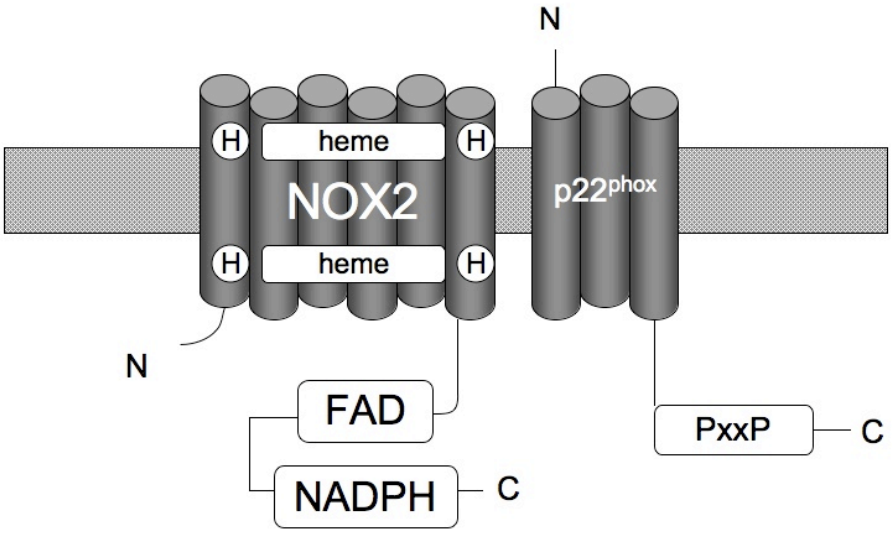


Figure 1.2 Structure of the Flavocytochrome b_{558}. Flavocytochrome b_{558} is composed of NOX2 colocalised with $p22^{phox}$ at the membrane. The core structure of NOX2 consists of six highly conserved transmembrane domains that contain two asymetrical hemes. The C-terminus of NOX2 contains conserved FAD and NADPH binding domains. This structure allows for the transfer of electrons from NADPH to FAD and subsequent heme redox centers to reduce molecular oxygen to O_2^-, once the complex is assembled. The structure of $p22^{phox}$ localised at the membrane, forming the flavocytochrome b_{558} complex with NOX2, allows for the binding of the cytosolic complex (through direct binding with $p47^{phox}$), at the C-terminus PxxP domain of $p22^{phox}$. This important binding domain secures the cytosolic complex to the membrane assembling the active NOX complex, for activation and generation of O_2^- (Groemping & Rittinger 2005; Nauseef 2004; Sumimoto, Miyano & Takeya 2005).

1.1.1.3 The NOX Enzyme Subunit p67^{phox}

The cytosolic NOX subunit p67^{phox} is a crucial component of the NOX2 enzyme system (Lambeth 2004), absolutely required for NOX2 enzyme activation and generation of O_2^- (Babior 2002; Takeya & Sumimoto 2006). Structurally, p67^{phox} is composed of a number of Tetratricopeptide Repeat (TPR) motifs at the N-terminus, an Activation Domain (AD) and two Src Homologue 3 (SH3) domains with a protein binding 'Phox and Bem 1' (PB1) module (Figure 1.4d). The TPR motifs mediate protein-protein interactions involved in the direct binding to Rac and the AD domain binds NOX2 at the membrane. The central SH3/PB1 domain is suggested to interact with the PB1 motif of p40^{phox} (Nauseef 2004), while the SH3/PB1 at the c-terminus interacts with the PRR of p47^{phox} (Takeya & Sumimoto 2003) (Figure 1.5 & 1.6). The protein-protein interaction domain of p67^{phox} enables cytosolic protein recruitment to the membrane and the activation domain facilitates interaction with NOX2 (Groemping & Rittinger 2005). Therefore, the role of p67^{phox} is one of oxidase activator, participating in electron transfer, through its direct interaction with NOX2 (El-Benna et al. 2005; Takeya & Sumimoto 2003). In fact it is thought that p67^{phox} is the component responsible for regulating the electron transfer from NADPH to FAD (Nisimoto et al. 1999). In order for this to occur however is the equally important process of p67^{phox} translocation to the membrane, which is dependent on its binding to the regulatory subunits, p47^{phox} or p40^{phox} (Lopes et al. 2004; Ueyama et al. 2007). This is particularly evident in patients that lack p47^{phox} and show impaired translocation of p67^{phox} to the membrane (Ueyama et al. 2007).

1.1.1.4 The NOX Enzyme Subunit p40^{phox}

The cytosolic NOX subunit p40^{phox} was initially described as a non-essential component of the oxidase (Lassegue & Clempus 2003). Recent studies in p40^{phox} deficient mice and inhibition of p40^{phox} phosphorylation, has been shown to inhibit NOX2 activation, indicating that the subunit is in fact an essential component of the NOX2 enzyme system (Ellson et al. 2006; Grandvaux, Elsen & Vignais 2001; Suh et al. 2006). Structurally, p40^{phox} is composed of an N-terminal phosphoinositide-interacting Phox (PX) domain for lipid binding, an SH3 domain, and a PB1 domain at the C-terminus (Figure 1.4b) that bind tightly to the PB1 region of p67^{phox} (Figure 1.5), securing the cytosolic complex (Sumimoto, Miyano & Takeya 2005) (Figure 1.6). It has therefore been hypothesized that p40^{phox} is responsible for maintaining the resting state of the NOX2 enzyme, stabilising the cytosolic components (DeLeo & Quinn 1996). Consequently, through signal transduction mechanisms, p40^{phox} separates from the other cytosolic subunits (p47^{phox} & p67^{phox}), instigating their translocation to the membrane (Pithon-Curi et al. 2002). Furthermore, p40^{phox} is thought to act as an adaptor component that recruits p67^{phox} to the membrane, to bind NOX2 for enzyme activation (Chen et al. 2007; Kuribayashi et al. 2002; Ueyama et al. 2007). It is proposed that p40^{phox} acquires specific protein targeting domains (PX) that in the absence of stimulation remain inaccessible, but once stimulated become unmasked and accessible to p67^{phox} binding and recruitment to the membrane (Ueyama et al. 2007). The association between these two enzyme subunits is evident in p67^{phox} deficient patients that also express a decrease in p40^{phox} (Matute et al. 2005; Vergnaud et al. 2000).

1.1.1.5 The NOX Enzyme Subunit p47^{phox}

The cytosolic NOX subunit p47^{phox} has been described as the oxidase organiser (Takeya & Sumimoto 2003), responsible for transporting the cytosolic complex to the membrane during oxidase activation (Babior 2002; El-Benna et al. 2005). Structurally, p47^{phox} is composed of a PX domain including a PxxP motif at the N-terminus, tandem SH3 domains, a polybasic phosphorylation target region or Autoinhibitory Region (AIR) and a PRR at the c-terminus (Nauseef 2004) (Figure 1.4c). When the resting cell is stimulated, p47^{phox} becomes phosphorylated and the entire cytosolic complex migrates to the membrane, where it associates with flavocytochrome b_{558} to assemble the active oxidase (Takeya & Sumimoto 2003). Like p40^{phox}, p47^{phox} has no intrinsic catalytic function (Diebold & Bokoch 2001) and these cytosolic components both appear to play a regulatory role that initiates the assembly of the active oxidase (Ueyama et al. 2007). In the cytosol, p47^{phox} binds the SH3 domain of p67^{phox} via its c-terminal PRR domain (Figure 1.5), initiating the assembly of the cytosolic and membrane components (Figure 1.6). Evidence of this has been shown in neutrophils that lack p47^{phox}, consequently resulting in the inability of p67^{phox} to transfer to the membrane (Dusi, Donini & Rossi 1996). Therefore, p47^{phox} is suggested to have a particularly important function, serving as an adapter protein, providing physical binding domains that facilitate the interaction of p67^{phox} with NOX2 (Diebold & Bokoch 2001; Li & Shah 2003) (Figure 1.6). However, the protein-protein interaction domains of p47^{phox} are inaccessible in the resting state masked by the AIR (Sumimoto, Miyano & Takeya 2005) (Figure 1.3).

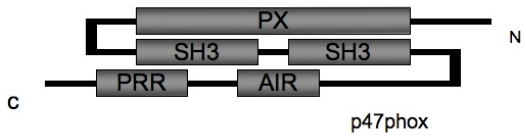


Figure 1.3 A model of the resting configuration of p47^{phox}. The AIR masks the protein-protein binding domains of p47^{phox}, rendering it inaccessible for protein binding. Phosphorylation at the AIR and PRR, converts the constrained configuration to an open and interactive formation for protein binding and assembly of the active complex. Specifically, the interactive configuration allows for the binding of p47^{phox} to p22^{phox}, securing the cytosolic protein complex at the membrane, initiating NOX complex assembly and activation (Groemping & Rittinger 2005; Sumimoto, Miyano & Takeya 2005).

Further to this important regulatory role, p47^{phox} has an equally important role in securing the cytosolic complex at the membrane, through its tandem SH3 domain binding to the PRR domain of p22^{phox} (Figure 1.5 & 1.6). The phosphorylation of p47^{phox} at the AIR induces a conformational change in the subunit state, exposing the tandem SH3 binding domain, rendering itself accessible to its membrane target, p22^{phox} (Babior 2002; Watanabe et al. 2006) (Figure 1.5). This is an important function for p47^{phox} as it secures the assembled components at the membrane, completing the enzyme for activation (Groemping et al. 2003; Watanabe et al. 2006) (Figure 1.6).

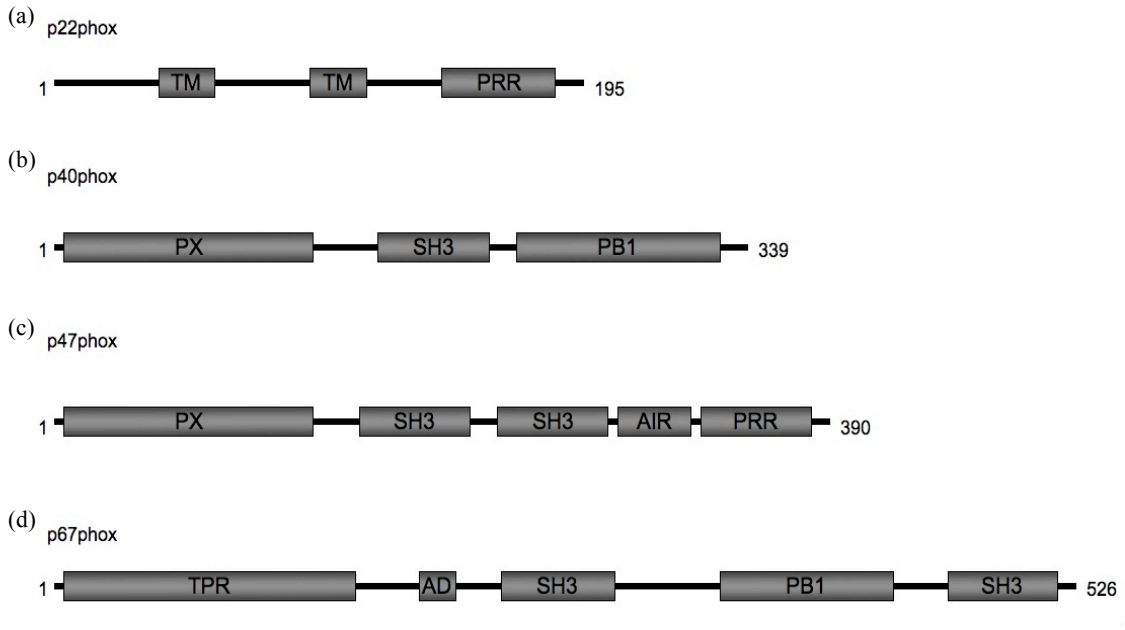


Figure 1.4 The proposed structure of the NOX enzyme subunits, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$. (a) $p22^{phox}$ is a 195 amino acid protein consisting of two successive TM domains and a protein-protein binding PRR/PxxP domain at the C-Terminus. (b) $p40^{phox}$ is a 339 amino acid protein consisting of an N-terminal PX domain, an SH3 domain and a C-Terminal PB1 protein-protein binding domain. (c) $p47^{phox}$ is a 390 amino acid protein consisting of an N-terminal PX domain, two successive protein-protein binding SH3 domains, an AIR domain for protein-binding inhibition and a C-terminal PRR protein-protein binding domain. (d) $p67^{phox}$ is a 526 amino acid protein consisting of a four TPR-motif containing domain, an AD domain for NOX2 binding and catalytic activation, two SH3 domains, one situated at the C-terminus for protein-protein binding and a PB1 domain also located toward the C-terminus for PB1 protein-binding (Groemping & Rittinger 2005; Sumimoto, Miyano & Takeya 2005).

1.1.1.6 Rac GTPase

In addition to the NOX2 subunits, phagocyte NOX has been suggested to also require the participation of a small GTP binding protein Rac, for enzyme activation (El-Benna et al. 2005; Takeya & Sumimoto 2003). Rac belongs to the Rho-family of small GTPases, which regulate a number of cellular signaling pathways (Groemping & Rittinger 2005). There are currently two known Rac GTPases expressed ubiquitously (Rac1) (Filippi et al. 2007) and restricted to hematopoietic cells (Rac2) (Fueller & Kubatzky 2008). Localised in the cytoplasm in resting cells, Rac exists bound to GDP associated with RhoGDI. Upon phagocytic activation, Rac dissociates from RhoGDI and GDP by binding GTP and migrates to the membrane along with the core cytosolic subunits, binding the NOX enzyme (Babior 2002; Nauseef 2004; Robinson 2008). However, the recruitment of Rac to the membrane is suggested to occur upon cell stimulation independent of the cytosolic components (Takeya & Sumimoto 2003). At the membrane, Rac is converted to the GTP-bound active state and is suggested to directly interact with $p67^{phox}$, by binding at the TPR domain (Figure 1.5 & 1.6), contributing to the necessary interaction and activation of $p67^{phox}$/NOX2 complex (Sumimoto, Miyano & Takeya 2005).

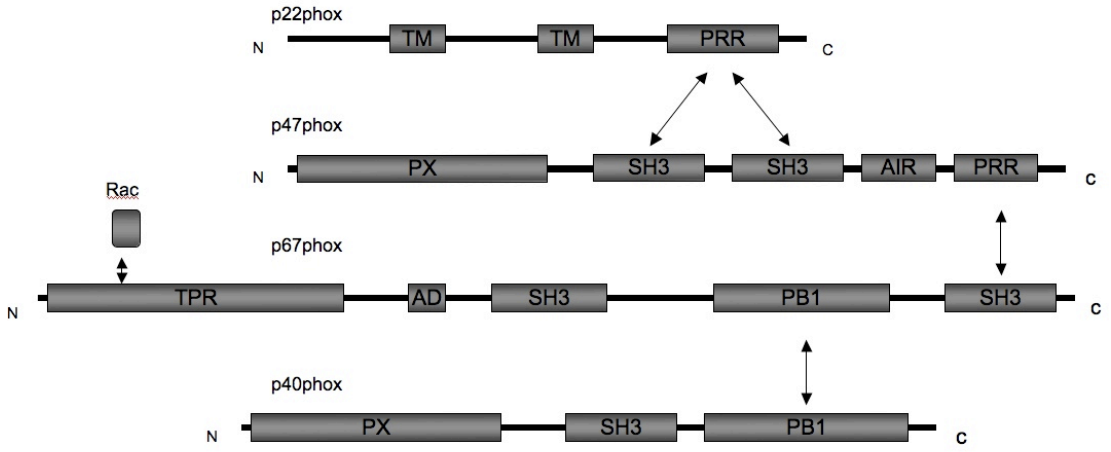


Figure 1.5 The proposed model of structural interaction between the NOX enzyme subunits p22^{phox}, p40^{phox}, p47^{phox} and p67^{phox} and Rac. Localised at the membrane, colocalised with NOX2, the PRR/PxxP domain of p22^{phox} binds with the dual SH3 domains of p47^{phox}. The PRR domain located at the C-terminus of p47^{phox} binds to the SH3 domain of p67^{phox}. The N-terminal TPR region of p67^{phox} binds Rac, while the PB1 domain binds to the PB1 domain of p40^{phox} (Opitz et al. 2007; Sumimoto, Miyano & Takeya 2005).

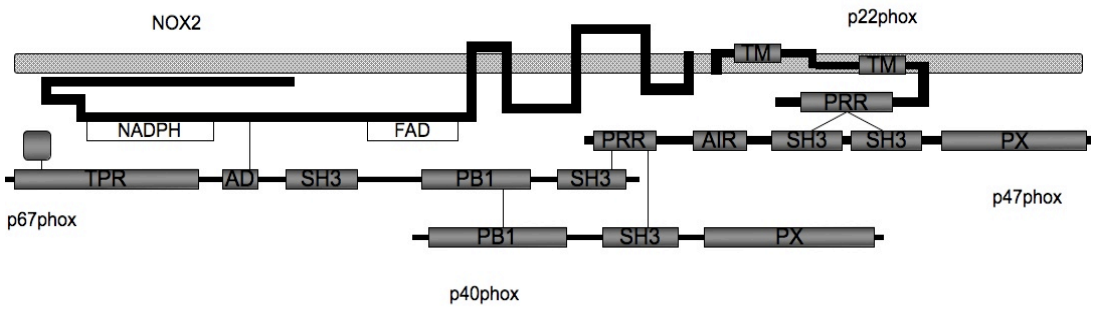


Figure 1.6 The proposed model of the assembly of the active NOX2 enzyme. The NOX2/p22^{phox} flavocytochrome b_{558} complex localised to the membrane, binds with the cytosolic complex p67^{phox}/p40^{phox}/p47^{phox}, through specific protein-protein binding. Upon stimulation and phosphorylation of p47^{phox} facilitates conformational change in the protein revealing protein-protein binding regions, initiating translocation of the cytosolic complex to the membrane for oxidase assembly. The tight binding of the dual SH3 domains of p47^{phox} to the PRR/PxxP domain of p22^{phox}, secures the cytosolic complex to the membrane to form the active complex. The C-terminal PRR domain of p47^{phox} binds both p67^{phox} and p40^{phox} to their SH3 domains and the binding of the PB1 domains of p67^{phox} and p40^{phox} secures the cytosolic complex. The N-terminal TPR region of p67^{phox} binds Rac, that translocates from the cytosol to join the active NOX complex at the membrane and is suggested to regulate the activity of the assembled oxidase (Groemping & Rittinger 2005; Leusen, Verhoeven & Dirk 1996; Sumimoto, Miyano & Takeya 2005).

1.1.1.7 Chronic Granulomatous Disease (CGD)

The phagocyte NOX enzyme is an important component of the innate immune response, for its role in respiratory burst. The absence of any one of the NOX2 enzyme subunits results in recurrent, often life-threatening, bacterial and fungal infections (Dinauer 2005). The importance of the enzyme became apparent with the discovery of NOX2 dysfunction in Chronic Granulomatous Disease (CGD). CGD is an immune deficiency caused by the inability of phagocytes to activate NOX2 and generate normal amounts of O_2^-, essential for the phagocytosis of microorganisms (Dinauer 2005; El-Benna et al. 2005). CGD is characterised by the formation of large granulomas that develop from un-phagocytosed bacteria by macrophages, due to a dysfunction in NOX2 (El-Benna et al. 2005). The inability for NOX2 to generate O_2^- is owed to the loss of one of the NOX2 subunits (Robinson 2008), demonstrating the importance of the enzyme system and regulatory function of the subunit components. While, defects or deficiencies in any one of the four NOX subunits, required for NOX2 enzyme activation, NOX2, $p22^{phox}$, $p47^{phox}$, or $p67^{phox}$, have been shown to induce consequences of NOX dysfunction (Lassegue & Clempus 2003; Sumimoto, Miyano & Takeya 2005; Vignais 2002; Watanabe et al. 2006), approximately 70% of CGD cases result from a mutation in the X-linked gene encoding NOX2 (Ott et al. 2006). A rare autosomal recessive form of CGD is caused by mutations in the gene encoding $p22^{phox}$ (Rae et al. 2000), suggestive of a dysfunction in the docking site for $p47^{phox}$, and therefore inability to stabilise the enzyme (Babior 2002; Sumimoto, Miyano & Takeya 2005). The remaining cases of autosomal recessive CGD involve genetic defects in either of the two regulatory proteins, $p47^{phox}$ or $p67^{phox}$ (Dinauer 2005; Watanabe et al. 2006). While, clinical manifestations are dependent on the

particular NOX subunit mutation, chronic lung, gastrointestinal and skin infections are common in CGD patients (Martire et al. 2008).

1.1.2 Non-phagocyte NOX Enzymes

It has been apparent in recent years that non-phagocytes such as fibroblasts, endothelial cells, vascular smooth muscle cells, cardiac muscle, neurons (Vaziri et al. 2004) and others, contain functional NOX enzymes, analogous to the phagocyte NOX2 enzyme system (Babior 2002; Li & Shah 2003) (Table 1.1). Despite the structural similarities, NOX enzymes found in non-phagocytes are functionally distinct from the widely studied phagocyte NOX enzyme (Jiang, Drummond & Dusting 2004; Li & Shah 2003). For example, it has been suggested that the active components in non-phagocyte NOX generate O_2^- through different translocation mechanisms to those of the phagocyte NOX enzyme (Arbogast & Reid 2004; Bayraktutan, Blayney & Shah 2000; Cheng, Ritsick & Lambeth 2004). In addition, neutrophil NOX generated O_2^- production during phagocytosis is thought to occur in the extracellular (phagosomal) compartment, whereas a substantial proportion of the O_2^- generated in non-phagocytes appears to be intracellular (Geiszt 2006; Krause 2007). However, the mechanism by which O_2^- is generated, via electron transfer from a reduced substrate to molecular oxygen, has been suggested to occur in a similar manner (Lassegue & Clempus 2003). In addition, the non-phagocyte NOX enzymes produce O_2^- at a level that is significantly less than that of the phagocyte NOX enzyme (1-10%) (Li & Shah 2003; Valko et al. 2007). Although differences in the NOX enzyme systems are apparent among various tissue types, in order to achieve oxidase activation, the enzymes require at least one of the five NOX homologues (Krause 2007; Lambeth, Kawahara & Diebold 2007; Sumimoto, Miyano & Takeya

2005). However, the additional subunits that appear to be essential for phagocyte NOX function may not be required for activation of the enzyme systems expressing the NOX homologues (Geiszt & Leto 2004; Takeya et al. 2003) (Figure 1.8). In addition, tissues expressing any one of the NOX homologues have shown patterns of complex configuration, cellular location and activation that are distinct from the phagocyte NOX enzyme (Bedard & Krause 2007).

1.1.2.1 The Homologues of NOX2

Currently, there are seven identified members of the mammalian NOX family homologues, where the founder member NOX2 instigated the discovery of six additional members, NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2 (Lambeth, Kawahara & Diebold 2007; Krause 2004). All NOX family members share a core structure consisting of six transmembrane domains, including two heme-binding regions, and a relatively long cytoplasmic C-terminus, which contains FAD and NADPH-binding regions (Krause 2004) (Figure 1.7). This structure allows for transport of electrons across membranes to reduce oxygen to O_2^- (Bedard & Krause 2007). However, the NOX homologues share only a percentage of the amino acid sequence identity to NOX2 and are also thought to share differences in their regulation and subunit dependency (Opitz et al. 2007; Sumimoto, Miyano & Takeya 2005).

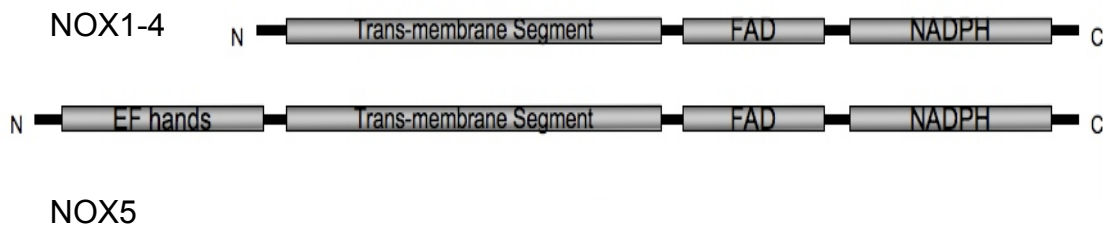


Figure 1.7 The core NOX homologue structure. All members share common features to the core NOX2 component, consisting of an N-terminal transmembrane segment and C-terminal FAD and NADPH binding domains. In addition to the core NOX homologue structure, NOX5 contains an additional N-terminal EF hand region for Ca^{+} binding (Geiszt 2006; Sumimoto, Miyano & Takeya 2005).

NOX1 was the first identified NOX2 homologue, sharing the highest percentage amino acid sequence identity of approximately 60% (Bedard & Krause 2007; Suh et al. 1999). NOX1 is abundantly expressed in colon epithelium but also expressed in a variety of cell types including vascular smooth muscle and endothelial cells (Bedard & Krause 2007; Geiszt 2006) (Table 1.1). It has been relatively confirmed that NOX1 generates O_2^- and requires the regulation of the cytosolic subunits known in the NOX2 enzyme system as $p47^{phox}$ and $p67^{phox}$, and have since been renamed NOX Organiser 1 (NOXO1) and NOX Activator 1 (NOXA1) for their respective roles in the enzyme system (Banfi et al. 2003; Takeya & Sumimoto 2003) (Figure 1.8). In addition to the high expression of NOX2 in phagocytes, NOX2 is also expressed in a variety of cell types, including smooth muscle, endothelial and skeletal muscle (Bedard & Krause 2007) (Table 1.1). NOX3 was discovered following the discovery of NOX1, and shown to share approximately 56% amino acid sequence identity with NOX2 (Cheng et al. 2001). Most abundantly expressed in the inner ear (Banfi et al. 2004a), NOX3 is also expressed in fetal and brain tissue (Bedard & Krause 2007)

(Table 1.1). While NOXO1 appears to be an essential component of the NOX3 enzyme system (Kiss et al. 2006), experimental evidence is yet to clarify the requirement for NOXA1 (Figure 1.8). NOX4 is a distant homologue of NOX2 sharing approximately 39% amino acid sequence identity and appears to only require the transmembrane component $p22^{phox}$ for enzyme activation and O_2^- production (Bedard & Krause 2007) (Figure 1.8). In addition, while the other NOX homologues are suggested to assemble at the plasma membrane, NOX4 has been suggested to localise on intracellular membranes (Martyn et al. 2006). NOX4 remains the least characterised of the NOX homologues (Krause 2007) and is proposed as the most widely expressed NOX homologue, functioning to generate O_2^- in a wide range of cells and tissues, including endothelium, smooth muscle, kidney, neurons, hepatocytes (Bedard & Krause 2007) and skeletal muscle (Cheng et al. 2001; Hidalgo et al. 2006) (Table 1.1). Sharing the lowest percentage amino acid sequence identity with NOX2 of approximately 27%, NOX5 has been described as the largest NOX homologue (Bedard & Krause 2007; Geiszt 2006). NOX5 does not require cytosolic NOX subunit regulation and is in fact suggested to function alone. Therefore, unlike NOX1-NOX4, NOX5 does not require any of the NOX components for enzyme activation and generation of O_2^- (Banfi et al. 2004b; Opitz et al. 2007) (Figure 1.8). However, in addition to the distinct NOX homologue structure of a transmembrane segment, FAD and NADPH binding regions, NOX5 contains a calcium binding domain or EF hands (Figure 1.7), as does DUOX1 and DUOX2 (Krause 2004). Therefore, NOX5 is thought to be regulated or dependent on calcium binding for enzyme activation (Banfi et al. 2004b; Geiszt 2006; Sumimoto, Miyano & Takeya 2005) (Figure 1.8). NOX5 has been shown to be expressed in a variety of tissues including fetal tissues, testes and ovaries (Bedard & Krause 2007) (Table 1.1).

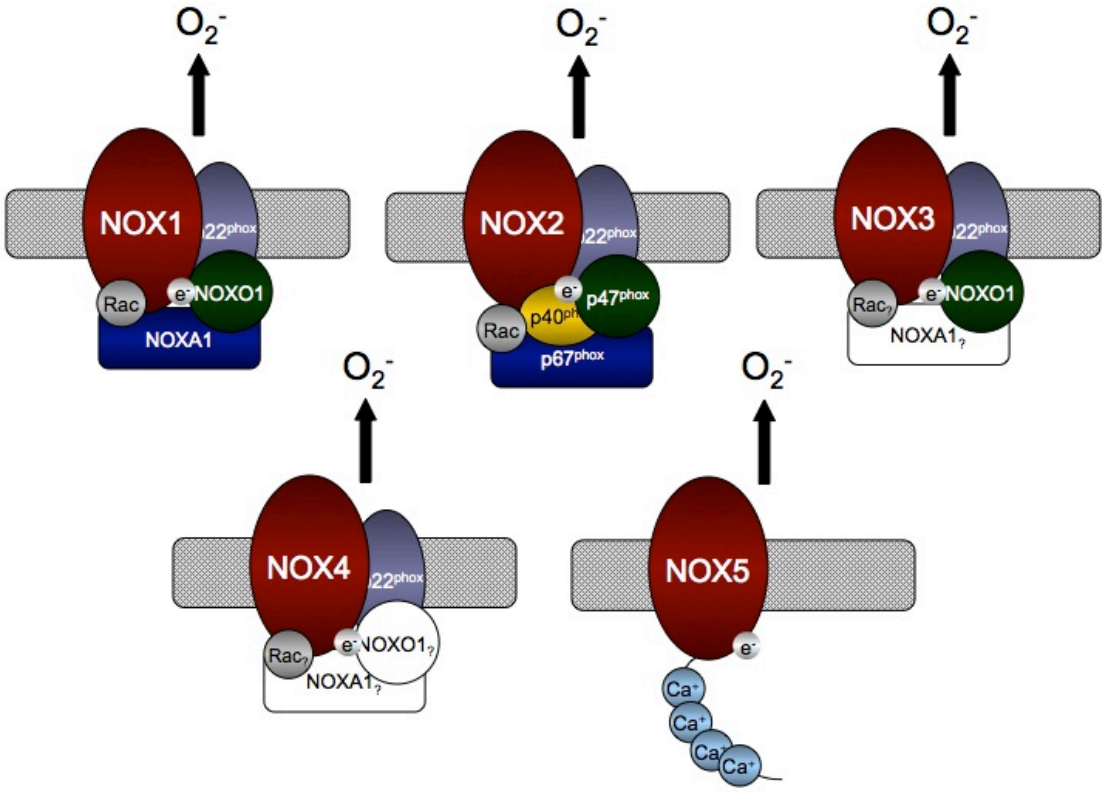


Figure 1.8 The core components required for the activation of the NOX homologues (NOX1-5) and generation of O_2^-. NOX1 localises at the membrane with $p22^{phox}$ and requires the assembly of the cytosolic organiser and activator proteins, NOXO1 and NOXA1, and Rac for electron transfer and generation of O_2^-. NOX2 represents the core structure of the NOX enzyme requiring the assembly of the membrane-bound NOX2/$p22^{phox}$ complex and the cytosolic $p47^{phox}$/$p40^{phox}$/$p67^{phox}$ complex and Rac for electron transfer and generation of O_2^-. NOX3 localises at the membrane with $p22^{phox}$ and requires the translocation of the cytosolic organiser NOXO1 for electron transfer and generation of O_2^-. It is unknown whether the NOX3 enzyme utilises the cytosolic activator NOXA1 or Rac. NOX4 localises with $p22^{phox}$ at the membrane and has been suggested to only require the membrane complex for electron transfer and generation of O_2^-, however the utilisation of the organiser and activator proteins and Rac for NOX4 enzyme activation is unknown. NOX5 localises at the membrane and does not require subunit component interaction for enzyme activation, but requires Ca^+ binding to its N-terminal EF-hand motif for electron transfer and generation of O_2^- (Bedard & Krause 2007; Opitz et al. 2007).

Research has shown NOX homologue expression in various tissues, and many tissue types have been shown to express more than one NOX homologue (Frey, Ushio-Fukai & Malik 2008; Lassegue et al. 2001). For example, endothelial cells in particular have been shown to express NOX1, NOX2, NOX4 and NOX5 (Frey, Ushio-Fukai & Malik 2008) and smooth muscle cells have been shown to express, NOX1, NOX2 and NOX4 (Lassegue et al. 2001), while NOX2 and NOX4 have been described in skeletal muscle tissue (Mofarrahi et al. 2008) (Table 1.1).

Table 1.1 Tissue distribution of the NOX homologues

NOX1	Colon, smooth muscle, endothelium, uterus, placenta, prostate
NOX2	Phagocytes, neurons, cardiomyocytes, skeletal muscle, hepatocytes, endothelium, smooth muscle
NOX3	Inner ear, fetal tissues, bone, brain
NOX4	Osteoclasts, endothelium, smooth muscle, hepatocytes, stem cells, fibroblasts, keratinocytes, melanoma cells, neurons, skeletal muscle
NOX5	Lymphocytes, testes, endothelium, smooth muscle, pancreas, placenta, ovary, uterus, stomach, fetal tissues

(Bedard & Krause 2007; Geiszt & Leto 2004; Javesghani et al. 2002; Mofarrahi et al. 2008)

1.2 Reactive Oxygen Species (ROS)

ROS can be found in virtually all intracellular organelles or compartments as a consequence of normal metabolic activity (Scandalios 2005). ROS are molecules derived from molecular oxygen by chemical reduction that contain one or more unpaired electrons, resulting in a considerable degree of free radical activity (Valko et al. 2007). Molecular oxygen is an essential, relatively unreactive, biological electron acceptor that serves vital roles in cellular functions (Scandalios 2005). However, during normal metabolic activity and redox-sensitive stimulation, oxygen can give rise to ROS (Scandalios 2005; Sumimoto, Miyano & Takeya 2005). ROS too are essential elements of life as they play a crucial role in a variety of physiologic processes (Dalton, Shertzer & Alvaro 1999; Valko et al. 2007). However, ROS can damage proteins, membranes and DNA, causing cellular damage and significantly compromising cellular function (Kowald, Lehrach & Klipp 2006). Furthermore, ROS can enter into reactions that, when uncontrolled, can become impaired and affect normal cellular function (Mates & Sanchez-Jimenez 1999; Scandalios 2005). ROS are generated as a result of cellular aerobic metabolism from the production of ATP in the mitochondria (Kerner et al. 2001; Morten, Ackrell & Melov 2006) and can therefore be generated in increased amounts when metabolic activity is high (Reid 2001). However, ROS can be generated from numerous sources, not only as a by-product of cellular metabolism released from the mitochondrial Electron Transport Chain (ETC), but also from specific ROS producing enzymes such as Xanthine Oxidase (XO), cytochrome p450, Cyclooxygenase (COX) and NOX enzymes (Frey, Ushio-Fukai & Malik 2008; Reid & Li 2001b; Vives-Bauza, Starkov & Garcia-Arumi 2007). Oxygen derived ROS include O_2^-, hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\bullet$)

(Vives-Bauza, Starkov & Garcia-Arumi 2007). However, O_2^- that is considered the primary ROS can further generate secondary ROS through molecular interaction (Nathan & Shiloh 2000; Valko et al. 2007) (Reaction 3).

Reaction (3) $O_2 \rightarrow O_2^- \rightarrow H_2O_2 \rightarrow OH^\bullet \rightarrow H_2O$

1.2.1 Superoxide Anion (O_2^-)

O_2^- is generated as a by-product of normal cellular respiration (Smith & Tisdale 2003) and as a primary product of oxidative enzyme activation (Dalton, Shertzer & Alvaro 1999; Vives-Bauza, Starkov & Garcia-Arumi 2007), through a one electron reduction of molecular oxygen (Hancock, Desikan & Neill 2001; Smith & Tisdale 2003) (Reaction 4). O_2^- is a reactive oxidant, as it is derived from molecular oxygen and contains unpaired electron(s) that renders the molecule reactive and potentially toxic (Macdonald, Galley & Webster 2003). This metabolite of oxygen is essential for normal metabolic and cellular function, however overexposure can damage DNA, proteins and lipids, consequently inflicting considerable damage on biological systems (Cejudo-Marin et al. 2004; Scandalios 2005). In addition, O_2^- acts as a precursor of a large number of highly reactive oxidizing agents (Dinauer 2005; Smith & Tisdale 2003), such as H_2O_2 and $OH^\bullet$.

Reaction (4) $O_2 + e^- \rightarrow O_2^-$

1.2.2 Hydrogen Peroxide (H_2O_2) & Hydroxyl Radical ($OH^{\bullet}$)

The dismutation of O_2^- by highly selective antioxidant enzymes generates a cascade of ROS production, including H_2O_2 (Reaction 5) and $OH^{\bullet}$ (Reaction 6) (Scandalios 2005; Vives-Bauza, Starkov & Garcia-Arumi 2007).

Reaction (5) $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

In addition to the dismutation of O_2^-, H_2O_2 is constantly generated in the mitochondria (Ji 2007) and can also be produced by peroxisomes (Valko et al. 2007). H_2O_2 is relatively stable and is therefore not highly destructive (Ji 2007), but like other ROS, does have the potential to cause considerable harm when generated in excess (Rhee 2006). Although H_2O_2 is quickly destroyed by specific antioxidants (described below), in situations where antioxidant systems are unable to cope, particularly when H_2O_2 is in excess, this ROS can become harmful to the cell (Scandalios 2005). Furthermore, excess H_2O_2 that is normally converted harmlessly to water, by the action of antioxidant enzymes (Reaction 8), can also give rise to $OH^{\bullet}$ in the presence of metal ions (Landis & Tower 2005; Macdonald, Galley & Webster 2003). Particularly under conditions of stress, when O_2^- and H_2O_2 are in excess, free iron is released from iron containing molecules, facilitating the production of $OH^{\bullet}$ (Valko et al. 2006) (Reaction 6). In addition, H_2O_2 has the ability to diffuse across most membrane barriers and has been implicated to play an important role in cellular signaling (Ji 2007; Rhee et al. 2005; Stone & Yang 2006).

Reaction (6) $O_2^- + H_2O_2 \rightarrow (Fe^{++} \text{ or } Cu^{++}) \rightarrow O_2 + HO^{\bullet} + OH^{\bullet}$

$OH^{\bullet}$ can be generated in the presence of both O_2^- and H_2O_2, through the Haber-Weiss reaction in the presence of transition metals or the Fenton reaction in the presence of iron (Reaction 6) (El-Benna et al. 2005; Scandalios 2005; Valko et al. 2006). $OH^{\bullet}$ has been described as one of the most reactive chemical species (Maier & Chan 2002) with a short half-life that reacts close to its site of formation (Hancock, Desikan & Neill 2001; Valko et al. 2007). In particular, $OH^{\bullet}$ is a very strong oxidizer that can attack phospholipids, nucleic acids and DNA, and is therefore capable of causing considerable cellular damage (Maier & Chan 2002). Therefore, the dismutation of O_2^-, by selective antioxidant enzymes, is an important regulator of cascading ROS generation, and maintenance of cellular oxidative homeostasis (Smith & Tisdale 2003; Valko et al. 2007).

1.3 Antioxidant Enzymes

The actions of oxygen-based free radicals, such as O_2^-, can be reduced by exposure to antioxidants, which function as radical scavengers through a series of enzyme based reactions (Blokhina, Virolainen & Fagerstedt 2003; Sastre, Pallardo & Vina 2000; Xia et al. 2003). Each cellular organelle or compartment has potential targets for ROS and therefore all cells possess an antioxidant defense system, providing adequate protection against the harmful effects of ROS (Devasagayam et al. 2004; Mayne 2003; Xia et al. 2003). Endogenous antioxidant enzyme defenses include, Superoxide Dismutase (SOD), catalase and Glutathione Peroxidase (GPx) (Vives-Bauza, Starkov & Garcia-Arumi 2007). These enzymatic antioxidants protect by converting highly reactive oxidants to less reactive species. In brief, SOD converts O_2^- to H_2O_2, which is further converted by catalase and GPx to water (Landis & Tower 2005; Scandalios 2005) (Figure 1.9).

1.3.1 Superoxide Dismutase (SOD)

The SOD enzymes are the first and most important line of antioxidant defense against the toxic effects of ROS (Cejudo-Marin et al. 2004; Zelko, Mariani & Folz 2002). Specifically, SOD catalyses the conversion of O_2^- to H_2O_2 that can in turn be converted by catalase and GPx reactions (Mates & Sanchez-Jimenez 1999) (Reaction 7). Therefore, SOD acts as an instigator for the cascade of ROS reducing antioxidant enzyme activity (Devasagayam et al. 2004). SOD begins with the oxidized form of the enzyme binding O_2^-, acquiring a proton and releasing oxygen. The reduced form of the enzyme then binds a second O_2^- anion and proton, to generate H_2O_2 and return to its oxidized state (Johnson & Giulivi 2005; Mates & Sanchez-Jimenez 1999) (Reaction 7).

Reaction (7) $O_2 + O_2^- + 2H^+ \text{—} (\mathbf{SOD}) \rightarrow H_2O_2 + O_2$

The SOD enzymes are an ubiquitous family of antioxidants thats primary function is to convert O_2^- to less reactive species (Johnson & Giulivi 2005). Localised in the mitochondria, cytoplasm, and extracellular spaces the SOD family antioxidant enzymes make up the major O_2^- scavenging system (Zelko, Mariani & Folz 2002). In humans, there are three distinct forms of SOD; Copper and Zinc (CuZn) containing CuZn-SOD (SOD1), localised to the intracellular cytoplasmic compartment, manganese (Mn) containing Mn-SOD (SOD2), localised to the mitochondria and the most recently characterised copper and zinc containing SOD found in the extracellular (EC) matrix, EC-SOD (SOD3) (Macdonald, Galley & Webster 2003; Zelko, Mariani & Folz 2002).

1.3.1.1 Superoxide Dismutase 1 (SOD1)

SOD1 is expressed in many cell types and tissues, localised in the cellular cytosol and functions to dismutate O_2^- (Zelko, Mariani & Folz 2002). Genetic inactivation of SOD1 results in mild phenotypes including infertility (Ho et al. 1998), age-associated hearing loss (Keithley et al. 2005) and early onset cataracts (Reddy et al. 2004). However, mice lacking SOD1 can develop significant pathologies with increasing age (Morten, Ackrell & Melov 2006) including loss of muscle mass, elevated levels of oxidative damage and a 30% reduction in lifespan (Muller et al. 2006). Furthermore, an overexpression of SOD1 has been shown to extend lifespan in Drosophila (Orr & Sohal 2003). Interestingly, an overexpression of a mutated form of SOD1 has been shown to be responsible for the motor neuron degeneration in Amyotrophic Lateral Sclerosis (ALS) patients and transgenic mouse models, which at least in part, demonstrates its importance as a cellular redox system regulator (Echaniz-Laguna et al. 2002; Harraz et al. 2008).

1.3.1.2 Superoxide Dismutase 2 (SOD2)

Unlike SOD1, SOD2 appears to be restricted to only a few cell types and tissues (Zelko, Mariani & Folz 2002). A major source of O_2^- is produced via the mitochondrial ETC and SOD2 is the primary antioxidant enzyme that functions to remove O_2^- from the mitochondria (Mates & Sanchez-Jimenez 1999). Therefore, SOD2 is particularly expressed in tissues abundant in mitochondria and of high metabolic activity, such as skeletal (Kerner et al. 2001) and cardiac muscle (Morten, Ackrell & Melov 2006). In studies of SOD2 knockout mice, it is evident that SOD2 is essential for survival, as these mice incur severe pathologies such as cardiomyopathy or neurodegeneration (Van Remmen et al. 2003), and mitochondrial dysfunction, as a

consequential buildup of endogenous ROS (Mates & Sanchez-Jimenez 1999; Morten, Ackrell & Melov 2006). Unlike a lack in SOD1 however, mice lacking SOD2 demonstrate lethal effects, either during development or within a few days of birth (Asikainen et al. 2002; Huang et al. 1997; Mates & Sanchez-Jimenez 1999).

1.3.1.3 Superoxide Dismutase 3 (SOD3)

SOD3 is expressed in relatively high amounts in the interstitial spaces of tissues and extracellular fluids of many cell types and tissues (Zelko, Mariani & Folz 2002). Therefore, SOD3 accounts for the majority of the SOD activity in plasma, lymph and synovial fluid (Mates & Sanchez-Jimenez 1999). SOD3 is highly expressed in specific cells and tissues of the heart, lung, blood vessels, kidney, smooth muscle, glial and endothelial cells (Nozik-Grayck, Suliman & Piantadosi 2005). Like SOD1, but unlike SOD2, genetic inactivation of SOD3 results in mild phenotypic changes (Morten, Ackrell & Melov 2006) that become particularly significant with age, such as age-associated endothelial dysfunction (Di Massimo et al. 2006).

1.3.2 Catalase & Glutathione Peroxidase (GPx)

Catalase and GPx play an important role in ROS scavenging and regulation of cellular H_2O_2, eliminating its accumulation and capturing it before it escapes the cell (Mates & Sanchez-Jimenez 1999). The production of H_2O_2, can result from O_2^- dismutation, instigating the scavenging of H_2O_2, by the antioxidant enzymes catalase and GPx (Kowald, Lehrach & Klipp 2006) (Reaction 8). Although catalase shares H_2O_2 scavenging with GPx, GPx has been suggested to be the major source of protection against low levels of H_2O_2, whereas catalase becomes more significant in protecting

against severe H_2O_2 (Vives-Bauza, Starkov & Garcia-Arumi 2007). However, in some cellular systems it is suggested that GPx is more efficient in catalyzing H_2O_2, as catalase has a lower affinity for H_2O_2 (Mates & Sanchez-Jimenez 1999).

Reaction (8) $O_2^- \text{—} (\mathbf{SOD}) \rightarrow H_2O_2 \text{—} (\mathbf{Catalase/GPx}) \rightarrow H_2O$

Catalase is found in relatively high concentrations in mammalian cells and functions to specifically reduce one molecule of H_2O_2 to water and molecular oxygen (Chelikani, Fita & Loewen 2004; Mates & Sanchez-Jimenez 1999) (Reaction 9). Catalase has been described as the most efficient of the antioxidant enzymes and cannot be saturated under any H_2O_2 concentration (Mates & Sanchez-Jimenez 1999; Scandalios 2005). Catalase is unique in its functioning to degrade H_2O_2 as it does so without consuming reducing equivalents and is therefore considered an energy-efficient antioxidant (Scandalios 2005; Vetrano et al. 2005; Vives-Bauza, Starkov & Garcia-Arumi 2007). Transgenic mice overexpressing human catalase (MCAT) demonstrate significant delays in age-associated conditions such as cardiac pathology, oxidative damage and mitochondrial deletions (Schriner et al. 2005).

Reaction (9) $2H_2O_2 \text{—} (\mathbf{Catalase}) \rightarrow 2H_2O + O_2$

GPx catalyzes the reduction of a variety of hydroperoxides, including H_2O_2, using GSH (Brigelius-Flohe 2006; Vives-Bauza, Starkov & Garcia-Arumi 2007) (Reaction 10). At least five GPx mammalian isoforms exist with GPx1 as the cytosolic form (Frey, Ushio-Fukai & Malik 2008) and GPx4 as the most widely expressed. The additional isoforms include GPx2 and GPx3, which are rarely detectable in most tissues and GPx5, which is the least described (Mates & Sanchez-Jimenez 1999). It is

important to note that GPx and/or catalase are not only important for the elimination of H_2O_2 accumulation itself, but also to avoid the production of the highly reactive, $OH^{\bullet}$ (Landis & Tower 2005; Scandalios 2005) (Reaction 6 & 8) (Figure 1.9).

Reaction (10) $2GSH + 2H_2O_2 \text{ — (}\mathbf{GPx}\text{)} \rightarrow GSSG + 2H_2O$

1.4 Oxidative Stress (OS)

Under normal physiological conditions, oxidant production and antioxidant dismutation is balanced (Macdonald, Galley & Webster 2003). In situations where this critical balance between oxidants and antioxidants is disrupted, Oxidative Stress (OS) arises. OS can result from an enhanced production of ROS or a reduction in the antioxidant system (Macdonald, Galley & Webster 2003; Scandalios 2005). That is, if ROS are generated inappropriately or antioxidant scavenging is insufficient to clear enhanced or even normal ROS production, ROS accumulates, consequently inducing a state of OS (McCord & Edeas 2005; Wei & Lee 2002). Furthermore, regulated increases in ROS induce temporary imbalances, representing a temporary shift in intracellular redox state toward a more oxidative environment (Valko et al. 2007). Such stress has been described when severely adverse environments or physiologic conditions overwhelm biological systems (Scandalios 2005). Changes in external and internal redox-sensitive stimuli can alter the activation of ROS producing enzymes, such as NOX, ultimately causing inappropriate ROS production (Frey et al. 2002; Hancock, Desikan & Neill 2001; Li & Shah 2003; Moe et al. 2006). The consequences of an imbalance in cellular oxidative state can cause severe metabolic malfunction and damage to biological molecules (Johnson & Giulivi 2005; Mates & Sanchez-Jimenez 1999). O_2^- and $OH^{\bullet}$ in particular are capable of causing severe

molecular damage and cell death (Scandalios 2005), while O_2^- and H_2O_2 can influence redox-sensitive signaling pathways, altering normal cellular function (Hancock, Desikan & Neill 2001; Scandalios 2005). Therefore, strict regulation in oxidase activity is essential for cell survival since inappropriate production of ROS can result in cellular dysfunction and death (Takeya & Sumimoto 2003).

Part of the cells stress response is to increase the activity of antioxidant enzymes when oxidant levels are high and in need of protection (Johnson & Giulivi 2005; Macdonald, Galley & Webster 2003). Antioxidant enzymes control the cellular redox state and are therefore key components in maintaining oxidative homeostasis (Frey, Ushio-Fukai & Malik 2008; Ji 2007). However, excess ROS production can alter the ability of the organisms' natural protective mechanism to cope, limiting endogenous antioxidant defense, consequently subjecting the cell to OS (Cai & Harrison 2000; Li & Shah 2003; Mayne 2003). Furthermore, OS can occur as a result of antioxidant depletion or dysfunction (Macdonald, Galley & Webster 2003; Scandalios 2005) in response to pathological conditions (Devasagayam et al. 2004; McCord & Edeas 2005) and general age-associated decline (Squier 2001; Wei & Lee 2002). Therefore, OS is determined by the balance between the generation of ROS and the antioxidant defense system (Kitiyakara et al. 2003). Cellular OS has been postulated to play a crucial role in the pathogenesis of a number of clinical disorders such as cancer (Gomes-Marcondes & Tisdale 2002), cardiovascular (Cai & Harrison 2000) and cerebral vascular (Jiang, Drummond & Dusting 2004) disease, neurodegeneration (Maier & Chan 2002), and general age-associated conditions (Golden & Melov 2001).

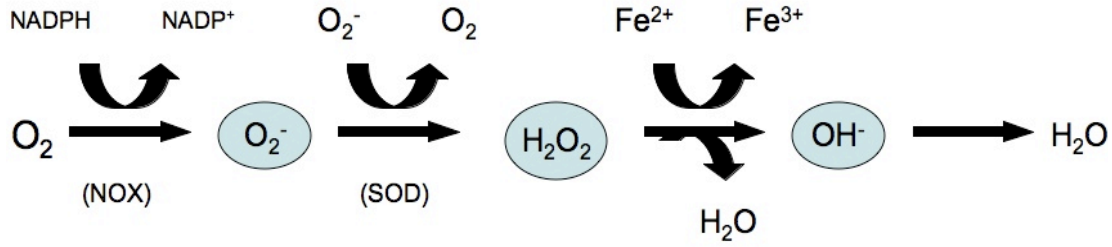


Figure 1.9 The generation of NOX-dependent ROS. The NOX enzymes catalyze the production of O_2^- by the one-electron reduction of molecular oxygen, using electrons donated from NADPH. The antioxidant enzyme SOD converts O_2^- to H_2O_2 and oxygen. H_2O_2 can be converted to H_2O however, in the presence of O_2^- and H_2O_2, and available Fe^{2+}, $OH^{\bullet}$ can be generated (El-Benna et al. 2005; Scandalios 2005; Valko et al. 2006).

1.4.1 Cellular Oxidative Damage

Excess ROS production and accumulation can induce severe cellular damage, leading to physiological dysfunction and cell death, consequently contributing to chronic disease development (Scandalios 2005; Zelko, Mariani & Folz 2002). Evidence of elevated markers of protein oxidation have been demonstrated in diseases such as Alzheimer's and Parkinson's disease, Duchene muscular dystrophy, ALS, rheumatoid arthritis and progeria (Mayne 2003). As has been mentioned earlier, phagocyte NOX plays a key role in host defenses against microbial pathogens and although this NOX enzyme is absolutely necessary for host survival, its activity requires strict regulation in order to prevent destruction of surrounding host tissue (Groemping & Rittinger 2005; Takeya & Sumimoto 2006). Phagocytic leukocytes become activated in response to stimulation that regulates the activity of NOX through either enforcing the

resting state or allowing translocation for the initiation of oxidase assembly and activation (Groemping & Rittinger 2005; Takeya & Sumimoto 2006). Phagocyte NOX generated O_2^- has therefore been implicated in inflammatory related disorders when production exceeds normal required levels (Groemping et al. 2003). The primary role of NOX in non-phagocytes is also for the generation of O_2^- in response to internal and external stimulation (Bedard & Krause 2007). It is therefore recognised that inappropriate activation can also induce cellular OS and damage to surrounding tissue (Groemping et al. 2003). In particular, OS has been implicated in various progressive degenerative conditions including spinal cord injury (Vaziri et al. 2004), hypoxic pulmonary hypertension (Liu et al. 2006), cardiovascular (Wingler et al. 2001) and cerebral vascular (Paravicini et al. 2004) disease and cancer (Mantovani et al. 2003), and O_2^- generated by NOX is proposed as the major source in these conditions.

The mitochondrial respiratory chain is suggested to constitute the greatest source of reactive oxidant production within the cell (Mates & Sanchez-Jimenez 1999; Smith & Tisdale 2003), consuming approximately 85% of oxygen utilised by all cells (Shigenaga, Hagen & Ames 1994). Mitochondrial ETC receives electrons from NADH and ultimately reduces oxygen to water by a four electron transfer by cytochrome c oxidase (Davidson & Schiestl 2001). Reduction takes place after a series of site-specific electron transfers through the inner mitochondrial membrane (Dalton, Shertzer & Alvaro 1999). Evidence of mitochondrial ROS production became evident with the characterisation of SOD2 that specifically functions to remove O_2^- from the mitochondria (Esposito et al. 1999). The proximity of ROS generation makes the mitochondria particularly susceptible to oxidative damage

(Macdonald, Galley & Webster 2003; Smith & Tisdale 2003). In addition to this, as the mitochondria is required primarily for the production of ATP, they are present in relatively high numbers in essentially all cells of the body (Giordano et al. 2003). Therefore, increased metabolic activity that generates excess ROS and/or impaired SOD2 function can cause significant mitochondrial damage, which has been implicated in various pathologies (Barreiro et al. 2005; Ohashi et al. 2006; Van Remmen et al. 2001). However, it is recognised that the site of oxidative damage is not necessarily the site of ROS generation and it is therefore important to elucidate the potential source of ROS production in order to understand its role in the cellular system (Krause 2007). Furthermore, ROS are being increasingly viewed as signaling molecules that can upset cellular homeostasis, through subtle variations in essential cellular processes and responses, and must therefore be considered beyond their involvement in mitochondrial dysfunction (McCord & Edeas 2005; Rhee et al. 2005).

1.5 Reactive Oxygen Species (ROS) as Cellular Signaling Molecules

Intracellular ROS are recognised as important components of various biological events for their involvement in redox-sensitive cellular signaling (Sumimoto, Miyano & Takeya 2005). Redox signaling describes the regulatory process of coordinated signaling, delivered through redox reactions. This is achieved through responses to extra and intra-cellular stimuli called cell signaling or signal transduction (Valko et al. 2007). In particular, ROS are signaling molecules that modulate the activity of diverse intracellular molecules (Dworakowski et al. 2006) and a number of intracellular signaling pathways, necessary for cell survival (Valko et al. 2007). Therefore, ROS

are not just harmful cellular damaging molecules but rather, when tightly regulated, function as important cellular molecules in critical cellular processes (Scandalios 2005). In particular, O_2^- and H_2O_2 have been described as central mediators involved in important cellular processes such as growth, differentiation, proliferation and apoptosis through signal transduction pathways (Hancock, Desikan & Neill 2001; Ji 2007; Scandalios 2005; Valko et al. 2007).

1.5.1 A Role for the NOX Enzymes in Cellular Signaling

Among the many ROS generating enzyme systems, NOX enzymes appear to be especially important in redox signaling and well suited for involvement in signal transduction (Dworakowski et al. 2006). NOX enzymes are known to be constantly fluctuating between an inactive and active state as a result of exposure to hormones, growth factors, cytokines and mechanical stress (Frey, Ushio-Fukai & Malik 2008; Li & Shah 2003). It is therefore suggested that NOX enzymes have an essential role in protein and gene regulation, within cells and tissues, in response to stimulus changes (Clark & Valente 2004). In particular, it has been clearly demonstrated that NOX-dependent ROS are involved in signal transduction, in response to stimulants (Li et al. 2005; Schieffer et al. 2000), including Phosphatidylinositol-3-OH Kinase (PI3K), Phospholipase A (PLA), Protein Kinase C (PKC), Aracodonic Acid (AA) (Russell, Eley & Tisdale 2007), and Angiotensin II (Ang II) (Wei et al. 2006). NOX is tightly controlled by these specific redox-sensitive stimulants, which re-inforces its important role in signaling cascades (Hancock, Desikan & Neill 2001). Receptor binding rapidly activates NOX, which is followed by an elevation of intracellular O_2^- and H_2O_2 levels and activation of redox-sensitive signaling (Li & Shah 2003). In particular, NOX generated ROS have been shown to regulate the expression of redox-sensitive genes

such as Tumour Necrosis Factor-alpha (TNF-α) and Ang II (Bedard & Krause 2007). H_2O_2 in particular, is recognised as a signaling molecule (Rhee 2006; Stone & Yang 2006) that can stimulate the expression of a variety of genes (Hancock, Desikan & Neill 2001), including inflammatory cytokines and antioxidant enzymes (Ji 2007). Part of the cells stress response is to increase the transcription of the SOD genes, which leads to increased activity, regulated by a variety of signals including upregulation in response to OS (Landis & Tower 2005). This is possible through activation of redox-sensitive transcription factors, downstream of selective oxidant signaling (Frey, Ushio-Fukai & Malik 2008). This has been demonstrated through gene expression profiles in various tissues under different stress conditions (Johnson & Giulivi 2005), including ROS-dependent transcription factor regulation of Nuclear Factor Kappa Beta (NF-kB) (Hancock, Desikan & Neill 2001; Valko et al. 2007).

1.5.2 The Role of NF-κB in Cellular Signaling

Signaling molecules that respond to changes in redox state include transcription factors, such as NF-κB and Activating Protein-1 (AP-1) (Scandalios 2005), protein kinases such as Mitogen-Activated Protein Kinase (MAPK) and the PI3K/Protein Kinase B (PKB or Akt) pathway (Valko et al. 2007), as well as antioxidant enzymes and cellular oxidases such as NOX enzymes (Dworakowski et al. 2006; Gauss et al. 2007). There is an abundance of evidence that suggests a role for ROS in gene transcription regulation (Allen & Tresini 2000; Fan et al. 2002; Kamata et al. 2002; True, Rahman & Malik 2000). In particular, NOX-derived ROS have been suggested to be involved in signaling the activation of transcription factors such as NF-κB (Brar et al. 2002) and AP-1, influencing various types of gene expression (Bedard & Krause 2007; Moore-Carrasco et al. 2006).

NF-κB is a ubiquitously expressed transcription factor that regulates the expression of a variety of genes, including those that promote cell survival, differentiation, inflammation and growth (Valko et al. 2007). NF-κB is a complex in the cytosol, bound to I-kB Kinase complex (IKK), which on stimulation is degraded and disassociated from NF-kB, initiating translocation from the cytosol to the nucleus where it functions to influence the expression of a variety of genes (Ji 2007). Activation of NF-κB occurs in response to extracellular stimulation, such as H_2O_2 (True, Rahman & Malik 2000), TNF-α (Li & Reid 2000) and Phorbol 12-Myristate 13-Acetate (PMA) (Kiningham et al. 2001) that transfer signals from the cell surface, downstream of intracellular mediators and activators, to the nucleus to stimulate gene expression (Ji 2007; Valko et al. 2007). Furthermore, ROS have been particularly implicated as second messengers involved in the activation of NF-κB redox-sensitive stimulation via intra and extra-cellular stimulation (Ji 2007; Valko et al. 2007). Therefore, abnormally high levels of ROS, usually in response to pathological conditions, may be responsible for permanent changes in signal transduction and gene expression, characteristic of many disease states (Valko et al. 2007). Therefore, cellular homeostasis may not only be upset by chemical insult, but also by subtle changes in ROS production, resulting in dysfunctional regulation of essential cellular processes and responses (McCord & Edeas 2005).

1.6 Reactive Oxygen Species (ROS) in Skeletal Muscle Tissue

ROS are produced inside skeletal muscle fibres under both normal (Reid et al. 1992) and pathological conditions (Javesghani et al. 2002; Russell, Eley & Tisdale 2007). Numerous sources of ROS have been identified in skeletal muscle including mitochondrial complex I and III, XO, COX and NOX (Jackson, Pye & Palomero 2007; Moylan & Reid 2007; Smith & Reid 2006), including both the NOX2 and NOX4 enzyme systems (Mofarrahi et al. 2008). In the resting muscle, O_2^- radicals are released at relatively low rates, however in response to increased muscle activity, the rate of O_2^- production can rise significantly (Javesghani et al. 2002; McArdle et al. 2004; Xia et al. 2003). Contractile activity results in an increase in oxygen consumption by the contracting skeletal muscle, leading to an increase in O_2^- production (Kinugawa et al. 2005; McArdle et al. 2004) and it is this increase in local OS that has been associated with muscle fatigue and wasting (Arbogast & Reid 2004; Linke et al. 2005; Xia et al. 2003). Although the major source of ROS in skeletal muscle is the mitochondrial ETC, the controlled release of ROS generated by the NOX enzymes and involvement in cellular signaling pathways (Hancock, Desikan & Neill 2001), suggests an important role for NOX in the skeletal muscle system (Kerner et al. 2001; McArdle et al. 2004) (Figure 1.10). With evidence of NOX2 and NOX4 expression in skeletal muscle tissue (Javesghani et al. 2002; Mofarrahi et al. 2008) (Figure 1.10), these NOX enzymes have been proposed to play an important mediating role in skeletal muscle growth pathways (Mofarrahi et al. 2008; Russell, Eley & Tisdale 2007).

Skeletal muscle ROS are tightly regulated by antioxidant enzymes that function specifically to eliminate ROS and oxidative associated tissue damage (Valko et al. 2006) (Figure 1.10). Furthermore, ROS themselves have been shown to regulate the expression of antioxidant enzymes in skeletal muscle, such as SOD (Hollander et al. 2001; Kiningham et al. 2001), catalase and GPx (Zhou, Johnson & Rando 2001), through NF-κB activation. Therefore, ROS appear to regulate the expression of antioxidants, while antioxidants convert ROS to less reactive molecules in order to avoid cellular OS. However, this tight regulation can be altered through redox-sensitive signaling pathways (Valko et al. 2007).

SOD is the major antioxidant enzyme in the skeletal muscle system for the protection of cells against attacks by ROS (Linke et al. 2005). A decrease in SOD expression has been shown to increase O_2^- activity in skeletal muscle, through decreased O_2^- scavenging (Kinugawa et al. 2005; Mantovani et al. 2003; Woodman, Price & Laughlin 2002). In addition, antioxidant administration has been shown to protect muscle by preventing muscle dysfunction and enhancing functional recovery from activity (Brar et al. 2003; Kim et al. 2003; Kinugawa et al. 2005; Perner et al. 2003; Wright et al. 2005). Furthermore, ROS have been implicated in conditions that induce severe metabolic changes such as injury, infection and sepsis, inducing catabolic protein breakdown (Hasselgren 2000). In particular, the Ubiquitin (Ub)-proteasome proteolytic pathway accounts for the majority of the bodies protein turnover (Mikhail et al. 2003), and ROS are suggested to play an essential role in this pathway (Gomes-Marcondes et al. 2003; Russell, Eley & Tisdale 2007). Therefore, changes in metabolic and redox-sensitive activators and responses in skeletal muscle can significantly alter skeletal muscle physiology, contributing to dysfunction and disease.

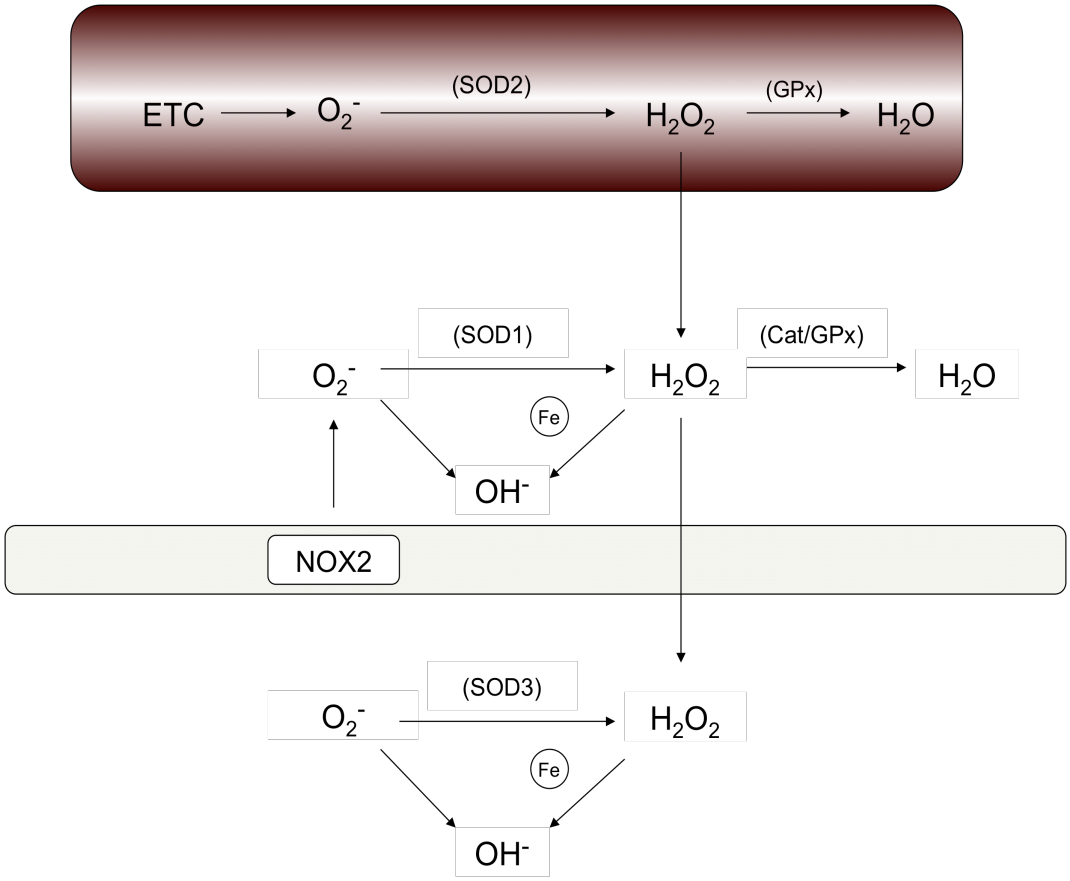


Figure 1.10 Diagramatic representation of potential ROS generation in skeletal muscle. ROS generated from the ETC is represented in red. O_2^- is generated as a by-product of mitochondrial metabolism where SOD2, localised in the mitochondria, regulates mitochondrial ROS by converting O_2^- to H_2O_2. GPx located in the mitochondria converts H_2O_2 to H_2O. However, H_2O_2 is highly permeable and can diffuse across the mitochondrial membrane in the cytosol or through the plasma membrane, where it can contribute to further redox reactions. Intracellular O_2^- is converted into H_2O_2 by SOD1, which can further be converted to H_2O by catalase (Cat) and/or GPx, but in the presence of O_2^- and Fe, can also give rise to $OH^{\bullet}$. NOX2 has been found to be expressed in skeletal muscle, localised to the plasma membrane, however it is still unknown whether the NOX2 complex generates O_2^- intracellularly or extracellularly in skeletal muscle cells. Intracellular O_2^- is converted into H_2O_2 by SOD1, which can further be converted to H_2O by Cat and/or GPx, but in the presence of O_2^- and Fe, can also give rise to $OH^{\bullet}$, while extracellular O_2^- is converted into H_2O_2 by SOD3, which can further be converted to H_2O by Cat and/or GPx, but in the presence of O_2^- and Fe, can also give rise to $OH^{\bullet}$ (Jackson, Pye & Palomero 2007).

1.7 Skeletal Muscle Wasting Cachexia/Sarcopenia

Cachexia, derived from the Greek word 'Kakos Hexis', meaning bad condition (Martignoni, Kunze & Friess 2003), is defined as decreased growth or protein synthesis and increased cell death or protein degradation (Giordano et al. 2003). The cachectic condition is characterised by a significant loss of body weight, of up to 30% (Tijerina 2004), as a result of significant depletion in muscle mass (Gomes-Marcondes et al. 2003; Whitehouse et al. 2001). Cachexia is commonly seen in aging skeletal muscle, known as sarcopenia, and multiple diseases involving wasting of host tissue (Tijerina 2004). These include conditions such as congestive heart failure (Filippatos, Anker & Kremastinos 2005), Autoimmune Deficiency Syndrome (AIDS) (Baronzio et al. 1999), sepsis (Sun et al. 2003), ALS (Leger et al. 2006) and cancer (Acharyya et al. 2004).

1.7.1 Aging Sarcopenia

Aging is associated with a general decline in physiological capacity of the major systems of the body, including the cardiovascular, respiratory, metabolic, neurological and musculoskeletal systems that indeed affect overall physiological function. The loss of skeletal muscle mass and function is one of the most dramatic phenotypic changes associated with aging (Mansouri et al. 2006), and is termed sarcopenia. Sarcopenia comes from the Greek word meaning 'poverty of flesh' (Karakelides, Nair & Gerald 2005), and is characterised by a significant breakdown of skeletal muscle protein (Kimball et al. 2004). In particular, it has been proposed that the regulation of gene expression related to protein synthesis and muscle atrophy is altered during the aging process (Goldspink 1998). Evidence of this has been demonstrated by studies in

aging humans that show a significant decline in muscle mass, with the most significant loss at approximately 65 years of age and steady rate of decline thereafter (Goldspink 1998; Pansarasa et al. 1999) (Figure 1.12), and a similar pattern of decline has also been shown in an animal study by Kimball et al (2004) (Figure 1.11).

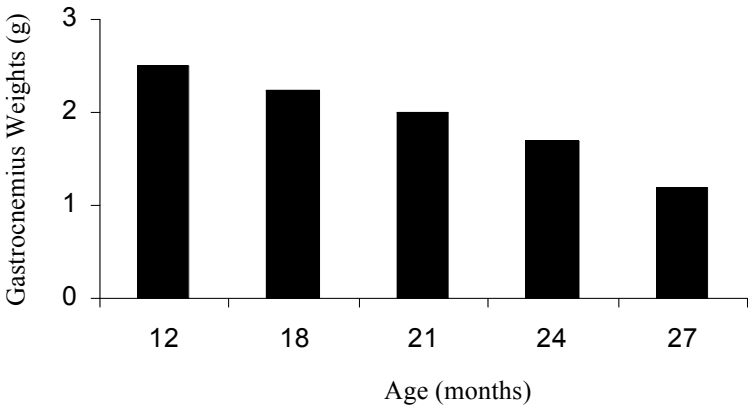


Figure 1.11 A graphic representation of the decline in skeletal muscle mass from rat gastrocnemius muscle during aging, adapted from Kimball et al (2004). Results show the decline in skeletal muscle mass (g) over 5 ages, including 12, 18, 21, 24 and 27 months of age. The pattern of decline shows the beginning of loss in skeletal muscle mass at 18 months and evidence of further decline thereafter.

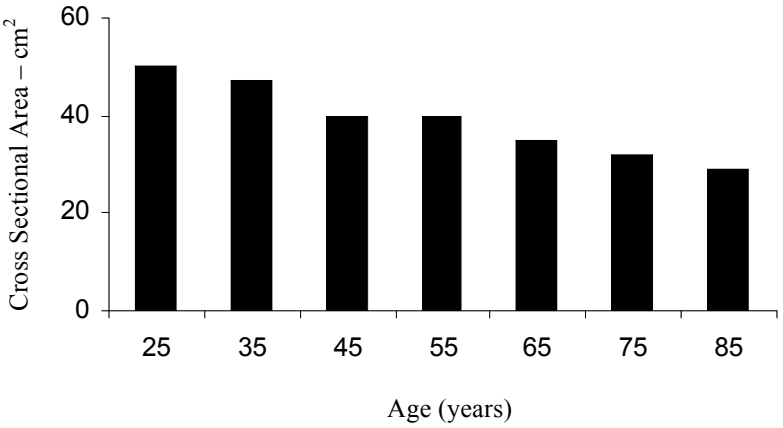


Figure 1.12 A graphic representation of the decline in muscle cross sectional area from human muscles of mastication during aging, adapted from Goldspink (1998). Results show the decline in skeletal muscle cross sectional area by CT measurement over the ages of 25, 35, 45, 55, 65, 75 and 85 years of age. The pattern of decline shows the beginning of loss in skeletal muscle mass at 45 years of age and evidence of further decline thereafter.

1.7.1.1 Incidence & Mortality of Aging Sarcopenia

Sarcopenia is the progressive decline in skeletal muscle mass during aging that has been reported to occur at a rate of 5% per decade, starting in the fourth decade (Short et al. 2003) with a prevalence of 6-15% in people older than 65 years of age (Melton et al. 2000). The general decline in muscle function during aging seems to result from a progressive loss of muscle mass that consequently results in a reduction in muscle strength and mobility (Pansarasa et al. 2002). Sarcopenia has been described as a multifactoral condition associated with inflammation, endocrine changes and OS (Marzani et al. 2008). These significant changes, evident during the aging process, have been proposed to contribute to changes in cellular function and general functional decline (Clavel et al. 2006; de Magalhaes, Cabral & Magalhaes 2005; Holzenberger et al. 2003; Pansarasa et al. 1999; Toth et al. 2005).

1.7.1.2 Current Research in Aging Sarcopenia

Experimental evidence supports the free radical theory of aging as a major contributor to the age-associated impairment at the cellular and tissue level (Pansarasa et al. 2002; Sastre, Pallardo & Vina 2000; Schriner et al. 2005). The free radical theory of aging has been implicated in the development of impaired cognitive function and motor skills that result in a decline in physiological function and quality of life (Sastre, Pallardo & Vina 2000). This theory suggests that endogenous ROS continually damage cellular macromolecules and that incomplete repair of such damage would lead to its accumulation over time, resulting in age-related deterioration (Scandalios 2005). Therefore the free radical theory demonstrates the involvement of ROS in physiological impairment and also suggests an important role for the primary antioxidant systems (Pansarasa et al. 2002; Schriner et al. 2005; Wei et al. 2006).

Aging has been associated with a significant increase in O_2^- production (Shigenaga, Hagen & Ames 1994) and a decrease in the expression of the O_2^- scavenger, SOD (Wei & Lee 2002). Furthermore, antioxidant supplementation has been shown to exert a protective effect on skeletal muscle, decreasing the progression of age-associated damage (Heaton et al. 2002; Khassaf et al. 2003; Morre, Lenaz & Morre 2000). In addition, Pansarasa et al (2002) demonstrated a significant decrease in total SOD activity in aged skeletal muscle. Therefore, it has been suggested that skeletal muscle aging may be a result of a reduced antioxidant system capacity and therefore an inability to successfully convert ROS into more inert species (Esposito et al. 1999; Gianni et al. 2004; Pansarasa et al. 1999; Wei & Lee 2002). However, skeletal muscle SOD and catalase have also been found to increase in adult mice, suggestive of an increased rate of O_2^- and therefore increased need for SOD and catalase scavenging (Vasilaki et al. 2006). Taken together, these studies suggest a role for ROS and antioxidant enzymes in skeletal muscle aging and indeed a significant contribution to age-associated degenerative dysfunction (Gianni et al. 2004; Wei & Lee 2002). Furthermore, the newly recognised role for ROS as cellular redox signaling molecules suggests additional changes in skeletal muscle as a result of alterations in redox-sensitive pathways (Kramer & Goodyear 2007). These alterations would undoubtedly have consequences in the cell, but may also specifically contribute to skeletal muscle wasting during aging (Toth et al. 2005).

Inflammation is a common factor in the aging process and high levels of pro-inflammatory cytokines have been implicated in skeletal muscle dysfunction (Visser et al. 2002). Inflammatory cytokines, such as Interleukin-6 (IL-6) and TNF-α, have shown marked increases during aging that have been suggested to act downstream of

NOX and redox-sensitive transcription factor NF-κB activation (Clark & Valente 2004; Csiszar et al. 2008). Furthermore, a marked increase in inflammatory cytokines and upregulation of the atrophy inducing Ub-proteasome pathway has been associated with sarcopenia (Clavel et al. 2006). Aging studies have also shown a decrease in the capacity of skeletal muscle to undergo hypertrophy (Blough & Linderman 2000; Funai et al. 2006; Haddad et al. 2005), of which ROS have been associated with (Abid et al. 2007; Mofarrahi et al. 2008; Wu et al. 2006). The association between elevated levels of inflammatory cytokines and skeletal muscle atrophy during aging may therefore result from inflammatory-stimulated ROS signaling of NF-κB activation and regulation of skeletal muscle atrophy/hypertrophy related genes that may involve NOX.

1.7.1.3 Oxidative Stress (OS) in Aging Sarcopenia

ROS production, OS and oxidative damage have been found to increase with age (Pansarasa et al. 1999). Specifically, aging has been associated with a consistent increase in ROS production and a general decline in physiological function, due to an accumulation of oxidatively damaged proteins (Ferrington, Husom & Thompson 2005; Squier 2001). This increase in ROS production, and accumulation of abnormal proteins from oxidative damage, progresses over the lifespan (Landis et al. 2004). Therefore, OS has been proposed to underlie a variety of disorders associated with the aging process (Heaton et al. 2002), including sarcopenia (Mosoni et al. 2004; Sundaram & Panneerselvam 2006). A correlation between ROS activity and age related changes in human skeletal muscle (Pansarasa et al. 1999), and age-dependent oxidative damage to DNA, lipids and protein (Mecocci et al. 1999) has been made. It is therefore suggested that the oxidative damage resulting from ROS in skeletal

muscle is a major contributor in the general physical decline, common in aging (Bejma & Ji 1999; Pansarasa et al. 2002). Furthermore, the ability of cells to actively detoxify ROS is suggested to become less efficient over time resulting in a pro-oxidant shift in the cellular redox environment (Wei & Lee 2002).

Indeed, mitochondrial OS plays an important role in the decline in physiological function during aging (Devasagayam et al. 2004; Mansouri et al. 2006; Schriner et al. 2005). Age related diseases, including cancer, type II diabetes and Alzheimer's and Parkinson's diseases show a clear correlation with mitochondrial dysfunction and increased levels of ROS (Morten, Ackrell & Melov 2006). The knowledge that mitochondrial ROS production increases with age, makes this a likely factor underlying the decline in skeletal muscle mass during aging (Devasagayam et al. 2004; Mansouri et al. 2006; Schriner et al. 2005). Further to this however, changes in the cellular redox environment would certainly alter normal cellular systems, in particular skeletal muscle growth pathways (Ji 2007). It has been proposed that OS could lead to muscle atrophy through a variety of factors, including an increase in apoptosis, proteolysis and/or alterations in skeletal muscle regeneration (Mansouri et al. 2006).

A decline in tissues repair and maintenance processes may be responsible for the age-associated deterioration of cellular structure and function (Landis & Tower 2005). Therefore, changes in the oxidative state and damage to tissue, associated with aging may not just result from ROS accumulation over time, but rather a complex system of changes that regulate cellular growth pathways. There is however, a clear association with aging and an altered cellular redox state (Bejma & Ji 1999; Mecocci et al. 1999;

Pansarasa et al. 1999; Wei & Lee 2002) that cannot be ignored. Interestingly, ROS have been proposed as central mediators involved in inflammatory and endocrine changes often associated with aging (Clark & Valente 2004; Csiszar et al. 2008; Wu et al. 2006). Furthermore, additional sources of ROS, involved in cellular signaling and skeletal muscle growth pathways, such as NOX, have also been shown to be altered during aging (Bejma & Ji 1999; Clark & Valente 2004; Ji 2007).

1.7.1.4 A Role for the NOX Enzymes in Aging Sarcopenia

Although there is limited research in the area of NOX in skeletal muscle, aging studies have demonstrated an age-associated increase in ROS production as a potential consequence of NOX subunit upregulation (Oudot et al. 2006; Salles et al. 2005) and oxidase activity (Hamilton et al. 2001). In addition, an overexpression of NOX4 has been proposed to lead to cellular senescence in fibroblasts (Geiszt et al. 2000), and degenerative dysfunction associated with aging (Paravicini et al. 2004; Wingler et al. 2001). OS has been associated with the cellular senescent phenotype in endothelial cells (Frey, Ushio-Fukai & Malik 2008) and NOX generated O_2^- is suggested to contribute to endothelial dysfunction (Chan et al. 2007; Hamilton et al. 2001; Jung et al. 2004) hypertension (Paravicini et al. 2004) (Beswick et al. 2001), arteriosclerosis and vascular aging (Oudot et al. 2006; Sorescu et al. 2002). Furthermore, NOX enzymes have been implicated in age-associated conditions such as diabetes (Nakayama et al. 2005) arthritis (Chenevier-Gobeaux et al. 2006), cataracts (Jiang et al. 2006) and motorneuron dysfunction (Harraz et al. 2008).

It is evident that ROS production, in skeletal muscle, increases with age (Cai et al. 2004a; Cakatay et al. 2003; Pansarasa et al. 2002; Vasilaki et al. 2006) and NOX enzymes have been suggested to contribute (Bejma & Ji 1999). Furthermore, studies have demonstrated a decrease in protein synthesis rate in aging skeletal muscle (Cuthbertson et al. 2005; Toth et al. 2005), which have been associated with NOX generated ROS signaling. Research has also demonstrated an increase in age-associated skeletal muscle proteolysis, as a result of increased Ub-proteasome pathway signaling (Cai et al. 2004a; Clavel et al. 2006; Reynolds et al. 2002) and NOX-dependent redox signaling (Russell, Eley & Tisdale 2007). Therefore, ROS, generated from NOX enzymes may play a key role in skeletal muscle growth pathways, contributing to age-associated skeletal muscle wasting sarcopenia.

1.7.2 Cancer-Induced Cachexia

While cachexia develops in a wide range of pathologies, cancer-induced cachexia has been reported to develop at a faster rate than any other known cachectic condition (Giordano et al. 2003). Cancer-induced cachexia is a condition of progressive skeletal muscle wasting that develops as a secondary condition to carcinogenesis (Busquets et al. 2004; Costelli et al. 2005). The catabolic response of skeletal muscle to tumour growth increases the breakdown of muscle proteins, characterised by a significant loss of body weight (Hasselgren, Wray & Mammen 2002). This loss of muscle mass is an important factor in the survival rate of cancer patients, as many physiological functions, such as respiratory function, is severely impaired (Tisdale 2001). As the function of the respiratory muscles deteriorate, there is an increased risk of pulmonary complications and infections that lead to extended periods of ventilatory support

(Hasselgren, Wray & Mammen 2002). Patients suffering cachexia can lose more than 30% of their original body weight (Tijerina 2004) and suffer a loss of general physiological function (Whitehouse et al. 2001), decreasing quality of life (Giordano et al. 2003) and eventually death from loss of respiratory (Hasselgren, Wray & Mammen 2002) and cardiac (Diffee et al. 2002) muscle function.

1.7.2.1 Incidence & Mortality of Cancer-Induced Cachexia

Approximately half of all patients with cancer experience cachexia (Diffee et al. 2002; Tijerina 2004) and almost a third of mortalities are estimated to result from cachexia, rather than the tumour itself (Acharyya et al. 2004). This high mortality rate among these cancer patients has been linked to their decreased response to therapy (Giordano et al. 2003; Mitch & Price 2001; Wang et al. 2003). Skeletal muscle cachexia is therefore largely responsible for many cancer deaths (Hasselgren, Wray & Mammen 2002). Interestingly, not all cancer patients develop cachexia and different cancers are more likely to induce the cachectic condition than other cancer types. Patients with pancreatic and stomach carcinomas have one of the highest incidence of cancer-induced cachexia whereas breast cancer, leukemia and sarcomas have been shown to have the lowest incidence (Giordano et al. 2003).

1.7.2.2 Current Research in Cancer-Induced Cachexia

It has been suggested that cancer-induced cachexia is a product of the bodies inflammatory response to tumours. In particular, an association has been made with the many inflammatory cytokines elevated in cancer patients, such as TNF-α and IL-6 (Cahlin et al. 2000; Ebrahimi et al. 2004; Sturlan et al. 2002). Pro-inflammatory

cytokines elicit a metabolic response very similar to that seen in the cachectic syndrome, induced by tumours (Younes & Noguchi 2000). Research has demonstrated an increase in TNF-α activation of Ub-dependent proteolysis in skeletal muscle during tumour growth (Diffee et al. 2002; van Royen et al. 2000). However, this alone does not explain the complex mechanism of wasting in cancer-induced cachexia (Giordano et al. 2003; Smith & Reid 2006). Rather, these studies are associated with a decrease in food intake, potentially triggered by the inflammatory products that contribute to cachexia through appetite suppression or anorexia (Busquets et al. 2005; Diffee et al. 2002; Monitto et al. 2001). Conflicting research has shown however, that cancer-induced cachexia is not associated with inflammatory cytokines (Smith, Greenberg & Tisdale 2004) or altered food intake and suggest the production of a catabolic factor, implicated in the development of cancer-induced cachexia (Busquets et al. 2005; Diffee et al. 2002; Monitto et al. 2001). Although, the catabolic mechanisms leading to the severe muscle wasting associated with cancer remains relatively unknown, research has suggested a role for ROS (Diffee et al. 2002; Li et al. 2003; Li & Reid 2000). Furthermore, a role for ROS in cytokine induced muscle wasting has been demonstrated (Jackman & Kandarian 2004).

A circulatory protein, Proteolysis Inducing Factor (PIF) was first described as a causative agent in cachexia when it was discovered in mice expressing the Murine Adenocarcinoma 16 (MAC16) tumour (Todorov et al. 1996). As this glycoprotein was initially discovered in the sera of mice bearing the cachexia inducing MAC16 tumour, but not in the sera of the mice bearing the non-cachectic MAC13 tumours, it was regarded as an important factor in the development of cancer-induced cachexia (Smith, Wyke & Tisdale 2004; Wyke, Smith & Tisdale 2003). Although, the

contribution of PIF to cancer-induced cachexia remains unknown, it is proposed to induce skeletal muscle proteolysis synergistically (Tisdale 2001), suggesting the involvement of additional catabolic factors. Skeletal muscle cachexia in patients with cancer is characterised by Ub-dependent breakdown of proteins, and PIF has been shown to induce this pathway of skeletal muscle atrophy (Lecker et al. 2004). Although the PIF/Ub pathway indeed plays a role in the development of cancer-induced cachexia (Smith & Tisdale 2003; Whitehouse & Tisdale 2002), a number of additional mechanisms are likely to be involved (Russell, Eley & Tisdale 2007; Tisdale 2004). Preproteasomal mechanisms, mediators, receptor binding, signaling pathways and activation of specific transcription factors are all important considerations (Hasselgren, Wray & Mammen 2002). ROS have recently been demonstrated to play a key role in cancer-induced cachexia with marked attenuation following antioxidant administration (Russell, Eley & Tisdale 2007). Russell et al (2007) demonstrated PIF and Ang II induced ROS signaling and the induction of the Ub-proteasome pathway in murine myotubes, and suggested that this is likely to be mediated through NOX enzyme activation.

1.7.2.3 Oxidative Stress (OS) in Cancer-Induced Cachexia

Cachexia has been associated with OS and the induction of oxidative damage and tissue breakdown (Gomes-Marcondes et al. 2003; Whitehouse et al. 2001). O_2^- has been shown to induce skeletal muscle wasting in many pathological conditions via catabolic mechanisms and is therefore suggested to contribute to muscle wasting, characteristic of cancer-induced cachexia (Gomes-Marcondes & Tisdale 2002; Whitehouse et al. 2001). However, the contribution of O_2^- in the development of cancer-induced cachexia is yet to be elucidated. Cancer patients have been shown to

present with elevated levels of serum ROS and lower antioxidant levels (Mantovani et al. 2003), indicative of a pro-oxidative shift. Further in animal studies, elevated levels of protein oxidation markers have been demonstrated in cancer-induced cachectic skeletal muscle (Barreiro et al. 2005; Russell, Eley & Tisdale 2007).

Skeletal muscle is highly vulnerable to protein catabolism (Giordano et al. 2003) and ROS such as O_2^-, H_2O_2 and OH^- are potential catabolic factors, capable of causing significant proteolysis (Li et al. 2003). ROS have been proposed to be involved in cellular signaling pathways including muscle atrophy (Diffee et al. 2002), through the Ub-proteasome pathway, but may also play a role in depressing protein synthesis (Russell, Eley & Tisdale 2007). In particular, cancer-induced cachexia has been associated with ROS induction of Ub-dependent proteolysis through transcription factor NF-κB activation (Wyke & Tisdale 2005; Zhou et al. 2003). Therefore, inappropriate ROS production, in response to cancer related stimuli, is a potential contributor to the secondary loss of muscle, presented in cancer-induced cachectic patients. However, limited research gives insight into the source of ROS and its role in cancer-induced cachexia.

1.7.2.4 A Role for the NOX Enzymes in Cancer-Induced Cachexia

Although limited research exists for the role of NOX in cachexia, a role for NOX enzymes in the regulation of skeletal muscle function has been suggested (Hidalgo et al. 2006; Javesghani et al. 2002; Mofarrahi et al. 2008; Wei et al. 2007). NOX enzymes have been shown to respond to external stimuli such as PIF, Ang II, TNF-α and various growth factors, all of which have been demonstrated to play a role in skeletal muscle protein catabolism, characteristic of cachexia (Clavel et al. 2006;

Russell, Sanders & Tisdale 2006). It has been made evident that under specific stress conditions, ROS can induce skeletal muscle proteolysis (Jackman & Kandarian 2004), not only by direct insult to skeletal muscle tissue, but also through its ability to influence muscle growth pathways (Li et al. 2003). Muscle cells treated with H_2O_2 have demonstrated increased protein breakdown and components of the Ub-proteasome pathway for proteolysis (Gomes-Marcondes & Tisdale 2002). In particular, cachexia has been associated with the up-regulation of atrophy related Ub ligases (Doucet et al. 2007; Kandarian & Jackman 2006; Lecker et al. 2004; Sacheck et al. 2004), and NOX-dependent ROS have been suggested to be involved in atrophy related transcriptional regulation (Barreiro et al. 2005; Cai et al. 2004b; Li et al. 2005). Furthermore, inhibition of the NOX activators, PIF or Ang II, shown to induce cachexia in skeletal muscle myotubes, has demonstrated marked attenuation of ROS (Russell, Eley & Tisdale 2007), suggesting a central mediating role for NOX in skeletal muscle atrophy. Therefore, in response to tumour related stimuli, NOX may play an important role in skeletal muscle catabolism, associated with cancer-induced cachexia development in cancer patients.

1.7.3 SOD1 Overexpression

A transgenic mouse model expressing the G93A human SOD1 mutation, has demonstrated neuronal and skeletal muscle degenerative pathology, characteristic of the human ALS condition (Harraz et al. 2008; Mahoney et al. 2006). Transgenic SOD1 mice have been extensively used in ALS research, where SOD1 is abundantly expressed, stable and active ($SOD1^{G93A}$ and $SOD1^{G37R}$) or marginally expressed and unstable ($SOD1^{G85R}$) (Turner & Talbot 2008). It is suggested that dominant SOD1

mutations result in a toxic 'gain-of-function', inducing the degenerative ALS condition (Dobrowolny et al. 2005; Harraz et al. 2008; Watanabe et al. 2001). This common genetic mutation in SOD1 therefore represents altered cellular ROS, through O_2^- dismutation and generation of H_2O_2 (Brooks et al. 2004; Mahoney et al. 2006). ALS is an age-dependent neurodegenerative disease that causes progressive motorneuron degeneration, skeletal muscle atrophy and paralysis (Higgins, Jung & Xu 2003). In particular, an increase in oxidative damage in neural tissue (Maier & Chan 2002) and changes in redox-sensitive cellular growth pathways, in skeletal muscle of ALS patients, has been reported (Dobrowolny et al. 2005). In addition, the changes in skeletal muscle in response to altered SOD1 has been proposed to play an important role in the pathogenesis of ALS (Mahoney et al. 2006) and is therefore a crucial component in the disease that deserves greater attention.

1.7.3.1 Incidence & Mortality of SOD1 Overexpression

ALS is a multisystem disorder and in addition to the progressive neurodegeneration, patients present with the typical phenotype of skeletal muscle degeneration and paralysis (Dobrowolny et al. 2005). Although the incidence of ALS is approximately 4-13 per 100,000 (Beleza-Meireles & Al-Chalabi 2009), it is the most frequent paralytic disease in adults (Grieb 2004). ALS is predominantly a sporadic disease (SALS) (Banci et al. 2008) and therefore of unknown etiology (Turner & Talbot 2008). However, approximately 10% of ALS cases are predominantly autosomal dominant inheritance or familial (FALS), the most important of which is a genetic mutation in the SOD1, accounting for 20% of familial cases (Beleza-Meireles & Al-Chalabi 2009; Mahoney et al. 2006). Dominant mutations in this enzyme in humans have shown symptoms of neurodegeneration and onset of ALS (Harraz et al. 2008),

and transgenic mice overexpressing SOD1 ($SOD1^{G93A}$) models this disease (Beleza-Meireles & Al-Chalabi 2009). Mice develop hindlimb weakness at approximately three months, progressing towards paralysis and premature death after four months (Turner & Talbot 2008). Adult onset degeneration in ALS patients progresses relentlessly within 3-5 years, ultimately causing respiratory paralysis and death (Grieb 2004).

1.7.3.2 Current Research in SOD1 Overexpression

The transgenic $SOD1^{G93A}$ mouse has been extensively used as a model of ALS, presenting reproducible similarities to the human ALS condition (Mahoney et al. 2006). Transgenic $SOD1^{G93A}$ mice develop severe motor neuron disease, reminiscent of ALS (Schon & Manfredi 2003), including hindlimb weakness and atrophy at approximately 100 days of age, progressing towards complete muscle paralysis and death at approximately 130-140 days (Mahoney et al. 2006). The importance of SOD1 became particularly evident in SOD knockout animal models. Mice lacking SOD1 demonstrate a progressive loss of muscle mass and significant decrease in average lifespan (Muller et al. 2006). Furthermore, SOD1 overexpression in Drosphila, demonstrate a decrease in cumulative oxidative damage and increased metabolic potential and lifespan (Sun et al. 2002). However, mouse models of SOD1 overexpression that model the human ALS condition, such as the $SOD1^{G93A}$ mutants, show a significant decline in lifespan and severe neurodegeneration and skeletal muscle wasting (Mahoney et al. 2006).

1.7.3.3 Oxidative Stress (OS) in SOD1 Overexpression

OS plays a key role in various degenerative conditions, including ALS (Aguirre et al. 2005; Vielhaber et al. 2000). Recently, Aguirre et al (2005) demonstrated significant oxidative damage in the cortex of $SOD1^{G93A}$ mice, while Vielhaber et al (2000), observed an increase in OS and associated damage in neuronal tissue of ALS patients. It has been reported that redox-dependent dissociation of SOD1 is impaired in SOD1 mutant models, which leads to sustained activation and H_2O_2 generation (Harraz et al. 2008). The pathological characteristics of $SOD1^{G93A}$ transgenic mice and the ALS condition include, gross atrophy, neurodegeneration, mitochondrial dysfunction, hypermetabolism and total SOD upregulation (Mahoney et al. 2006), each of which are associated with elevated levels of ROS (Dobrowolny et al. 2008; Esposito et al. 1999; Wu et al. 2006). Mohoney et al (2006) observed compensatory elevated antioxidant levels, including SOD1 activity, in gastrocnemius muscle of $SOD1^{G93A}$ mice, and reports of antioxidant supplementation in $SOD1^{G93A}$ mice have shown clinical benefits. Taken together, altered SOD1 enzyme function appears to be associated with a significant increase in ROS production. Although ROS appear to be implicated in tissue degeneration, and may play a role in $SOD1^{G93A}$ associated muscle degeneration, what remains to be elucidated are the cellular oxidative consequences of SOD1 modification in skeletal muscle. The SOD enzymes are not only important antioxidants for the dismutation of O_2^-, but also play a key role in regulating oxidative enzymes and redox-sensitive signaling pathways (Valko et al. 2007). ALS has been associated with OS and ROS generating systems, including mitochondrial ETC, NOX enzymes and SOD1 mutation itself (Harraz et al. 2008), and are likely to play an important role in skeletal muscle atrophy, associated with ALS.

1.7.3.4 A Role for the NOX Enzymes in SOD1 Overexpression

Although limited research exists for skeletal muscle degeneration in response to SOD1 overexpression, studies have demonstrated a role for NOX in neurodegeneration in ALS (Harraz et al. 2008; Liu et al. 2009; Wu et al. 2006). Recently, a study by Liu et al (2009) demonstrated a role for NOX-dependent ROS production in the facilitation of neurotoxic inflammatory responses in microglia, in $SOD1^{G93A}$ mutants. Harraz et al (2008) demonstrated direct regulation of NOX activity by SOD1 that was defective in $SOD1^{G93A}$ mutant mice, leading to an increase in NOX2-dependent O_2^- production in transgenic mouse tissue and cell lines. With the knowledge that NOX enzymes function primarily to generate O_2^- (Bedard & Krause 2007), and that SOD1 is the primary antioxidant enzyme that dismutates O_2^- in the cellular cytosol (Zelko, Mariani & Folz 2002), it is not surprising that the NOX enzymes play a role in a condition where SOD1 is significantly altered. Furthermore, skeletal muscle vulnerability to ROS (Hancock, Desikan & Neill 2001; Ji 2007) and the role that NOX generated ROS is proposed to play in skeletal muscle signaling pathways, suggests a potentially important role for NOX in skeletal muscle degeneration in the $SOD1^{G93A}$ mutation. Therefore, a dysfunction in SOD1 would certainly have consequences related to NOX enzymes that may contribute to the degeneration of skeletal muscle in SOD1 mutant ALS.

A significant reduction in NOX2 activity significantly alters the progression of ALS disease in $SOD1^{G93A}$ transgenic mice (Marden et al. 2007) with delays in neurodegeneration and extended life span (Wu et al. 2006), have been demonstrated. The study by Wu et al (2006) also demonstrated the potential role that NOX

generated ROS play in inhibiting the IGF-1/Akt survival pathway in motor neurons. This pathway is particularly important in skeletal muscle also, as it is the major pathway of muscle hypertrophy and regeneration (Costelli et al. 2005; Lai et al. 2004; Stitt et al. 2004). It is therefore suggested that this pathway of inhibition in skeletal muscle is contributing to the degeneration in response to SOD1 mutation. Indeed, genetic modification of SOD1 function, such as that present in the $SOD1^{G93A}$ model of ALS, would influence OS in most tissues, including skeletal muscle, consequently altering cellular redox systems such as NOX enzymes. It is therefore proposed that NOX enzymes are involved in the development of SOD1 associated neurodegeneration and ALS, and is therefore of interest to investigate this primary O_2^- generating system in the $SOD1^{G93A}$ mutant model.

1.8 Summary

NOX enzymes are primary generators of O_2^-, and the functional NOX enzyme systems have recently been described in a variety of cells and tissues. ROS are reactive molecules that can cause considerable oxidative damage to biological structures, but also play a key role in cellular redox-sensitive signaling pathways. With the help of antioxidants, ROS such as O_2^- are converted to less reactive species such as H_2O_2, however can give rise to other highly reactive species, such as $OH^{\bullet}$. It is important to recognise these ROS as necessary molecules for normal cellular function, but that can be produced inappropriately and cause direct oxidative damage and significant alterations in cellular redox-sensitive pathways, important for normal cellular function. ROS can be stimulated by a variety of redox-sensitive intra and extra-cellular stimulants that regulate ROS producing enzyme systems such as NOX,

and this regulation can be altered under stress conditions, particularly in response to disease. OS has been proposed to play a key role in a number of progressive degenerative diseases such as cardio and cerebral vascular dysfunction particularly, and has also been implicated in skeletal muscle wasting. The NOX2 and NOX4 enzyme systems have been described in skeletal muscle, however their role in this tissue is relatively undefined. As a primary generator of O_2^- and initiator of cascading ROS production, NOX enzymes are indeed important regulators of cellular redox homeostasis and OS in skeletal muscle. Furthermore, the role of NOX-dependent ROS as central mediators of intracellular signaling pathways, in response to redox-sensitive stimuli, has been proposed in skeletal muscle atrophy and hypertrophy pathways that can be altered in response to disease.

Age-associated skeletal muscle wasting or sarcopenia is described as one of the most dramatic phenotypic changes during the aging process and evidence suggests that accumulation of ROS over time is a key contributor to the decline in skeletal muscle mass. Cancer-induced cachexia has been described as the fastest developing skeletal muscle wasting condition of all cachectic associated pathologies, and has been strongly associated with the induction of the Ub-proteasome pathway, which may be mediated through intracellular ROS signaling. It is evident that the regulation of ROS and ROS generating enzyme systems play an important role in maintaining cellular redox homeostasis and may therefore play a key role in the development of skeletal muscle wasting in response to disease. It is therefore important to consider the regulation of cellular ROS through antioxidant scavenging, which can also be altered in response to disease. Experimental studies have demonstrated the importance of the

primary antioxidant systems SOD, through SOD transgenic animal models. Of particular interest is the primary intracellular O_2^- converting enzyme SOD1, which has been shown to induce age-associated pathologies in SOD1 knockout mice and extended survival when SOD1 is overexpressed. However, an additional transgenic mouse model of $SOD1^{G93A}$ mutation overexpresses SOD1 and demonstrates severe skeletal muscle wasting, a general phenotype characteristic of the human ALS condition.

1.9 Research Aims & Hypothesis

A large body of evidence exists for the involvement of OS in aging-associated, cancer-induced cachectic and SOD1 overexpression-associated skeletal muscle wasting however, the source of ROS is yet to be elucidated. In addition, while the role of the NOX enzyme systems in skeletal muscle is not fully understood, its contribution to oxidative associated tissue dysfunction suggests a role for NOX in skeletal muscle pathology. The involvement of NOX enzymes in skeletal muscle wasting associated with aging, cancer and SOD1 overexpression has not been described, and investigation of the NOX enzymes in skeletal muscle wasting will not only give insight into this system under pathology, but also its potential role in the skeletal muscle system. Therefore, these studies aim to investigate the NOX enzymes, previously shown to be expressed in skeletal muscle tissue, in conditions of skeletal muscle wasting. In particular, these studies will utilize well-established animal models of aging, cancer-induced cachexia and $SOD1^{G93A}$ that demonstrate significant skeletal muscle wasting. Within these models, we aim to investigate the NOX2 and

NOX4 enzyme systems and the associated subunit components, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ and Rac1, in skeletal muscle. To compliment the investigation of the O_2^- generating NOX enzymes, these studies will investigate the primary antioxidant enzyme systems; SOD1, SOD2 and SOD3, and catalase and GPx, for the conversion of O_2^- and subsequent conversion of H_2O_2. In addition to the oxidative and antioxidative systems, we aim to determine changes in O_2^- levels in skeletal muscle from each of the animal models and appropriate controls. Furthermore, the involvement of the cellular redox-sensitive transcription factor NF-κB in muscle atrophy and hypertrophy pathways, suggests its potential role in these models of skeletal muscle wasting, and will therefore be investigated in these studies.

The first hypothesis being tested in these studies is that NOX enzyme systems are upregulated in skeletal muscle in response to aging, cancer and SOD1 overexpression, consequently generating increased levels of O_2^-, in wasting skeletal muscle. The second hypothesis being tested in these studies is that while antioxidants may respond to the increase in ROS in these conditions, the response is not sufficient enough to compensate for the increases in ROS, consequently contributing to OS and tissue damage in age-associated sarcopenia, cancer-induced cachexia and SOD1 overexpression. Furthermore, while direct oxidative damage by ROS is indeed a potential contributor to skeletal muscle protein breakdown, we cannot ignore the evidence for the role of NOX generated ROS, in NF-κB activation and influence on skeletal muscle hypertrophy and atrophy pathway in these models of skeletal muscle wasting. Therefore, the third hypothesis being tested in these studies is that transcription factor NF-κB gene expression is upregulated in skeletal muscle, contributing to potential changes in skeletal muscle hypertrophy and atrophy

pathways, through NOX-dependent redox signaling, in age-associated sarcopenia, cancer-induced cachexia and SOD1 overexpression.

Chapter 2 Methods

2.1 Experimental Animals

Animal models of skeletal muscle wasting used in this study included a model of aging sarcopenia, cancer-induced cachexia and the SOD1^{G93A} model of ALS. All animal experiments were carried out with ethical approval. Animal experiments for the aging study and cancer-induced cachectic study 2 were reviewed and approved by the Victoria University Animal Experimental Ethics Committee (AEEC). Applications for Victoria University AEEC approval were considered on August 15, 2005 and approved for the period of September 1, 2005 until September 1, 2008. Approval was for experiments carried out and described in detail in the AEEC application for the use of BALB/c mice in the aging study (AEETH 14/05) and BALB/c *nu/nu* mice in the cancer-induced cachectic study 2 (AEETH 07/05). Animal models were developed as described below and skeletal muscle was collected for analysis. For the cancer-induced cachectic study 1 and SOD1^{G93A} study, animal models were obtained from existing studies carried out by Aston University (Pharmaceutical Sciences Research Institute, Aston University, Birmingham, UK) and The Howard Florey Research Institute (Melbourne, Australia) under their respective research department ethical approval.

2.1.1 Animal Model of Aging Sarcopenia

All animal procedures carried out in this study were approved by the Victoria University AEEC (AEETH 14/05). Female BALB/c mice (n=78) used in this study were purchased from the Animal Resources Centre (ARC), Western Australia. 62 Mice were purchased at 9 months of age and assigned to three different age groups 12 (n=18), 18 (n=20), and 24 (n=24) months. An additional 16, female BALB/c mice were purchased at 5 months of age and assigned to the 6 month old age group. All mice were maintained in the Victoria University Animal House until they reached the required age for tissue collection. Mice were maintained under controlled environmental conditions, 12 hour light/dark cycle, 21 ± 2°C, 30% humidity, in conventional cages with *ad libitum* access to standard chow and water, throughout the course of the study. Mice were monitored daily and any changes in body weight, physical appearance, behaviour, activity or signs of illness were recorded. Mice suffering from illness or pathologies throughout the study and on dissection were eliminated from the study. At 6, 12, 18 or 24 months of age, mice were anaesthetised using pentobarbital sodium (70mg/kg) for tissue collection. Tissues collected included, heart, liver, adipose tissue, diaphragm, quadriceps, gastrocnemius, tibialis anterior and soleus muscle. The tissues were dissected, weighed and immediately snap frozen in liquid nitrogen (BOC, Australia) and stored in a -80ºC freezer for experimental analysis.

2.1.2 Animal Model of Cancer-Induced Cachexia

The Murine Adenocarcinoma (MAC) model, initially derived from tumours induced by administration of 1,2-dimethylhydrazine, has been established as a model of colon cancer and cancer-induced cachexia. The MAC model is unique as it utilises two histologically similar colon cancer cell lines, MAC16 and MAC13. The two cell lines grow differently in culture (described below) and are shown to exhibit differences in their induced pathologies, once injected into mice. Mice injected with the MAC16 cell line establish tumour growth with the secondary condition of muscle wasting, cancer-induced cachexia, measurable by significant weight loss and skeletal muscle wasting. In contrast, mice induced with the MAC13 cell line establishes robust solid tumour growth, but without the development of cancer-induced cachexia. Using the contrasting cell lines in these mice provides a comparable and controlled model for the cancer-induced cachectic condition.

2.1.2.1 MAC13 and MAC16 Cell Culture

The MAC13 and MAC16 cells were donated from Professor Michael. J. Tisdale (Pharmaceutical Sciences Research Institute, Aston University, Birmingham, UK) and maintained at -80°C for later use. Cells were grown in culture for implantation into donor MAC13 and MAC16 mice to develop a model of cancer and cancer-induced cachexia respectively. Cells were maintained in culture with subsequent passages, to establish optimal growth, and only healthy rapidly dividing viable cells were used to inject into mice. General cell culture techniques were used to culture cells (described below), in order to maintain cells and to store frozen for later use. Growth media used to culture both MAC13 and MAC16 cells was RPMI1640 with L-glutamine (GIBCO,

USA) with added 10% Fetal Bovine Serum (FBS) (Invitrogen, USA) and 0.5% penicillin streptomycin (Invitrogen, USA). The MAC13 and MAC16 cells grow differently in culture, as the MAC13 cells grow as a monolayer attached to the culture flask whereas the MAC16 cells grow mostly in suspension.

2.1.2.1.1 Cell Preparation

The MAC13 and MAC16 cell lines were thawed in a 37ºC water bath and once almost completely thawed were transferred to a sterile cell culture fume hood. The cells were transferred to a sterile centrifuge tube containing 10ml of growth media with 20% FBS, using a sterile Pasteur pipette. The cell suspension was centrifuged gently at 200xg for 5 minutes at 25ºC, the media was removed and the cell pellet was resuspended in 10ml of fresh growth media containing 20% FBS. The cell suspension was then transferred to a 50cm (100ml) flask and placed in a cell culture humidifier (37ºC and 5% CO_2). Cells were resuspended in fresh growth media within 24hrs after thawing and then every 48hrs thereafter. Cells were maintained in a cell culture humidifier (37ºC and 5% CO_2) until they reached 80% confluency, before they were subcultured and prepared for injection into mice or frozen for storage (described below). MAC13 and MAC16 cells were resuspended in fresh growth media every 48 hours. The MAC13 cells grow as a monolayer attached to the culture flask, and therefore the media was removed from the flask and the cells were resuspended in 10mls of fresh growth media. The majority of MAC16 cells grow in suspension and therefore in order to resuspend cells in fresh growth media without removing viable cells, 5mls of growth media and suspended cells were removed from the flask and 5mls of fresh growth media was added.

For subculture, MAC13 cells require trypsinization however, as the MAC16 cells grow mostly in suspension, trypsinization is not necessary. With the detachment of MAC13 cells by trypsinization, both the MAC13 and MAC16 flasks containing 10ml of cells in growth media were transferred into subsequent flasks containing fresh growth media (1:10 dilution) and the newly generated flasks were returned back to the cell culture humidifier (37ºC and 5% CO_2). Flasks containing MAC13 cells were removed of growth media and cells were washed twice in calcium and magnesium free Phosphate Buffered Saline (PBS) (Sigma, USA), to remove any remaining growth media from the flask. Following this, 3ml of 0.5% Trypsin EDTA (Sigma, USA) was added to the cell flask and placed in the cell culture humidifier (37ºC and 5% CO_2) for 3mins or until cells detached. To inactivate the trypsin, 7ml of growth media was added to the flask.

Aliquots of cells were frozen and stored in a -80°C freezer for later use. Only cells that were healthy and rapidly dividing were frozen for optimal recovery. On the day of freezing MAC13 cells were detached from the flask by trypsinization and deactivated with the addition of fresh growth media as described above. Flasks containing both MAC13 and MAC16 cells in 10ml of growth media were transferred to a sterile centrifuge tube and gently centrifuged at 400xg for 5 minutes at 25°C. The supernatant containing media and dead cells was removed and the remaining pellet was resuspended in freezing media containing 10% Dimethyl Sulfoxide (DMSO) (Sigma, USA), 20% FBS and immediately after the addition of freezing media, 0.5ml of this cell suspension was transferred to freezing vials and placed in a -80ºC freezer for storage.

To test for cell viability and the concentration of cells in the media, a Trypan blue stain was performed using a general Trypan blue cell viability method. Aliquots of the cell suspension were diluted in Trypan Blue dye (1:20). The Trypan blue diluted cell suspension was placed on a hemacytometer chamber and viewed under a microscope. Cells were counted and calculated to obtain viable concentration to be injected into donor mice (dead cells stain blue, while live cells are birefringent). The calculation for cell viability was carried out as follows, the number of live cells per 1x1x0.1mm areas = cells x $1*10^{-4}cm^3$ ($1*10^{-4}ml$). With a 1:20 dilution the number of cells in two 1mm squares divided by 10 = cells x 10^6/ml.

(2 squares/ 2 x 20 x 10,000 / 1,000 = 10^6 cells/ml)

(2 squares/ 10 x 10,000 / 1,000 = 10^6 cells/ml)

(2 squares/ 100 x 10,000 / 1,000 = 10^6 cells/ml)

(2 squares/ 10 = millions/ml)

2.1.2.1.2 Preparation of Cells for Implantation

Cells were injected into a donor BALB/c *nu/nu* mice when they reach 80% confluency. Cells were removed from the flask as previously described, MAC13 requiring trypsinization, placed in a centrifuge tube and centrifuged at 400xg for 5minutes at 25ºC. The supernatant containing media and dead cells was removed and the pellets were resuspended in 1ml of isotonic saline (0.9%). 100ul of each cell suspension containing approximately 1 x 10^6 cells was injected subcutaneously into the thigh of donor mice using 27G needles (27G x 0.5, Livingston International, USA).

2.1.2.2 Cancer-Induced Cachexia Study 1

The MAC16 model was first established and used as a model for studying the condition of cancer-induced cachexia by Professor Michael J Tisdale (Pharmaceutical Sciences Research Institute, Aston University, Birmingham, UK). Female NMRI mice from the inbred Aston University colony were used as an animal model that has previously been shown to establishing tumour growth and cancer-induced cachexia, once injected with the MAC16 cell lines (Hussey & Tisdale 2000; Whitehouse et al. 2001). Skeletal muscle quadriceps from MAC16-induced mice (n=10) establishing significant weight loss and cachexia, along with NMRI controls (n=10) that were not induced with cancer, were donated from Professor Michael J Tisdale (Pharmaceutical Sciences Research Institute, Aston University, Birmingham, UK). Female NMRI mice from the inbred Aston University colony were injected with the MAC16 cell line. NMRI mice injected with MAC16 cells and weight matched controls were maintained in the Aston University colony with *ad libitum* access to standard chow and water. MAC16 cells were grown in culture (as described above) and implanted subcutaneously into the hindlimb of donor NMRI mice. Donor NMRI mice are used to grow MAC16 tumours and develop cachexia to optimal conditions, that is, tumour growth and significant weight loss of up to 25%, to represent a model of cancer-induced cachexia. Fragments of the MAC16 tumour, maintained in donor mice that established weight loss between 20-25%, were implanted subcutaneously into the flank of recipient NMRI mice. Body weights were recorded for the MAC16-induced and control mice daily and tumour volume was measured in MAC16-induced mice daily by means of calipers. Mice injected with the MAC16 tumours were considered to be good models of cancer-induced cachexia when they presented with solid tumour growth by 9-12 days post implantation and significant weight loss of up to 25%

original body weight. Mice were sacrificed at day 25 at varying degrees of weight loss (12-25%). Tissue was collected from the MAC16-induced and control NMRI mice, immediately snap frozen in liquid nitrogen and stored at -80ºC. Skeletal muscle quadriceps from MAC16 (n=10) and control (n=10) mice were obtained and used for our analysis.

2.1.2.3 Cancer-Induced Cachexia Study 2

In addition to the NMRI mouse model, the BALB/c *nu/nu* immunosuppressed mouse strain has previously been shown to establish tumour growth and cancer-induced cachexia once induced with the MAC16 cell lines (Monitto et al. 2001). Female BALB/c *nu/nu* mice utilized in this study were purchased from ARC, Western Australia. All animal procedures carried out in this study were approved by the Victoria University AEEC (AEETH 07/05). The MAC model for cancer-induced cachexia was chosen for this study as it enables the utilisation of the two histologically similar colon cancer cell lines, MAC16 and MAC13. The MAC13-induced mice were used as a murine model of cancer and the MAC16-induced mice as a murine model of cancer-induced cachexia. Both MAC13 and MAC16 cells were donated from Professor Michael J. Tisdale (Pharmaceutical Sciences Research Institute, Aston University, Birmingham, UK) and grown in culture to 80% confluency as described above. A total of 100μl of this cell suspension was injected subcutaneously into the thigh of each donor mouse.

2.1.2.3.1 MAC13 & MAC16 Cell Implantation

As previously described by Bing et al (2000), donor mice were used to implant tumour fragments into subsequent recipient mice to be used as models of both cancer (MAC13) and cancer-induced cachexia (MAC16). Cells from culture were implanted subcutaneously into the thigh of donor mice and monitored daily while recordings of food intake, body weight and tumour size were obtained. MAC13 donor mice that developed tumours by day 9-12 and no weight loss, approximately 25 days thereafter, were considered to represent a model of cancer. MAC16 donor mice that developed tumours by day 9-12 with progressive weight loss, of up to 25% their original body weight thereafter, were considered to represent a model of cancer-induced cachexia. Tumours from MAC13 and MAC16 donor mice were used to implant recipient BALB/c *nu/nu* mice in order to establish and maintain a tumour line, which consistently produced cancer and cancer-induced cachexia, respectively. Tumours were dissected from donor mice and re-injected subcutaneously into the flank of the recipient BALB/c *nu/nu* mice.

Mice were monitored daily and data from food intake, body weight and tumour size were recorded. MAC13 tumour cell recipient mice that developed tumours by day 9-12 and no weight loss up to 30 days thereafter were used as a model of cancer in this study. MAC16 tumour cell recipient mice that developed tumours by day 9-12 with progressive weight loss, of up to 25% their original body weight thereafter, were used as a model of cancer-induced cachexia in this study. In addition, 12 female BALB/c *nu/nu* mice were purchased from ARC (Western Australia) and used as a control. All mice in this study were maintained under controlled environmental conditions, 12

hour light/dark cycle, 21 ± 2°C, 30% humidity, in conventional cages with *ad libitum* access to standard chow and water, throughout the course of the study. All mice used in this study, control (n=12), MAC13-induced (n=13) and MAC16-induced (n=16) mice were anaesthetised using pentobarbital sodium (70mg/kg) for tissue collection. Tissues collected included, heart, liver, diaphragm, quadriceps, gastrocnemius, tibialis anterior and soleus muscle. The tissues were dissected, weighed and immediately snap frozen in liquid nitrogen (BOC, Australia) and stored in a -80ºC freezer for experimental analysis.

2.1.3 Animal Model of SOD1 Overexpression ($SOD1^{G93A}$)

$SOD1^{G93A}$ mice hemizygous for $SOD1^{G93A}$ transgene were used in this study as a model of altered SOD1 function. The $SOD1^{G93A}$ mutant mouse model displays a high SOD1 transgene copy number and exhibits the ALS phenotype. That is, progressive neurodegeneration and severe hindlimb dysfunction that eventually progresses to complete paralysis and death at approximately 19-23 weeks of age. Transgenic mice carrying the G93A human SOD1 mutation and Wild Type (WT) human SOD1 transgene were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were bred at The Howard Florey Institute (Melbourne, Australia) and offspring were genotyped by Polymerase Chain Reaction (PCR) assay of DNA extracted from tail tissue and comparisons were made with mice with an overexpression of the WT human SOD1 gene. All mice used in this study were maintained under controlled environmental conditions, 12 hour light/dark cycle, 21 ± 2°C, 30% humidity, in conventional cages with *ad libitum* access to standard chow and water throughout the course of the study. $SOD1^{G93A}$ transgenic mice were clinically scored for disease

progression as follows; Stage I, onset of hindlimb tremor; Stage II, onset of hindlimb paresis; Stage III, severe hindlimb paresis with abnormal gait; Stage IV, hindlimb paralysis and Stage V, death (Turner et al. 2003). $SOD1^{G93A}$ transgenic mice (n=10) were anaesthetised at Stage III along with $SOD1^{WT}$ control littermates (n=10), using pentobarbital sodium (70mg/kg) for tissue collection. Skeletal muscle quadriceps, were dissected from $SOD1^{G93A}$ and $SOD1^{WT}$ control mice and immediately snap frozen in liquid nitrogen (BOC, Australia) and stored in a -80ºC freezer for experimental analysis.

2.2 Analysis Methods

2.2.1 Tissue Collection

All tissues collected from mice were weighed and immediately snap frozen in liquid nitrogen (BOC, Australia) and stored at -80ºC. In order to investigate skeletal muscle wasting in aging sarcopenia, cancer-induced cachexia and a model of SOD1 overexpression, skeletal muscle quadriceps were chosen to carry out the experimental analysis. The quadriceps muscle was chosen because it has a mixed fiber type and represents one of the largest muscle groups, which was required for multiple analysis procedures, for these studies. Upon dissection, mice were anaesthetised using pentobarbital sodium (70mg/kg) and the heart, liver, diaphragm, right and left quadriceps, gastrocnemius, tibialis anterior and soleus muscles were collected. These additional tissues were collected for potential analysis if required. The gastrocnemius muscles were used to measure O_2^- via histological analysis, for the aging study only, due to lack of abundance of quadriceps muscles after subsequent method

development. The gastrocnemius muscle when used as a whole i.e. both red and white also represents a larger mixed fibre type muscle. All experimental procedures were carried out on frozen skeletal muscle samples.

2.2.2 Reverse Transcription-Polymerase Chain Reaction (RT- PCR)

2.2.2.1 RNA Extraction

Frozen quadriceps muscles were weighed (50mg) and homogenised in 500µl of Tri-Reagent (Molecular Research Center, USA) RNA isolation reagent, in tubes containing Zirconia-Silica homogenisation beads (Daintree Scientific, Australia) and placed in a fast prep homogeniser (Thermo Scientific, Sweden) on 6 speed for 40 seconds. The samples were placed on ice for 5 minutes and homogenization was repeated. The homogenate was stored at room temperature for 5 minutes to permit dissociation of nucleoprotein complexes. The homogenate was supplemented with 0.1ml of 1-Bromo-3-Chloropropane (BCP) 99% (Molecular Research Center, USA) and shaken vigorously for 15 seconds. The resulting mixture was stored at room temperature for 15 minutes before being centrifuged at 12,000xg for 15 minutes at 4ºC. The resulting mixture separates into a lower red phenol-chloroform phase, interphase and colourless upper aqueous phase. RNA remaining in the aqueous phase was transferred to a fresh tube and mixed with 0.5ml of Isopropyl alcohol (ICN Biomedicals, USA) to precipitate the RNA. Samples were stored at room temperature for 10 minutes and centrifuged at 12,000xg for 8 minutes at 4ºC. The supernatant was removed and the RNA precipitate pellet, formed at the bottom of the tube, was washed by vortexing with 1ml of ethanol (75%). The washed pellet containing ethanol was then centrifuged at 7,500xg for 5 minutes at 4ºC. The ethanol was

removed from the resulting RNA pellet, which was then air-dried for 5 minutes. RNA was dissolved in 10µl of Nuclease Free Water (NFW) by passing the solution a few times through a pipette tip before a 10 minute incubation was performed at 55ºC. RNA (5µl) was diluted in milli Q water (1995µl) and measured for purity and RNA concentration by spectrophotometry (Bio-Rad, USA). Absorbance was read at 260/280nm and the concentration of RNA was calculated for RT-PCR analysis.

For optimal results RNA was DNase treated using RQ1 (RNA-Qualified) RNase-Free DNase (Promega, Australia) that degrades both double and single-stranded DNA. The DNase digestion was designed as follows; depending on RNA concentration RNA was diluted in NFW to a final concentration of 1µg/µl and 5µl was added to a mix of 1µl of RQ1 RNase-Free DNase 10X reaction buffer (Promega, Australia) and 1 unit/mg RNA of RQ1 RNase-Free DNase (Promega, Australia) and made up to 10µl final volume with NFW. The reaction mixture was incubated at 37ºC for 30 minutes and the reaction was stopped by the addition of 1µl of RQ1 DNase Stop Solution (Promega, Australia) and incubated at 65ºC for 10 minutes to inactivate the DNase.

2.2.2.2 cDNA Synthesis

The DNase treated RNA was reverse transcribed using the Reverse Transcription (RT) System kit (Promega, Australia). A 20µl reaction was made including 4µl of $MgCl_2$ (25mM) diluted in NFW to optimal concentration; 2µl of RT Buffer (10X), 2µl of dNTP mixture (10mM), 0.5µl of Recombinant RNasin Ribonuclease Inhibitor, 15U of AMV RT, 0.5µg of oligo(dT)15 primer RNA (1µg) and made up to a final volume of 20µl with NFW. The reaction mix was placed in a thermal cycler (Bio-Rad, USA) and incubated at 42ºC for 15 minutes, 95ºC for 5 minutes and then cooled

at 5ºC for 5 minutes. The resulting cDNA was stored at -20°C for subsequent analysis.

2.2.2.3 Polymerase Chain Reaction (PCR)

2.2.2.3.1 Cancer-Induced Cachexia Study 1

Primer sequences used were the same as those used previously (Furukawa et al. 2004; Park et al. 2005) and additional primers were designed using Primer 3 software (Table 2.1). Each primer was used to confirm homologous binding to the desired mRNA of the muscle samples. Real-time PCR was performed using Light Cycler Real-Time PCR detection system (Roche, USA) with PCR reactions performed using LightCycler FastStart DNA Master SYBR Green I kit (Roche, USA). Primers were made up to 100µM stocks and diluted further to a working concentration of 3µM in NFW. Magnesium ($MgCl_2$) concentration was optimized for each primer pair by titration (1µM – 5µM) to obtain the best PCR mix. Real-time PCR mix included $MgCl_2$ (25mM) diluted to the required concentration with NFW, 2µl of each of the forward and reverse primers (3µM), 2µl of SYBR green, 2µl of cDNA (1:20) and NFW to a final volume of 20µl. To compensate for variations in input RNA amounts and efficiency of reverse transcription, cyclophilin and 18s ribosomal mRNA was quantified and all results were normalised to these values. To obtain replicatable data/analysis each sample was run in duplicate and housekeeping genes in triplicate. PCR thermal cycler parameters were set to standard mode of 10 minute incubation at 95°C before repeat cycling at 95°C for 15 seconds, followed by a 1 minute incubation at 60°C for 40 cycles. Fluorescent emission data were captured and mRNA levels were analysed using the Critical Threshold (C_T) value (Schmittgen et al. 2000). The $2^{\Delta}C_T$ was calculated by subtracting the ${}^{\Delta}C_T$ for 18s ribosomal mRNA from the ${}^{\Delta}C_T$ for

the gene of interest in the control and experimental samples. The relative expression of the gene of interest was calculated using the expression $2^{\Delta}C_T$ and reported as arbitrary units of gene expression.

Table 2.1 Primer sequences used for PCR analyses.

Gene	Forward Primer	Reverse Primer
NOX2	TTGGGTCAGCACTGGCTCTG	TGGCGGTG TGCAGTGCTATC
p22phox	GTCCACCATGGAGCGATGTG	CAATGGCCAAGCAGACGGTC
p67phox	CTGGCTGAGGC CATCAGACT	AGGCCACTGCAGAGTGCTTG
p47phox	GATGTTCCCCATTGAGGCCG	GTTTCAGGTCATCAGGCCGC
p40phox	GCCGCTATCGCCAGTTCTAC	GCAGGCTC AGGAGGTTCTTC
SOD1	TGAACCAGTTGTGTTGTCAGG	TCCATCACTGGTCACTAGCC
SOD2	TGGCTTGGCTTCAATAAGGA	AAGGTAGTAAGCGTGCTCCCACAC
SOD3	AGGTGGATGCTGCCGAGAT	TCCAGACTGAAATAGGCCTCAAG
18S	GTAACCCGTTGAACCCCATT	ATCCAATCGGTAGTAGCG
Cyclophilin	CAGACGCCACTGTCGCTTT	TGTCTTTGGAACTTTGTCTGCAA

2.2.2.3.2 Aging Study, Cancer-Induced Cachexia Study 2 & SOD1^{G93A} Study

PCR reactions for the aging study, cancer-induced cachectic study 2 and the SOD1^{G93A} study were performed using 7500 Real-Time PCR System (Applied Biosystems, USA) and TaqMan Gene Expression Assay (Applied Biosystems, USA) pre-designed probe and primer sets for quantitative gene expression analysis (Table 2.2). This method of PCR analysis is a more advanced and reliable method and was therefore used in these studies. TaqMan Gene Expression Assays contain FAM dye-labeled TaqMan Minor Groove Binder (MGB) probe (final concentration of 250nM) and forward and reverse primers for the sequence of interest (final concentration of

900nM each). cDNA concentration was optimised for most efficient PCR detection via cDNA titration (1-20µl) for each primer set. The PCR reaction mix components were as follows, 2.5µl of TaqMan Gene Expression Assay (20x); 25µl of TaqMan Gene Expression Master Mix, cDNA template optimised to each primer set (2-12µl) and NFW to a final volume of 50µl. To compensate for variations in input RNA amounts and efficiency of reverse transcription, GAPDH mRNA was quantified for the cancer-induced cachectic study 2 and the SOD1^{G93A} study, and all results within these studies were normalised to these values. Due to changes in GAPDH between age groups, 18s ribosomal mRNA was quantified for the aging study and all results within this study were normalised to these values. PCR thermal cycling conditions were set to standard mode of 10 minute incubation at 95°C before repeat cycling at 95°C for 15 seconds, followed by a 1 minute incubation at 60°C for 40 cycles. Fluorescent emission data were captured and mRNA levels were analysed using the C_T value (Schmittgen et al. 2000). The $2^{\Delta}C_T$ was calculated by subtracting the $^{\Delta}C_T$ for GAPDH or 18s ribosomal mRNA from the $^{\Delta}C_T$ for the gene of interest in the control and experimental samples. The relative expression of the gene of interest was calculated using the expression $2^{\Delta}C_T$ and reported as arbitrary units.

Table 2.2 ABI custom primer descriptions used for PCR analyses.

Gene	ABI Primers	Gene	ABI Primers
NOX2	Mm00432775_m1	SOD1	Mm01344233_g1
NOX4	Mm00479246_m1	SOD2	Mm00449726_m1
p22phox	Mm00514478_m1	SOD3	Mm00448831_s1
p40phox	Mm00476300_m1	GPx1	Mm00656767_g1
p47phox	Mm00447921_m1	Catalase	Mm00437992_m1
p67phox	Mm00726636_s1	GAPDH	Mm99999915_g1
RAC1	Mm01201656_m1	ACTB	Mm00607939_s1
NF-kB1	Mm00476361_m1	18s	Hs02387368_g1

2.2.3 Superoxide Dismutase (SOD) Enzyme Activity

2.2.3.1 Protein Quantification (Bradford Method)

SOD activity procedures were standardised for protein measurement using the Bradford method, with a Protein Assay Kit (Bio-Rad, USA). Dye reagent was diluted 1 part with 4 parts distilled water and filtered through Whatman #1 filter paper to remove particles. Five dilutions of the protein standard using Bovine Serum Albumen (BSA) diluted in NFW were prepared at concentrations of 0.1, 0.2, 0.4, 0.6, 0.8 mg/ml. Each standard and sample homogenate was assayed in duplicate. 100µl of each standard and sample prepared was added to 5ml of diluted dye reagent, vortexed and incubated at room temperature for 5 minutes. Absorbance (595nm) was measured for all duplicate standards and samples immediately after the 5 minute incubation to control for increases in absorbance overtime. Results of the standard absorbances were used to prepare a standard curve and samples were calculated for protein concentrations according to the standard concentrations.

2.2.3.2 Cancer-Induced Cachexia Study 1

SOD activity in the cancer-induced cachectic study 1 was measured by spectrophotometric assay based on epinephrine autoxidation, described previously (Sun & Zigman 1978). Epinephrine undergoes autoxidation in air to form adrenochrome, which absorbs light at 320nm. SOD inhibits epinephrine oxidation and therefore the amount of inhibition is in direct proportion to SOD activity.

2.2.3.2.1 Tissue Preparation

Frozen muscle aliquots (100mg) were homogenised in 10ml of ice-cold potassium phosphate buffer (100mM) with BSA, commonly used for tissue homogenization procedures. Potassium phosphate buffer was prepared as follows; KH_2PO_4 (0.2M), K_2HPO_4 (0.8M), EDTA (10mM), BSA (0.05%) in dH_20 (pH 7.4). Muscle aliquots in buffer were homogenised using a 15ml glass homogenization tube placed on ice and rotated at approximately 50 rpm for 10 minutes and centrifuged at 400xg for 10 minutes at 4ºC to remove insoluble connective tissue and the supernatant was collected for SOD analysis. SOD activity was measured by spectrophotometric assay based on epinephrine autoxidation described previously (Sun & Zigman 1978). Muscle homogenates were added to cuvettes containing epinephrine (30mM, pH 1.3) and sodium bicarbonate buffer $NaHCO_3$ (pH 10.2) with EDTA and the change in absorbance at 320nm was measured over 3 minutes at 25ºC. The amount of activity was calculated and standardised for protein using the Bradford method (Bio-Rad, USA).

2.2.3.2.2 Superoxide Dismutase (SOD) Activity Assay

Sample homogenates (100µl) were added to UV visible cuvettes, with 2ml of sodium bicarbonate ($NaHCO_3$) buffer with EDTA, adjusted to a pH of 10.2 with KOH, and 20µl of epinephrine (30mM), adjusted to a pH of 1.3 using HCl. To maintain consistency of reaction time for all samples, once sample homogenates were added, each sample was vortexed 4 times and after exactly 5 minutes, the change in absorbance (320nm) over 3 minutes was measured. All assays were performed at 25ºC in duplicate.

The amount of activity, which causes 50% inhibition of epinephrine oxidation, is equal to 1 unit; therefore activity was calculated using the following equation:

$$\text{Activity} = \frac{(^{\Delta}\text{OD.min blank} - {}^{\Delta}\text{OD.min sample})}{{}^{\Delta}\text{OD.min blank}} / (0.5 \times \text{TDF})$$

Total Dilution Factor (TDF) = (homogenate (µl/1) x ((buffer (ml) + epinephrine (ml))/ volume of homogenate (ml))

SOD activity was standardised for protein using the Bradford method (Bio-Rad, USA) standard procedure explained previously. To calculate SOD activity in terms of protein levels for each sample the following equation was used:

$$\text{SOD Activity} = \frac{\text{units/ml}}{\text{mg total protein/ml}}$$

2.2.3.3 Aging Study, Cancer-Induced Cachexia Study 2 & SOD1^{G93A} Study

SOD activity was measured in the aging study, cancer-induced cachectic study 2 and SOD1^{G93A} study, using the 706002 Superoxide Dismutase Assay Kit (Cayman, USA). The SOD Assay Kit measures a tetrazolium salt for the detection of O_2^- radicals generated by ROS producing enzymes. This measure of SOD activity is a more advance and reliable method and was therefore used in these studies.

2.2.3.3.1 Tissue Preparation

Tissue samples were homogenised in ice-cold HEPES buffer (20mM) (10ml/g of tissue), consisting of EGTA (1mM), mannitol (210mM) and sucrose (70mM) (adjusted to a pH of 7.2) using a hand held remote homogeniser (Omni International TH, USA). The tissue homogenate was centrifuged at 1,500xg for 5 minutes at 4°C to remove connective tissue and the supernatant was collected for SOD analysis. To separate the cytosolic and mitochondrial enzymes, the supernatant was centrifuged at 10,000xg for 15 minutes at 4°C, where the resulting supernatant contains the cytosolic SOD (SOD1) and the pellet contains the mitochondrial SOD (SOD2). The mitochondrial pellet was further homogenised in ice-cold HEPES buffer (20mM) (5ml/g of tissue).

2.2.3.3.2 Superoxide Dismutase (SOD) Activity Assay

Both the cytosolic and mitochondrial fractions were assayed and measured for SOD activity. Each reagent provided by the SOD Assay Kit was prepared as per the manufacturers protocol, yielding Assay Buffer, Sample Buffer, Radical Detector, SOD Standard and XO. SOD standards were prepared by diluting 20µl of the SOD standard with 1.98ml of sample buffer to obtain a SOD stock standard and the SOD stock was diluted to yield different concentrations with sample buffer (Appendix 1). The assay was run using a 96 well plate ELISA reader (2550 EIA Reader, Bio-Rad, USA) at 460nm at 25°C. SOD standards and samples (10µl) were placed in wells in duplicate using a repeat pipette (Eppendorf, South Pacific). 200µl of radical detector was added and the reaction was initiated by adding 20µl of XO to each well. The plate was then carefully shaken, covered and incubated at 25°C for 20 minutes, before

reading the absorbance (460nm). SOD activity was standardised for protein using the Bradford method (Bio-Rad, USA) standard procedure, described earlier.

To calculate SOD activity, the average absorbance of each standard and sample was calculated using Microsoft Excel. Standard A's absorbance, containing no SOD stock (Appendix 1), was divided by itself and by all the other standard and sample absorbances to yield the Linearized Rate (LR). The LR was plotted as a function of final SOD Activity (U/ml) from the table (Appendix 1) for a typical standard curve. SOD activity was calculated using the equation from the linear regression of the standard curve substituting the LR for each sample.

2.2.4 Detection of O_2^- by DHE Fluorescence Staining

Dihydroethidium (DHE) is a O_2^- sensitive dye, commonly used for the measure of OS in cryosectioned tissue. DHE is cell permeable and reacts with O_2^-, converting DHE into ethidium fluorescence (Serrander et al. 2007). Skeletal muscle gastrocnemius, from the aging study, and quadriceps muscles from the cancer-induced cachectic study 2 and $SOD1^{G93A}$ study, were used for the measure of O_2^- levels in frozen skeletal muscle tissue. A cross section of the muscle was taken at the belly and the muscle piece was immediately placed into the cryostat (Microm HM 505E, Zeiss, Germany) and fixed onto the chuck using Tissue-Tek Optimum Cutting Temp (O.C.T) compound. Muscle was cut into 5-10µm thick sections, placed onto slides and frozen at -20°C for subsequent DHE staining. To maintain consistency and eliminate bias, sample sections (5µm) were analysed in duplicate and each group within each study was stained and analysed together. To explain further, slides from

each age group within the aging study were stained with DHE, photographed and analysed together. Before subsequent staining with DHE, slides were brought to room temperature. DHE (5μM) was applied to the skeletal muscle cross sections (5μm), and incubated in a light protected oven at 37°C for 30 min. The DHE was washed from the sections with PBS and fluorescence was assessed using Axiocam HBO 50/AC fluorescence microscopy (Zeiss, Germany). The DHE stained sections were viewed using a 10x objective lens and subsequent photographs of the muscle sections were taken to view the sections in their entirety. The intensity of ethidium fluorescence detection of O_2^- was measured with MCID software (Imaging Research Inc, Bundoora, Australia) with excitation at 480 nm and emission at 560 nm, and expressed as arbitrary units of fluorescence (Azumi et al. 2002; Miller et al. 2002; Williams & Allen 2007).

2.3 Statistical Analysis

Statistical analysis was performed using SPSS statistical package (version 15.0) for all studies. Results are expressed as mean ± Standard Error of the Mean (SEM). Differences were determined by one-way Analysis Of Variance (ANOVA), with Tukey HSD as posthoc for both the aging study and the cancer-induced cachectic study 2, while an independent t-test was used for both the cancer-induced cachectic study 1, and the $SOD1^{G93A}$ study. Results from all studies were considered statistically significant if *p*-values were equal to or <0.05.

Chapter 3 Aging Study

3.1 Introduction

One of the most dramatic phenotypic changes associated with aging, is age-associated muscle wasting sarcopenia (Fulle et al. 2004; Mansouri et al. 2006). The progressive age-associated loss of skeletal muscle mass and function leads to significant weakness, frailty, disability and an overall decrease in quality of life (Castaneda 2002; McArdle, Vasilaki & Jackson 2002). To date, many theories have been proposed to explain the degradation of skeletal muscle associated with aging, however it is most likely that one or a number of catabolic factors are involved (Castaneda 2002). It is well known that aging is associated with an increase in oxidative status and that OS is involved in many age related diseases (Pansarasa et al. 1999; Ferrington, Husom & Thomson 2005; Heaton et al. 2002; Landis et al. 2004; Mosoni et al. 2004; Squier 2001). The evidence of cellular damage leading to lipid peroxidation, protein carbonylation and DNA damage in aged skeletal muscle (Mecocci et al. 1999; Muller et al. 2006; Sundaram & Panneerselvam 2006), indeed suggests a role for ROS. Therefore, the oxidation of skeletal muscle proteins is likely to be a primary contributor to age related muscle decline.

3.1.1 Oxidative Stress (OS) in Aging Sarcopenia

OS has been proposed as a potential contributor in the development of sarcopenia (Mosoni et al. 2004; Sundaram & Panneerselvam 2006). It is well known that ROS are generated during skeletal muscle cellular metabolism and are capable of causing considerable harm to cells and tissues under conditions of increased metabolic activity

(Ji, Gomez-Cabrera & Vina 2006; Vasilaki et al. 2006). O_2^- is generated in skeletal muscle as a by-product of cellular metabolism, but is capable of exceeding beyond it's normal cellular production during increased activity (Fulle et al. 2004; McArdle et al. 2005) and in many pathological conditions (Geiszt et al. 2000; Harraz et al. 2008; Oudot et al. 2006). The primary oxidant O_2^- can lead to the generation of additional ROS, such as H_2O_2 and HO^-, that when produced inappropriately, is responsible for cellular OS and associated damage to cells and tissues (Babior 1997; Mansouri et al. 2006; Supinski & Callahan 2007). Skeletal muscle is particularly vulnerable to an overproduction of O_2^-, and therefore H_2O_2 and HO^-, due to its high metabolic activity (Sundaram & Panneerselvam 2006), but also accumulates over time (Landis et al. 2004). Although ROS are generated from a variety of sources, the O_2^- generating NOX enzymes have recently been proposed to contribute to aging (Krause 2007).

3.1.2 The NOX Enzymes in Skeletal Muscle

It is well known that the mitochondria is a primary site of ROS generation, as a by-product of cellular metabolism (Kerner et al. 2001), however the knowledge that the NOX enzymes are primary O_2^- producing systems (Lambeth 2004; McArdle et al. 2004), brought about much speculation for its role in skeletal muscle. Also, the discovery of the NOX homologues for the catalytic subunit NOX2 (Bedard & Krause 2007; Geiszt 2006) found the expression of NOX2, and the associated subunit components, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ in skeletal muscle (Cheng et al. 2001; Javesghani et al. 2002), as well as the GTP binding protein Rac1 (El-Benna et al. 2005; Groemping & Rittinger 2005) and NOX4 (Mofarrahi et al., 2008). While studies have demonstrated a role for NOX2 in the skeletal muscle system, NOX4 has

been linked to cellular senescence in fibroblasts (Geiszt et al. 2000) and vascular dysfunction (Paravicini et al. 2004; Wingler et al. 2001). The role of NOX enzymes, in general, in skeletal muscle remains relatively undefined, however the enzyme system has been implicated in a variety of muscle wasting conditions (Barreiro et al. 2005; Busquets et al. 2005; Fulle et al. 2004; Mahoney et al. 2006). Therefore it is proposed that NOX may contribute to an increase in ROS production and damage to skeletal muscle during aging, characteristic of sarcopenia.

3.1.3 Antioxidant Enzymes in Aging Sarcopenia

It has been established that NOX enzymes have the potential to cause considerable harm to healthy cells and tissues (Geiszt et al. 2000; Griendling, Sorescu & Ushio-Fukai 2000), including skeletal muscle (Javesghani et al. 2002). The consequence of NOX enzyme activation not only generates the potentially destructive O_2^-, but also mediates a cascade of generating ROS, through endogenous antioxidant activity (Ji 2007). Indeed, the cascading events can lead to a harmful cocktail of reactive oxidants, however the antioxidant enzymes are important regulators of ROS (Harraz et al. 2008) and maintenance of cellular oxidative balance (Johnson & Giulivi 2005; Scandalios 2005). Antioxidant enzymes are particularly important in skeletal muscle, due to the high metabolic activity and potential for inappropriate ROS generation (Ji 2007; McArdle, Vasilaki & Jackson 2002). However, it has been proposed that aging may have consequential effects on skeletal muscle signaling pathways and responses to stimuli that may compromise their important role in regulating cellular oxidation (Ji 2007). Contrasting studies have investigated skeletal muscle antioxidant function during aging, proposing both a compensatory increase in antioxidant activity (Gianni

et al. 2004; Pansarasa et al. 1999) as well as evidence to suggest antioxidant dysfunction, where a compensatory response is impaired (Esposito et al. 1999; Hollander et al. 2000; Wei & Lee 2002). Nonetheless, with evidence to suggest ROS accumulation during aging and skeletal muscles high vulnerability to ROS and potential damage (Haycock et al. 1996), it is of great importance to investigate skeletal muscle antioxidant function during aging.

3.1.4 A Role for the NOX Enzymes in Aging Sarcopenia

In addition to OS, aging is also associated with chronic inflammation and many inflammatory diseases often result in protein oxidation and catabolism (Mosoni et al. 2004). Pro-inflammatory cytokines, such as TNF-α and IL-6 have demonstrated a stimulatory effect on NOX enzyme activation, therefore suggesting an inflammatory mediated oxidative signaling mechanism (Frey et al. 2002; Gauss et al. 2007). Cytokine binding to plasma membrane receptors triggers Rac-mediated NOX enzyme activation and O_2^-/H_2O_2 generation (Rhee 2006). It is therefore possible that the increase in OS, associated with aging, is a consequence of cytokine-dependent NOX enzyme activation. Another important regulatory factor, proposed to be involved in this complex pathway (Gauss et al. 2007) and in skeletal muscle growth pathways (Cuthbertson et al. 2005), is transcription factor NF-κB. The role of ROS in NF-κB activation (Brar et al. 2002; Clark & Valente 2004; Gauss et al. 2007) further supports this potential pathway of muscle wasting during aging. Due to the relationship between aging, inflammation and OS, it is possible that the cycle of generating ROS from cytokine-induced NOX enzyme activity is potentially activated in aging skeletal muscle.

Alterations in oxidative balance has been proposed as a major contributor to aging and age-associated disease (Oudot et al. 2006; Sundaram & Panneerselvam 2006; Wei & Lee 2002), and evidence suggests its potential involvement in the destruction of skeletal muscle proteins, characteristic of sarcopenia (Cakatay et al. 2003; Fulle et al. 2004). However, the key contributors to the imbalance in cellular oxidative state and subsequent consequences in the cell during aging, remain undefined. There is however evidence to suggest that the NOX enzyme systems in skeletal muscle are likely sources of ROS that contribute to the severe wasting of skeletal muscle, during aging. Therefore, this study aims to investigate the ROS generating NOX and antioxidant enzyme systems in aging skeletal muscle. In particular, this study aims to determine changes in the important NOX enzyme subunits; NOX2, NOX4, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac1, and O_2^- levels, as well as the primary antioxidants; SOD1, SOD2, SOD3, catalase and GPx, for the conversion of O_2^- and subsequent H_2O_2, in skeletal muscle during aging. Further to these important cellular systems, we aim to investigate the expression of the redox-sensitive transcription factor NF-kB, proposed to play a key role in muscle growth pathways.

3.2 Research Methods

3.2.1 Animal Model of Aging Sarcopenia

All animal procedures carried out in this study were approved by the Victoria University AEEC (AEETH 14/05). Female BALB/c mice were purchased from ARC, Western Australia. Mice were purchased at 9 months of age and assigned to one of 3 groups and maintained until 12 (n=18), 18 (n=20) and 24 (n=24) months of age. In addition, 5 month old mice (n=16) were purchased and maintained for one month (6 months of age) before tissue collection. Mice were maintained under controlled environmental conditions; 12 hour light/dark cycle, 21 ± 2°C, 30% humidity, in conventional cages with *ad libitum* access to standard chow and water throughout the course of the study. Mice were monitored daily and any changes in body weight, physical appearance, behaviour, activity or signs of illness were recorded. Mice suffering from illness or pathologies on dissection were eliminated from the study. Data from these recordings were used to calculate animal survival rates. At 6, 12, 18 or 24 months of age, mice were anaesthetised using pentobarbital sodium (70mg/kg) and skeletal muscle tissue was collected, weighed and immediately snap frozen in liquid nitrogen and stored at -80ºC for later use.

3.2.2 Reverse Transcription-Real-Time PCR

RNA was extracted from frozen quadriceps muscle using Tri Reagent (Molecular Research Centre, USA), according to the manufacturer's protocol. Total RNA concentration was determined spectrophotometrically at 260nm. Prior to RT all RNA samples were DNase treated (Promega, Australia) and then first-strand cDNA was

generated from 1µg RNA using AMV RT (Promega, Australia). The cDNA was stored at -20°C for subsequent analysis. Pre-designed TaqMan Gene Expression Assays (Applied Biosystems, USA) were used containing specific primers and probes for the genes of interest (Table 2.2). Real-time PCR was performed using Applied Biosystems 7500 detection system and PCR reactions were performed using TaqMan Gene Expression Master Mix (Applied Biosystems, USA). Briefly a real-time PCR mix of 50% TaqMan Gene Expression Master Mix (Applied Biosystems, USA) and 0.5% TaqMan Gene Expression Assay Mix (20x), and cDNA, optimised to specific Gene Expression Assays, was run for 40 cycles of PCR in a total volume of 25µl. To compensate for variations in input RNA amounts and efficiency of reverse transcription, 18s ribosomal mRNA was quantified and all results were normalised to these values. Fluorescent emission data was captured and mRNA levels were analysed using the C_T value (Schmittgen et al. 2000). The $2^{\Delta}C_T$ was calculated by subtracting the ${}^{\Delta}C_T$ for 18s ribosomal mRNA from the ${}^{\Delta}C_T$ for the gene of interest in the control and experimental samples. The relative expression of the gene of interest was calculated using the expression $2^{\Delta}C_T$ and reported as arbitrary units.

3.2.3 Superoxide Dismutase (SOD) Activity Assay

Frozen muscle aliquots (approximately 100mg) were placed in ice-cold HEPES buffer (20mM) containing; EGTA (1mM), mannitol (210mM), and sucrose (70mM) and adjusted to a pH of 7.2 (10ml/g). Muscle aliquots were homogenised in buffer, using a glass on glass homogeniser, and centrifuged at 1,500xg for 5 minutes at 4ºC, to remove insoluble connective tissue. To separate the cytosolic and mitochondrial fractions, the supernatant was centrifuged at 10,000xg for 15 minutes at 4ºC and the resulting supernatant, containing the cytosolic fraction, was collected for SOD1

enzyme. The remaining pellet containing the mitochondrial fraction was resuspended and homogenised in ice-cold HEPES buffer for SOD2 enzyme analysis. SOD1 and SOD2 activity was measured in cytosolic and mitochondrial muscle fractions respectively by spectrophotometric assay, with the use of a commercially available kit (706002 Superoxide Dismutase Assay Kit; Cayman Scientific). The amount of activity was calculated and standardised for protein using the Bradford method (Bio-Rad, USA).

3.2.4 Detection of O_2^- by DHE Fluorescence Staining

Skeletal muscle O_2^- was measured using O_2^- sensitive DHE dye. As a reliable method for NOX activity in skeletal muscle is yet to be established, we determined O_2^- by the popular DHE method, through histological examination. DHE is cell permeable and reacts with O_2^- converting DHE into ethidium fluorescence (Serrander et al. 2007). DHE (5μm) was applied to gastrocnemius cryosections (5-10μm) and incubated in a light protected oven at 37°C for 30 minutes. The DHE was washed from the sections with PBS and fluorescence was assessed using fluorescence microscopy. Each section was photographed in three sections to obtain the whole tissue section. The density of ethidium fluorescence detection of O_2^- from the whole section was measured with MCID imaging software (Imaging Research Inc) and expressed as arbitrary units of fluorescence (Azumi et al. 2002; Miller et al. 2002; Williams & Allen 2007).

3.2.5 Statistical Analysis

Statistical analysis was performed using SPSS statistical package (version 15.0). Results are expressed as mean ± SEM. Differences were determined by one-way ANOVA with Tukey HSD as posthoc and results were considered statistically significant if *p*-values were equal to or <0.05.

3.3 Results

3.3.1 Animal Model of Aging Sarcopenia

Female BALB/c mice were maintained until they reached 6, 12, 18 or 24 months of age. Survival rates were recorded and results indicated a decline in survival from approximately 14 months of age. Animal survival data over the course of the study found a 60% survival rate at age 18 months and a 50% survival rate at 24 months of age (Figure 3.1).

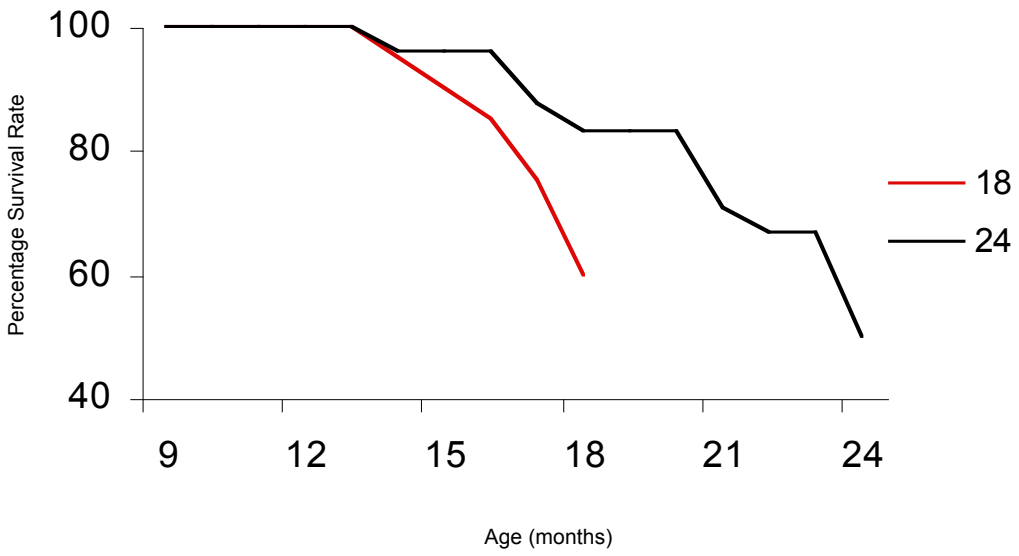


Figure 3.1 Animal survival rates in the 18 and 24 month old age groups, expressed as a percentage of live animals for each group. Female BALB/c animals purchased at 9 months of age were assigned to the 18 month old group (n=20) and 24 month old group (n=24). We show here that animal survival declined from approximately 14 months of age in both groups and continued to decline thereafter. A 60% decline in animal survival was observed in the 18 month old group, while a 50% decline in survival rate was observed the 24 month old group.

Body weight and skeletal muscle weights were recorded at tissue collection and evaluated as a measure of muscle mass in all four age groups. Skeletal muscle weight increased significantly at 12 months compared to 6 months ($p<0.001$) (Figure 3.2). While there were no differences observed in skeletal muscle weight at 18 and 24 months compared to 6 months, a significant decline was observed at 18 ($p<0.001$) and 24 months ($p<0.001$) compared to 12 months (Figure 3.2). In addition, there was no significant difference in skeletal muscle weights between 18 and 24 months of age.

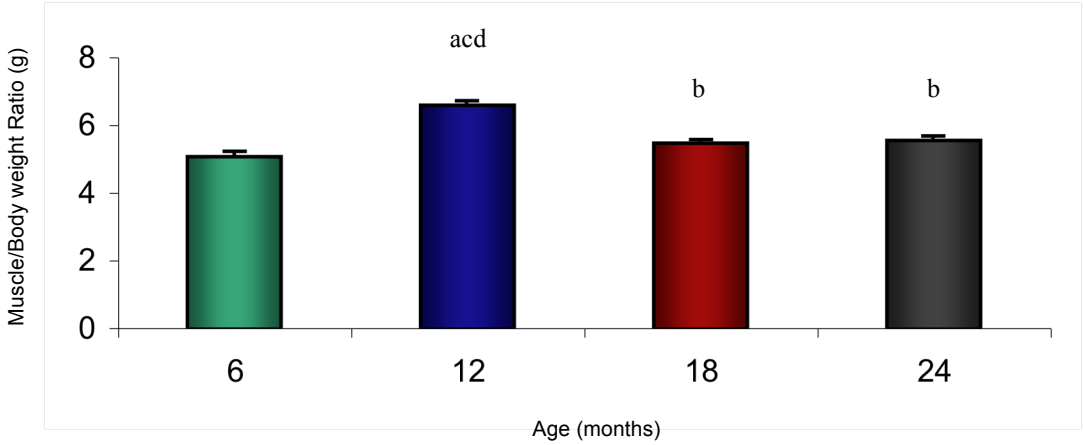


Figure 3.2 Quadriceps muscle weights, as an indicator of age-associated changes in skeletal muscle mass. Muscle/body weight ratio is expressed as a ratio of body weight for each age group. The values represent the mean ± SEM. Statistically significant differences ($P<0.05$) between groups are indicated by lower case letters, where 6m/o = a, 12m/o = b, 18 m/o = c and 24 m/o = d.

3.3.2 Reverse Transcription-Real-Time PCR

3.3.2.1 NOX Subunit Gene Expression

NOX subunit gene expression was measured in quadriceps muscle from the four age groups. Results are expressed as units of gene expression for each of the NOX subunits. Results indicated an increase in the mRNA expression of NOX2 in skeletal muscle at 18 months compared to the younger, 6 (p=0.027) and 12 (p=0.023) months, and the older 24 months (p=0.047) (Figure 3.3). The mRNA expression of NOX4 was also higher in skeletal muscle at 18 months compared to 6 months (p=0.021) (Figure 3.4). Similarly, an increase in the mRNA expression of the cytosolic NOX subunit, $p40^{phox}$ was observed in skeletal muscle at 18 months compared to 6 months (p=0.030) (Figure 3.8). An increase in the mRNA expression of $p67^{phox}$ was also found in skeletal muscle at 24 months, compared to the younger 6 (p=0.007), 12 (p=0.001) and 18 (p=0.032) month old mice (Figure 3.6). No change was observed in the mRNA expression of the additional NOX subunits, $p22^{phox}$ (Figure 3.5) and $p40^{phox}$ (Figure 3.7) or Rac1 (Figure 3.9).

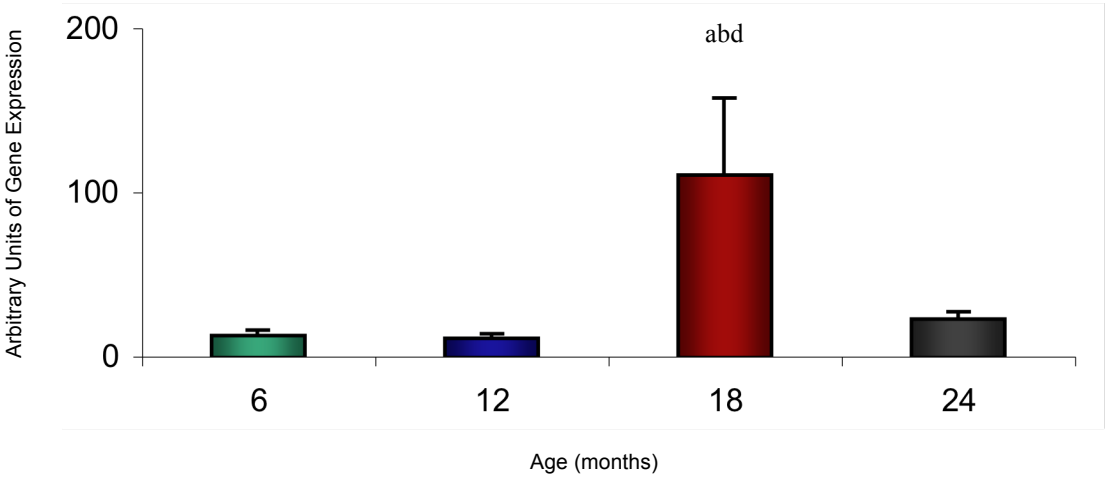


Figure 3.3 The mRNA levels of NOX2 in aging skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters, where 6m/o = a, 12m/o = b, 18 m/o = c and 24 m/o = d.

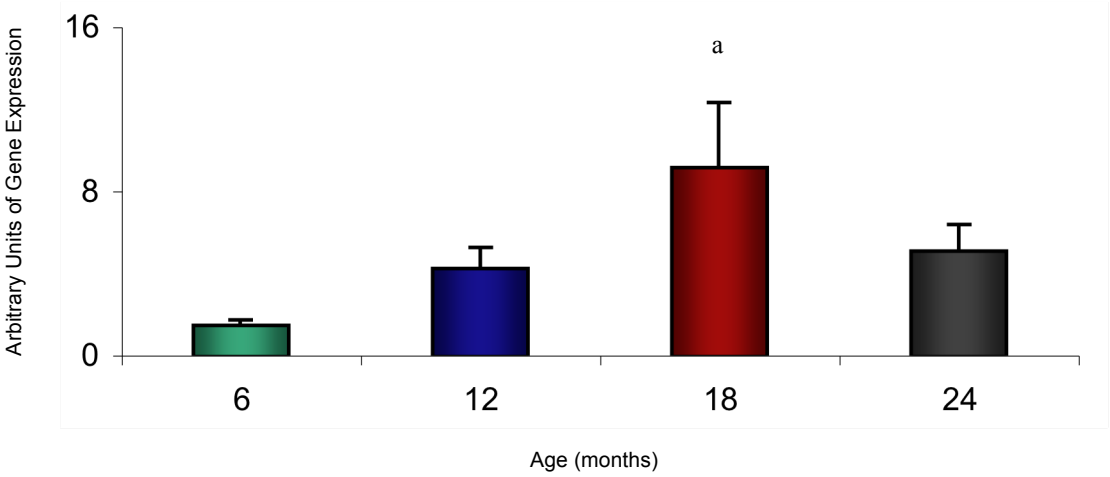


Figure 3.4 The mRNA levels of NOX4 in aging skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters, where 6m/o = a, 12m/o = b, 18 m/o = c and 24 m/o = d.

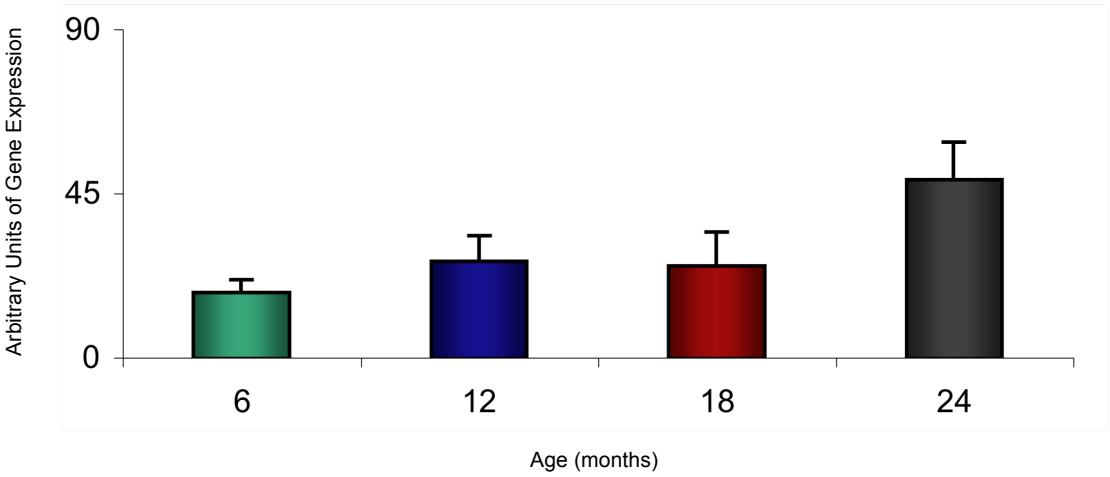


Figure 3.5 The mRNA levels of p22^{phox} in aging skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters, where 6m/o = a, 12m/o = b, 18 m/o = c and 24 m/o = d.

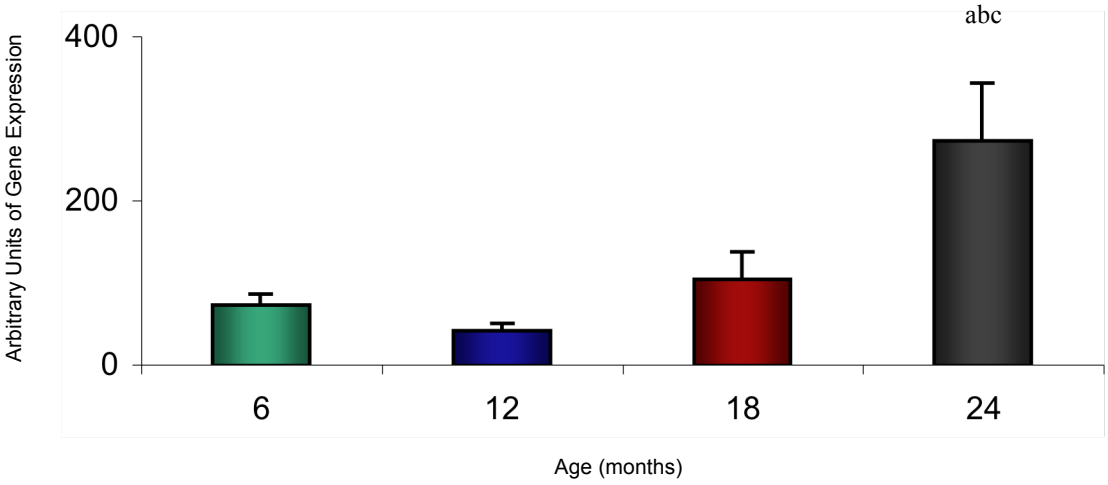


Figure 3.6 The mRNA levels of p67^{phox} in aging skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters, where 6m/o = a, 12m/o = b, 18 m/o = c and 24 m/o = d.

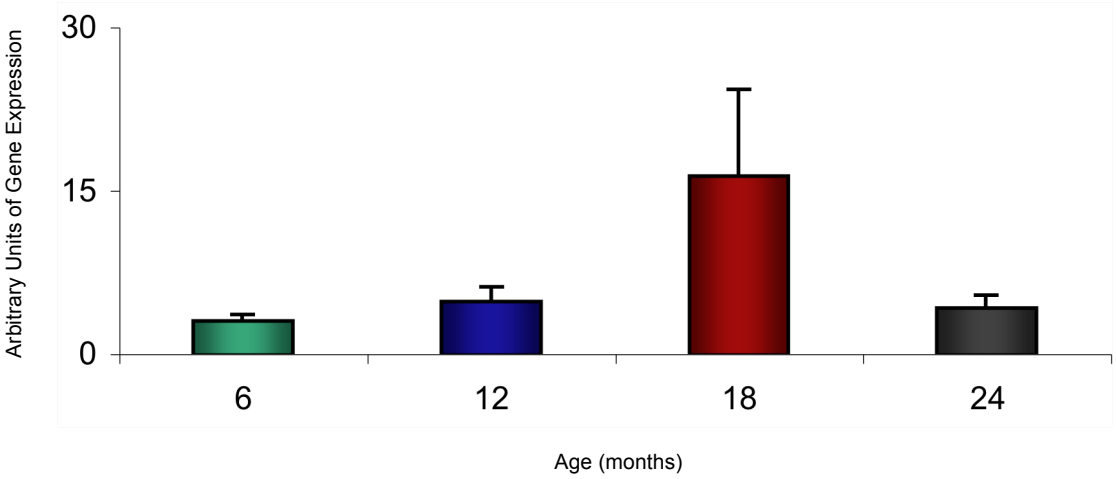


Figure 3.7 The mRNA levels of p47^{phox} in aging skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters, where 6m/o = a, 12m/o = b, 18 m/o = c and 24 m/o = d.

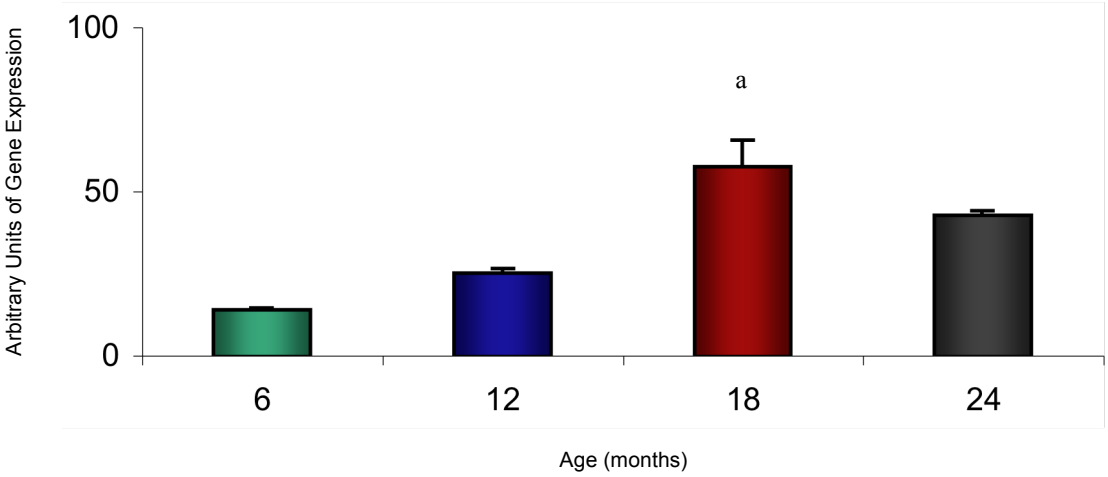


Figure 3.8 The mRNA levels of p40^{phox} in aging skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters, where 6m/o = a, 12m/o = b, 18 m/o = c and 24 m/o = d.

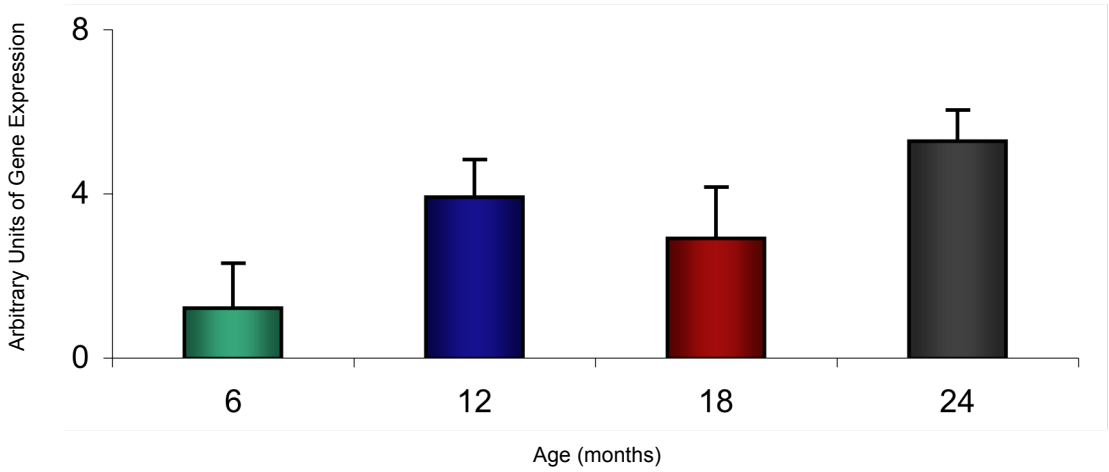


Figure 3.9 The mRNA levels of Rac1

in aging skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences ($P<0.05$) between groups are indicated by lower case letters, where 6m/o = a, 12m/o = b, 18 m/o = c and 24 m/o = d.

3.3.2.2 Antioxidant Enzyme Gene Expression

Changes were observed in the mRNA levels of the antioxidant enzyme SOD isoforms, SOD1 and SOD2. The mRNA expression of the cytosolic SOD1 and mitochondrial SOD2 antioxidant enzymes were significantly higher in skeletal muscle at 24 months compared to the younger 6 (p=0.005) (p=0.016), 12 (p=0.010) (p=0.006) and 18 (p=0.043) (p=0.012) month old mice (Figure 3.10 & 3.11). However, no change was observed in the mRNA expression of the extracellular SOD3 antioxidant enzyme (Figure 3.12). In addition, the mRNA expression of GPx was higher in skeletal muscle at 24 months compared to 6 months (p=0.031) (Figure 3.13). However, no change was observed in the mRNA expression of catalase in skeletal muscle between the age groups (Figure 3.14).

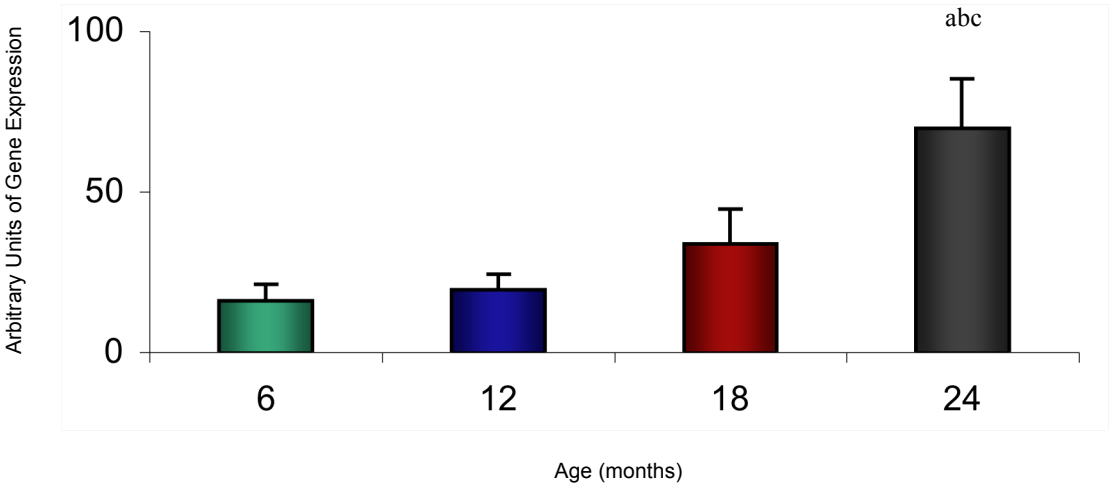


Figure 3.10 The mRNA levels of SOD1 in aging skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters, where 6m/o = a, 12m/o = b, 18 m/o = c and 24 m/o = d.

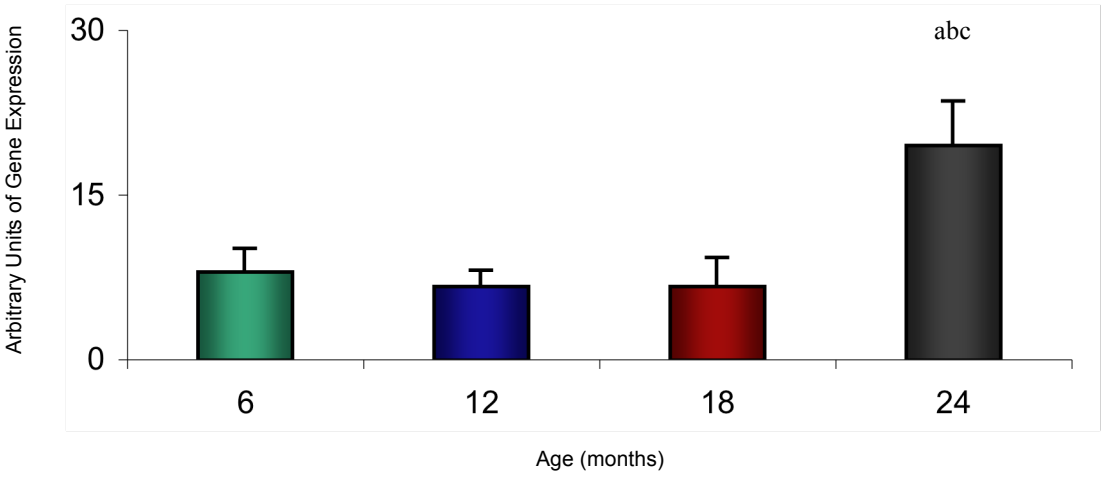


Figure 3.11 The mRNA levels of SOD2 in aging skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters, where 6m/o = a, 12m/o = b, 18 m/o = c and 24 m/o = d.

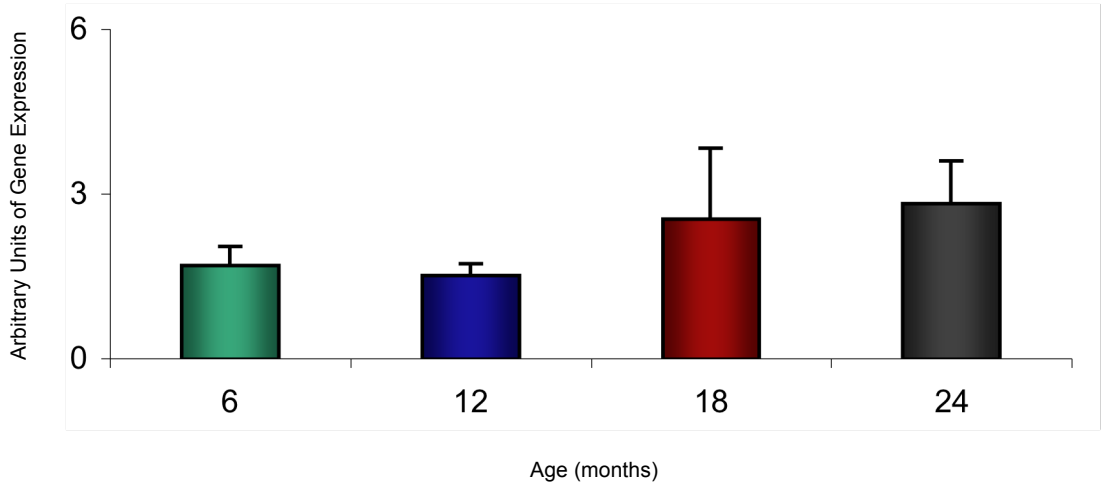


Figure 3.12 The mRNA levels of SOD3 in aging skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters, where 6m/o = a, 12m/o = b, 18 m/o = c and 24 m/o = d.

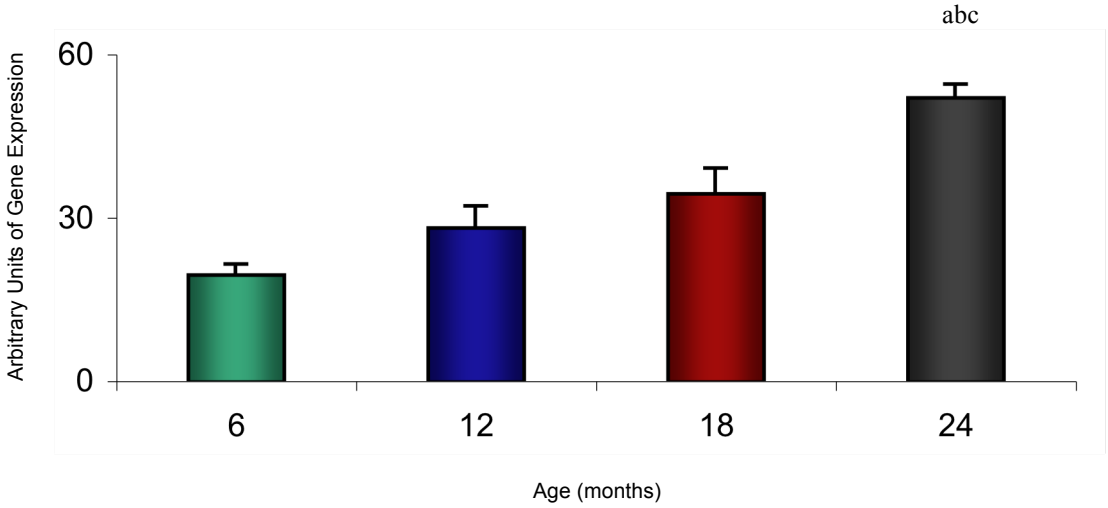


Figure 3.13 The mRNA levels of GPx in aging skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters, where 6m/o = a, 12m/o = b, 18 m/o = c and 24 m/o = d.

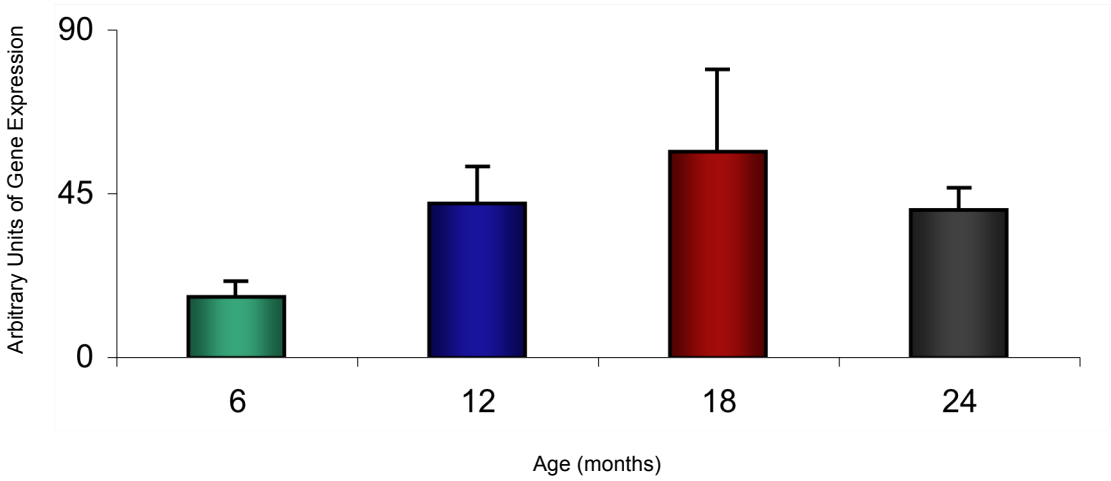


Figure 3.14 The mRNA levels of catalase in aging skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters, where 6m/o = a, 12m/o = b, 18 m/o = c and 24 m/o = d.

3.3.2.3 NF-κB Gene Expression

No change in the gene expression of transcription factor NF-κB was observed in skeletal muscle between the age groups (Figure 3.15).

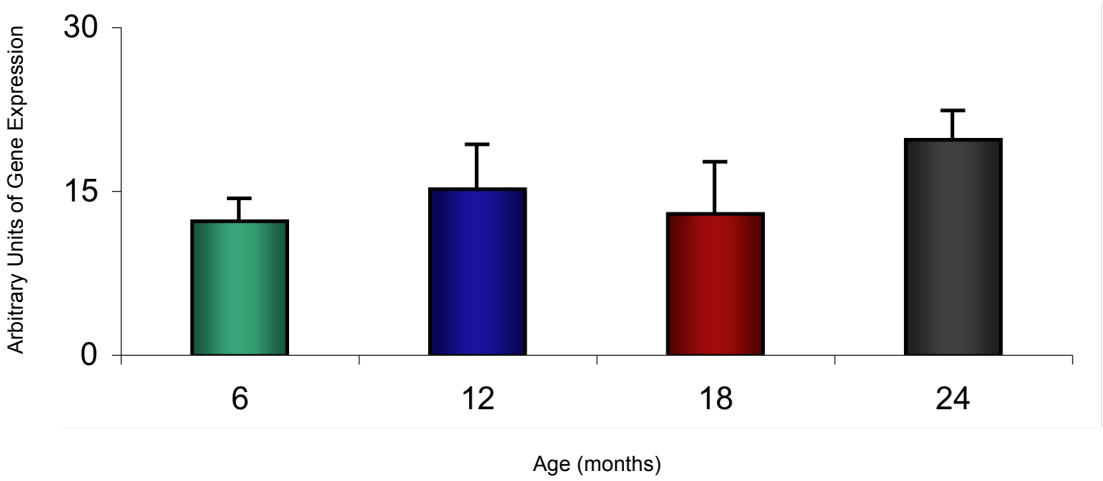


Figure 3.15 The mRNA levels of NF-κB in aging skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences ($P<0.05$) between groups are indicated by lower case letters, where 6m/o = a, 12m/o = b, 18 m/o = c and 24 m/o = d.

3.3.3 Superoxide Dismutase (SOD) Activity Assay

SOD activity was examined in the cytosolic and mitochondrial skeletal muscle fractions as a measure of SOD1 and SOD2 activity. SOD1 activity levels were significantly lower in skeletal muscle at 12 months compared to 6 (p=0.005), 18 (p<0.001) and 24 (p<0.001) months (Figure 3.16). In addition, SOD1 activity levels were elevated in skeletal muscle at 18 months compared to 6 (p<0.001), 12 (p<0.001) and 24 (p=0.011) months (Figure 3.16). Interestingly, a significant decline was observed in SOD2 activity in aging skeletal muscle with significantly less SOD2 activity at 24 months, when compared to the younger 6 (p<0.001) and 12 (p<0.001) month old muscle (Figure 3.17).

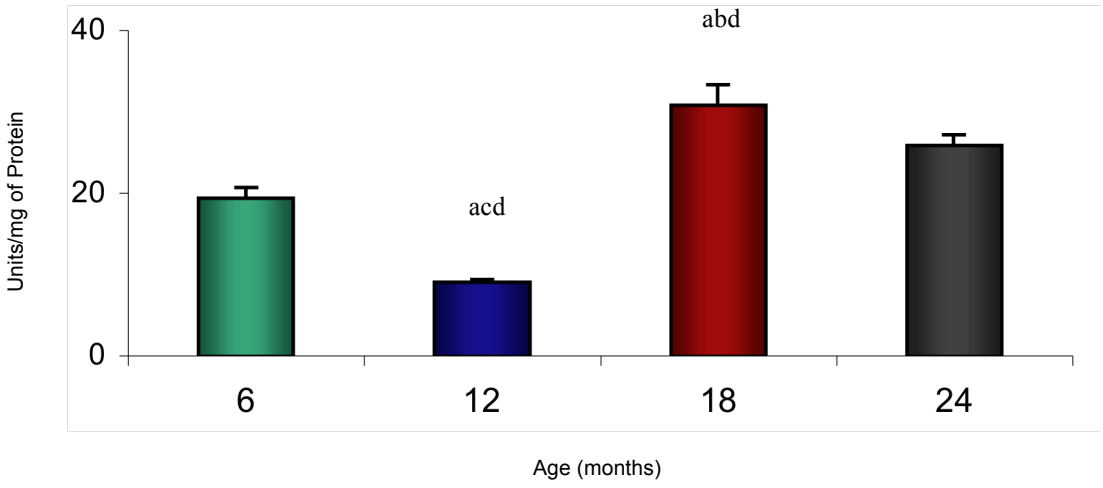


Figure 3.16 SOD1 activity in aging skeletal muscle, expressed as units per mg of protein. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters, where 6m/o = a, 12m/o = b, 18 m/o = c and 24 m/o = d.

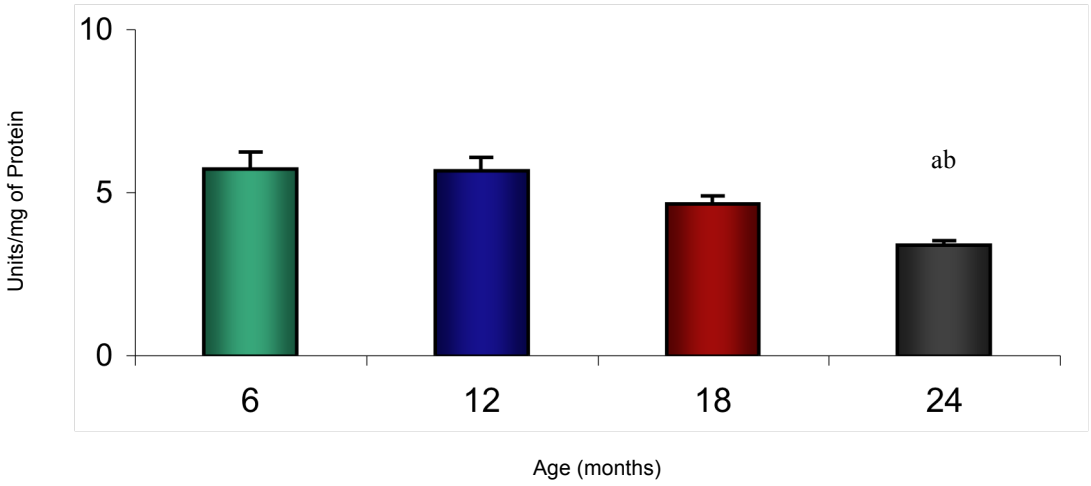


Figure 3.17 SOD2 activity in aging skeletal muscle, expressed as units per mg of protein. The values represent the mean ± SEM. Statistically significant differences ($P<0.05$) between groups are indicated by lower case letters, where 6m/o = a, 12m/o = b, 18 m/o = c and 24 m/o = d.

3.3.4 *Detection of O_2^- by DHE Fluorescence Staining*

To investigate levels of O_2^-, we used *in situ* DHE staining in cryosections of skeletal muscle tissues from each of the four age groups. A marked increase in ethidium fluorescence was found throughout skeletal muscle at 24 months, when compared to 6 (p=0.001) and 12 (p<0.001) months (Figure 3.18 & 3.19).

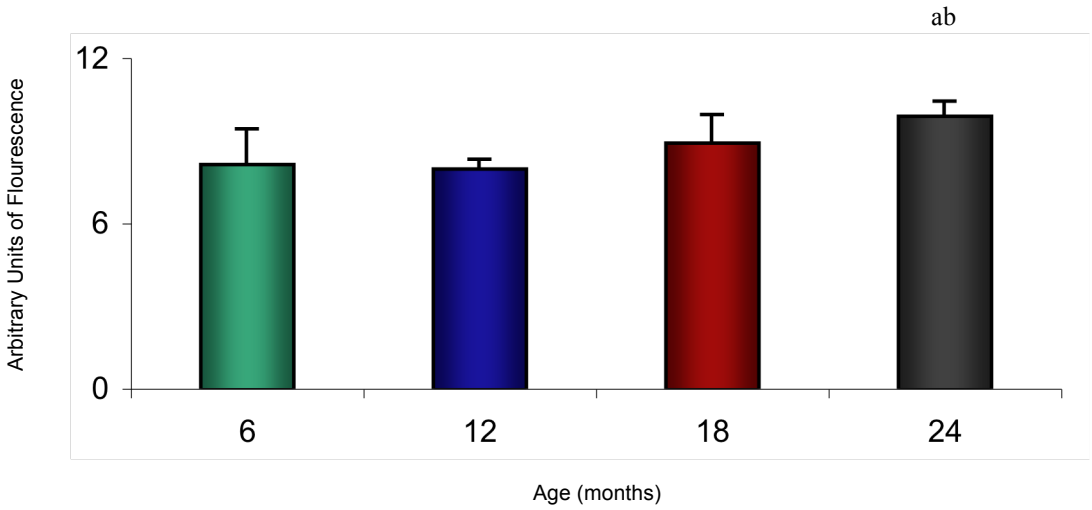


Figure 3.18 O_2^- levels in aging skeletal muscle, by histological DHE examination, expressed as arbitrary units of fluorescence. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters, where 6m/o = a, 12m/o = b, 18 m/o = c and 24 m/o = d.

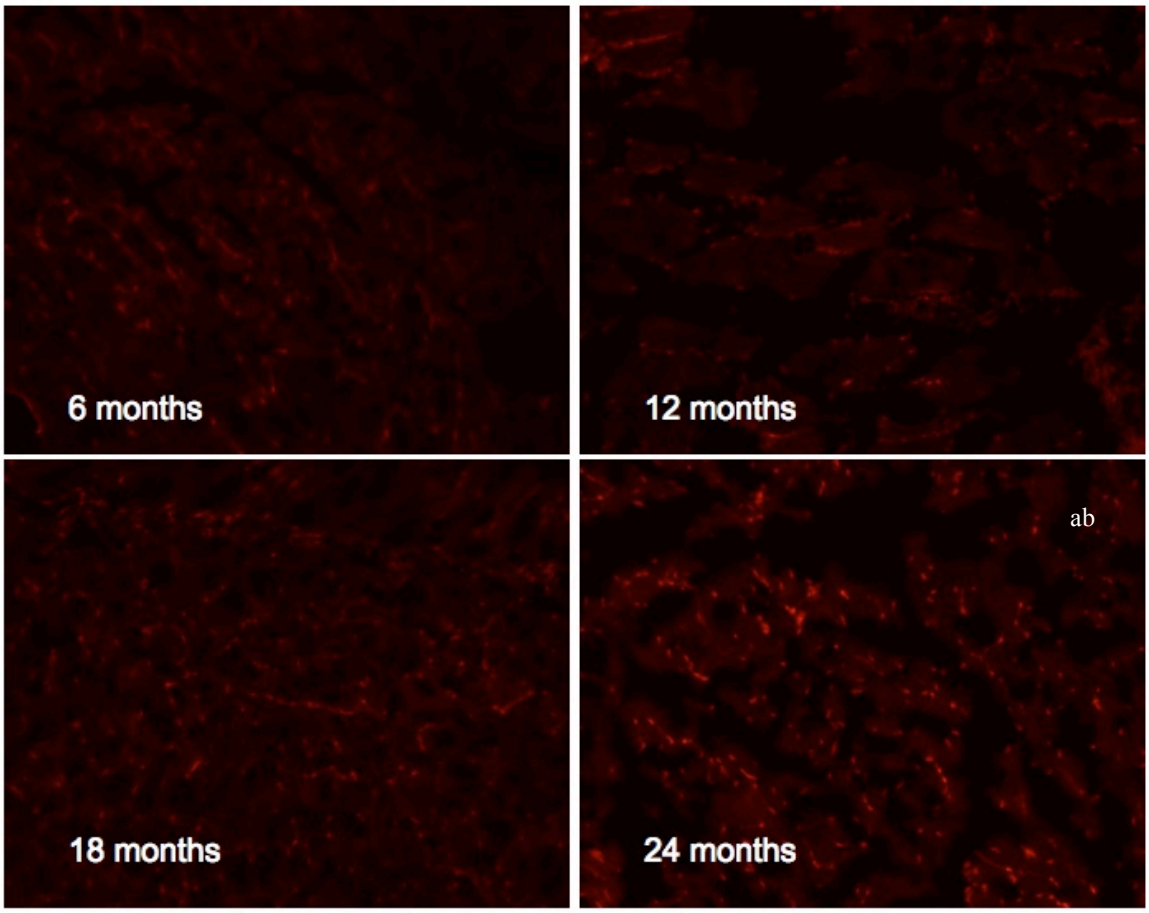


Figure 3.19 Histological DHE fluorescence detection, as a measure of O_2^-, in aging skeletal muscle. Statistically significant differences (P<0.05) between groups are indicated by lower case letters, where 6m/o = a, 12m/o = b, 18 m/o = c and 24 m/o = d.

3.4 Discussion

Sarcopenia is age-associated loss of muscle mass that appears to progressively favour protein degradation over protein synthesis (Roubenoff & Castaneda 2001; Yarasheski 2003). However, the mechanisms involved in the loss of skeletal muscle mass during aging, remains unclear. Aging research has converged on a common theory of free radical damage, focusing on the imbalance of oxidative generation and antioxidant capacity, which ultimately causes a shift in homeostasis. Furthermore, numerous aging studies have demonstrated a general decline in antioxidant compensation and ability to cope with increases in ROS production and overall cellular function, as a common feature of the aging process (Esposito et al. 1999; Hollander et al. 2000; Pansarasa et al. 2002; Sundaram & Panneerselvam 2006; Wei & Lee 2002). However, what remains to be elucidated are the key players involved in initiating and promoting cellular oxidative damage and muscle responses to cope, repair and function to maintain muscle integrity during aging. We investigated skeletal muscle at intervals over a 24 month period, and were therefore able to demonstrate the progression of specific cellular oxidative changes with advancing age.

This was an expansive study that investigated potential ROS generating and antioxidant enzyme systems in skeletal muscle during aging. While skeletal muscle mass increased at 12 months compared to 6 months, we observed a significant decline in skeletal muscle mass at 18 months and beyond, demonstrating an age-associated decline that is indicative of age-associated sarcopenia. While sarcopenia was first evident at 18 months, oxidative systems appear to be well regulated in skeletal muscle at this time. The significant decline in skeletal muscle mass at 18 months, was

matched by a marked increase in the gene expression of NOX enzyme subunits, with no change in O_2^- levels, when compared to the younger 6 and 12 months, that could be explained by the increase in SOD enzyme activity. While sarcopenia was still evident at 24 months, we observed no further decline in skeletal muscle mass. Interestingly, differential changes in the NOX subunits and primary antioxidant profiles were accompanied by a marked increase in O_2^- levels in skeletal muscle at 24 months compared to 18 months.

A significant increase was observed in the catalytic and regulatory NOX enzyme subunits, NOX2 and $p40^{phox}$, as well as NOX4 mRNA in skeletal muscle at 18 months, when compared to the younger 6 and 12 months. Despite the potential for increased NOX enzyme activity in skeletal muscle at 18 months, our results did not demonstrate an increase in O_2^- levels. The increase in SOD1 activity in skeletal muscle at 18 months compared to the other age groups, is not only suggestive of an increase in O_2^- production, but also its ability to actively dismutate O_2^- and avoid accumulation. These results suggest a potentially important role for the NOX2 and NOX4 enzymes in skeletal muscle during aging that appears to be involved in the age-associated muscle loss that we observed at this time. However, the mechanism(s) and to what extent these NOX enzyme systems contribute to the loss of muscle mass during aging is unknown.

Further to this however, we demonstrated a significant increase in the NOX subunit $p67^{phox}$ in skeletal muscle at 24 months compared to the younger 6, 12 and 18 months and a significant increase in levels of skeletal muscle O_2^-, compared to the younger 6 and 12 months. These results suggest an increase in NOX enzyme activity potential

and production of O_2^- at 24 months, compared to 6 and 12 months. Collectively, these results indicate an role for the NOX2 and NOX4 enzymes in aging skeletal muscle, demonstrated by the significant increase in the regulatory and catalytic NOX subunits, at 18 and 24 months, when significant decline in muscle mass was evident. Furthermore, despite the increase in SOD1 and SOD2 gene expression, this study demonstrated a significant decrease in skeletal muscle SOD1 activity at 24 months, compared to 18 months, and a significant decline in skeletal muscle SOD2 activity compared to the younger 6 and 12 months. With the localisation of SOD1 in the cytosol and recent evidence for its presence in the mitochondrial intermembrane space, SOD1 is an important enzyme that regulates the intracellular oxidative environment and cellular homeostasis (Schon & Manfredi 2003; Vives-Bauza, Starkov & Garcia-Arumi 2007). The mitochondrial localisation of SOD2, indicates SOD2 as the primary antioxidant for the dismutation of mitochondrial O_2^- generation. Accumulation of O_2^- in the mitochondria and the cytosol would indeed alter the cellular oxidative environment and contribute to cellular oxidative damage. It is important to note that these important SOD antioxidant enzyme systems regulate the generation of cascading ROS such as H_2O_2 and $OH^{\bullet}$. The decrease in skeletal muscle SOD1 and SOD2 activity at 24 months is indicative of a functional decline in antioxidant protection and a likely contributor to the accumulation of O_2^-, particularly compared to the younger 6 and 12 month old skeletal muscle. Therefore, while the protective antioxidant system appears to successfully maintain skeletal muscle oxidative balance in the younger age groups, our results indicate a prooxidative shift at 24 months, which points towards a decline in antioxidant protection.

This study supports the involvement of the NOX and antioxidant enzyme systems in aging skeletal muscle, however does not appear to be indicative of a role for NOX-generated O_2^- in cumulative oxidative damage to skeletal muscle, that has been associated with sarcopenia. The decline in muscle mass at 18 months is indicative of age-associated sarcopenia, however cellular O_2^- does not appear to be the primary contributor to skeletal muscle damage in this study. The changes in NOX, as a primary generator of O_2^- and instigator of potential cascading ROS, at 18 and 24 months however, does indicate a role for this system in aging skeletal muscle. Therefore, we propose that NOX and antioxidant enzyme systems play an alternative intracellular signaling role, in skeletal muscle during the aging process. Collectively, these results not only suggest differences in cellular oxidative responses with age, but may also give some insight into the complex signaling initiation, promotion and maintenance of skeletal muscle physiology through the involvement of ROS.

3.4.1 Muscle Mass as an Indicator of Aging Sarcopenia

Skeletal muscle is the largest mass of protein in the body and the loss of muscle mass is therefore directly related to protein loss and is recognised as a valuable indicator of protein homeostasis and muscle wasting (Castaneda 2002). We demonstrated a decrease in skeletal muscle mass at 18 and 24 months when compared to 12 months. Our study supports others that have demonstrated significant age-associated muscle loss and important cellular changes in aging skeletal muscle (Attaix et al. 2005; Kimball et al. 2004; Pansarasa et al. 1999). Most significant, Pansarasa et al (1999) suggested that the time of most importance in terms of muscle changes and associated damage is at 65 years of age. In addition, Kimball et al (2004) demonstrated in male

Sprague-Dawley rats, skeletal muscle equilibrium between 6-18 months, with age related decline in skeletal muscle mass at 18 months. Evidence of sarcopenia in these rats was observed from 21-27 months, with a maximum life expectancy of 28 months of age. These studies greatly support the time of significant change to skeletal muscle that we observed in our mouse model, at 18 months of age.

Recent studies have suggested a complex system of cellular changes that lead to the disruption of important growth pathways in aging skeletal muscle that suggest a role for ROS. Toth et al (2005) demonstrated a significant decline in protein synthesis rates in aging skeletal muscle from human subjects (Mean age of approximately 70 years) that was associated with an increase in TNF-α and decrease in Insulin-like Growth Factor-1 (IGF-1). ROS have been shown to be stimulated under pro-inflammatory stimuli such as TNF-α and involved in the suppression of IGF-1, an important factor for muscle protein synthesis or hypertrophy (Stitt et al. 2004). Similarly, Cuthbertson et al (2005) demonstrated a depression in anabolic signalling pathways in skeletal muscle from elderly men suffering significant muscle wasting that was associated with inflammation, ROS-associated damage and a decrease in IGF-1. In addition to these studies, Clavel et al (2006) demonstrated an increase in age-associated proteolysis, as a result of Ub-proteasome pathway induction and a decrease in the IGF-1/Akt signaling pathway for muscle protein synthesis, in skeletal muscle from old rats. These studies are important in understanding the changes that contribute to an imbalance in skeletal muscle protein breakdown and protein synthesis pathways during aging and may therefore be of significance to our study.

Although we demonstrated a significant decline in muscle mass at 18 and 24 months, what was interesting was the maintenance of this decline from 18 to 24 months. Previous studies have demonstrated a compensatory mechanism in skeletal muscle that may occur in response to muscle insult and onset of skeletal muscle wasting. Edstrom et al (2006) recently demonstrated a significant downregulation in atrophy related genes in rat skeletal muscle at 30 months of age when compared to 4 and 12 months. The potential decrease in muscle atrophy at 30 months could result as a compensatory response to earlier muscle loss that this particular study did not investigate. Our study suggests that muscle wasting is evident at 18 months and that muscle mass appears to be maintained thereafter. This maintenance of muscle mass was shown in the aging study by Kimball et al (2004) that while muscle synthesis and muscle growth was positively related during earlier age, changes occurred beyond 21 months, when sarcopenia was first evident. In addition, while muscle mass began to decrease in rats beyond 21 months, markers of protein synthesis were significantly elevated at 24 and 27 months. Our study suggests important cellular responses in skeletal muscle following age-associated loss at 18 months, for the maintenance of muscle integrity at 24 months or regeneration following prior insult. Furthermore, the changes we observed in the NOX enzyme subunits and antioxidant enzymes is suggested to play an important role in muscle physiology during aging.

3.4.2 The Role of the NOX Enzymes in Aging Sarcopenia

We aimed to determine a role for NOX in aging skeletal muscle. This study demonstrated a significant increase in the gene expression of the regulatory and catalytic subunits of the NOX enzyme system in skeletal muscle at 18 months, with an increase in the expression of NOX2, NOX4 and $p40^{phox}$ subunits. Specifically, we

showed a 10 fold increase in the mRNA of the rate limiting NOX2 enzyme subunit NOX2, at 18 months. We also demonstrated an increase in the regulatory NOX2 enzyme subunit $p40^{phox}$, in skeletal muscle at this time. $p40^{phox}$ has been demonstrated as an essential component of the NOX2 enzyme for its regulatory role in initiating the recruitment of the cytosolic subunits to the membrane for enzyme activation (Ellson et al. 2006; Suh et al. 2006). The primary function of NOX enzymes are to generate O_2^-, however the role of this particular ROS in skeletal muscle is yet to be elucidated. Certainly, it is with interest that the potential increase in O_2^-, as a result of the changes in the expression of NOX enzyme subunits, was matched by an increase in SOD1 activity. Such findings, suggest in part a very well regulated response to O_2^- production within skeletal muscle to prevent damage, but also allows speculation as to why such changes in the expression profile of NOX enzyme subunits was evident at this time, and to what extent these changes contributed or were causative to the changes in skeletal muscle mass.

In addition to these changes in NOX subunit gene expression at 18 months, further changes in NOX subunit expression was evident at 24 months, with a significant increase in $p67^{phox}$. $p67^{phox}$ is an important cytosolic component of the NOX2 enzyme for its direct interaction with NOX2 and activation of the enzyme (Takeya & Sumimoto 2003). The increase in this important subunit, for its catalytic function in skeletal muscle, at 24 months is indicative of an increase in NOX2 enzyme activation that may have contributed to the increase in O_2^- levels, observed at this time. Interestingly, these changes in skeletal muscle do not appear to alter skeletal muscle mass, despite the significant loss at both 18 and 24 months. This result is suggestive of additional roles for the O_2^- generating enzyme system in skeletal muscle atrophy

and hypertrophy pathways. Bell et al (2005) demonstrated a novel protective role for NOX generated O_2^- in myocardiac remodeling. Similarly, Looi et al (2008) demonstrated an important role for the NOX2 enzyme in cardiac remodeling with a significant decrease in cardiac hypertrophy in NOX2-/- mice. In addition, Bendall et al (2002) observed marked attenuation of Ang II-induced hypertrophy in cardiac muscle of NOX2-/- mice, suggesting a direct functional role for NOX2 in cardiac hypertrophy. These studies demonstrate an important role for NOX in muscle regeneration. Therefore, NOX enzymes are better described as a system that plays various roles within the cell that may lead to deleterious effects, but may also mediate protective responses in order to maintain muscle integrity in response to damage.

In addition to the changes observed in the NOX2 enzyme, this study demonstrated an increase in skeletal muscle NOX4 mRNA levels at 18 months. This increase in NOX4 suggests a potential role for this NOX enzyme system in aging skeletal muscle. In contrast to the NOX2 enzyme, NOX4 has been suggested to only require $p22^{phox}$ for oxidase activation and generation of O_2^- (Ambasta et al. 2004; Geiszt 2006). It has also been suggested that NOX4 does not reside in the plasma membrane, rather its localisation is within intracellular organelles and generates O_2^- intracellularly (Krause 2007; Martyn et al. 2006). Therefore, the increase in skeletal muscle NOX4 gene expression at 18 months may have contributed to intracellular O_2^- production and oxidative cellular damage, as well as mediating various intracellular signaling pathways, leading to changes in muscle during aging. However, the localisation of NOX4 in skeletal muscle is yet to be defined and therefore its role in skeletal muscle aging remains unknown. It is also important to note that because NOX4 potentially generates O_2^- within subcellular compartments, such as the

endoplasmic reticulum, it has been suggested to be undetectable by DHE (Serrander et al. 2007). Indeed, an increase in NOX4 generated O_2^- production in intracellular organelles has the potential to cause damage to important cellular structures and therefore overall cellular function. While, this study suggests a role for NOX4 in aging skeletal muscle and its potential contribution to the loss of muscle mass, evident at 18 months, it also indicates the importance of further investigation into the role of NOX4 in skeletal muscle and its potential involvement in skeletal muscle aging.

3.4.3 Skeletal Muscle Atrophy in Aging Sarcopenia

It is well known that ROS are mediators of damage to cellular structures, which ultimately compromise cellular function. ROS also act as second messengers in intracellular signaling cascades and are involved in regulating redox-sensitive signaling pathways (Valko et al. 2006). Although we cannot ignore the significant decline in skeletal muscle mass and increase in the regulatory and catalytic NOX2 and NOX4 subunits observed at 18 months, our results suggest NOX enzyme involvement in intracellular signaling pathways in aging skeletal muscle, rather than a contributor to direct cellular oxidative damage. It is well known that with advancing age comes alterations in gene regulation (Gallegly et al. 2004) and this study supports age-associated changes in skeletal muscle oxidative systems that appear to be involved in the consequential changes to skeletal muscle physiology. Skeletal muscle requires two particularly important and complex growth pathways for protein synthesis and protein breakdown in order to ultimately maintain muscle homeostasis (Glass 2005). It is therefore important to consider these pathways, normally in balance with one another, that may become alternatively regulated and eventually favour protein breakdown over protein synthesis, with advancing age (Clavel et al. 2006). However, what is

interesting and remains unknown are the complex mechanisms that lead to the shift to protein breakdown in skeletal muscle during aging, however this study may suggest the involvement of NOX-dependent ROS signaling.

Inflammation is a key contributor to the aging process and is implicated in many age-associated conditions (Csiszar et al. 2008; Salles et al. 2005; Ungvari et al. 2007; Visser et al. 2002). In particular, pro-inflammatory cytokines play a key role in various muscle wasting conditions (Argiles, Busquets & Lopez-Soriano 2005; Reid & Li 2001b) including sarcopenia (Visser et al. 2002). A study by Welle et al (2003) suggested age-associated activation of inflammatory pathways, via gene expression profiles, in aged human skeletal muscle. Further to this, Langen et al (2006) demonstrated significant skeletal muscle atrophy and impaired muscle regenerative capacity in a TNF-α amplified transgenic model of Chronic Obstructive Pulmonary Disease (COPD). Similarly, Visser et al (2002) determined a significantly higher level of plasma IL-6 and TNF-α cytokines, associated with lower muscle mass, in well functioning healthy older men and women. NOX enzymes have been shown to respond to several pro-inflammatory cytokines, inducing ROS and often cellular OS (Li & Shah 2003; Moe et al. 2006; Ungvari et al. 2007) that leads us to speculate about its role in this environment. Interestingly, Yoshida and Tsunawaki (2008) reported that vascular NOX2 and NOX4 enzymes, induced by TNF-α, were responsible for enhanced ROS and endothelial dysfunction. Similarly, Moe et al (2006) demonstrated NOX4 involvement in increased O_2^- production in vascular smooth muscle, under inflammatory conditions, suggesting a role for the NOX4 enzyme in cardiovascular disease. Furthermore, there is evidence to suggest that

cytokines, commonly elevated during aging, are involved in skeletal muscle atrophy, mediated by H_2O_2 signaling (Supinski & Callahan 2007).

Langen et al (2006) suggested that TNF-α-induced muscle atrophy and suppression of muscle regenerative capacity, may be possible through NF-κB activation. Transcription factor NF-κB has been shown to regulate the expression of a number of key components of skeletal muscle atrophy (Cai et al. 2004b; Guttridge 2004). Clark and Valente (2004) suggested an important role for NOX-dependent ROS signaling in cytokine-induced NF-κB activation and gene regulation, as a key contributor to the age-associated degenerative changes in tissue. In particular, NF-κB has been shown to induce the expression of skeletal muscle atrophy related genes, Muscle Ring Finger-1 (MuRF-1) and Atrogin-1 (Cai et al. 2004b), which mediate ubiquitination of myofibrillar proteins and subsequent degradation (de Palma et al. 2008). Interestingly, Hunter and Kadarian (2004) demonstrated a decrease in muscle atrophy in NF-κB1 knockout mice following skeletal muscle unloading, supporting the involvement of NF-κB in skeletal muscle atrophy related pathways. Furthermore, Clavel et al (2006) found a marked upregulation of genes involved in the Ub-proteasome pathway, MuRF1 and Atrogin-1 in the skeletal muscle of aged rats. This study also observed a decrease in the IGF-1/Akt signaling, a key regulatory pathway for muscle hypertrophy (Appendix 2), and elevated levels of TNF-α in aged rat skeletal muscle, which is suggestive of the potential involvement of these cellular signaling pathways in the development of sarcopenia. Further to this, evidence suggests that NOX enzymes are key regulators of NF-κB activation, through redox-sensitive signaling pathways (Clark & Valente 2004; Moe et al. 2006; Ungvari et al. 2007) (Appendix 2). Csiszar et al (2008) demonstrated an increase in inflammatory cytokines; IL-6 and

TNF-α during aging, which was suggested to induce downstream NOX enzyme and subsequent ROS stimulated NF-κB activation in endothelial cells. In addition, Piao et al (2005) suggested NOX2 stimulates muscle differentiation, downstream of P13K/p38MAPK pathway, by activating NF-κB via ROS signaling (Appendix 2). Collectively, these studies give evidence for an important signaling role for NOX-dependent ROS in NF-κB-dependent gene regulation, downstream of inflammatory cytokines. The increase in inflammatory cytokines, common in aging, and the role of NF-κB in the regulation of important skeletal muscle growth pathways, makes this NOX-dependent ROS signaling pathway significant in aging skeletal muscle. While we did not observe any changes in NF-κB gene expression in skeletal muscle between the age groups, further investigation into this transcription factor, for its role in skeletal muscle growth pathways, is of great interest in aging skeletal muscle.

This study demonstrated an increase in NOX enzyme subunits at 18 months that is suggestive of a potential increase in NOX activation and O_2^- production. While there were no changes in skeletal muscle O_2^- levels at 18 months, an increase in SOD1 activity was observed, indicative of an increase in the need for O_2^- protection, which we can speculate may be generated by NOX. Further to this, these results suggest the successful conversion of O_2^- to H_2O_2. Therefore, while SOD1 is keeping O_2^- in check, eliminating potential for O_2^- accumulation, increased amounts of H_2O_2 could be consequently generated, suggesting an increase in H_2O_2 signaling potential, in skeletal muscle at 18 months that may be the key contributor to the loss of muscle mass. In further support of this, we did not demonstrate compensatory changes in the gene expression of the antioxidant enzymes, catalase and GPx, for the conversion of excess H_2O_2. H_2O_2 in particular, has been shown to stimulate NF-κB activation

(Kamata et al. 2002; Ungvari et al. 2007), and therefore NOX-dependent O_2^- production and active dismutation by SOD, could be involved in the downstream H_2O_2/NF-κB signaling, shown to influence atrophy related gene expression (Cai et al. 2004b; Hunter & Kandarian 2004). Furthermore, this pathway could activate downstream of elevated pro-inflammatory cytokines (Csiszar et al. 2008; Supinski & Callahan 2007; True, Rahman & Malik 2000) and lead to the loss of muscle mass observed at 18 months. We can only speculate from our current knowledge of the potential upstream signaling regulators of NOX enzymes that initiate potential downstream activation of atrophy related processes that may have lead to the changes observed in skeletal muscle during aging.

3.4.4 Oxidative Stress (OS) in Aging Sarcopenia

Aging in general is associated with an increase in OS with a steady rise over the lifespan (Sundaram & Panneerselvam 2006). A study by Pansarasa et al (1999) suggested a correlation between ROS activity and age related changes in human skeletal muscle, while a similar study by Mecocci et al (1999) demonstrated age dependent oxidative damage to DNA, lipids and protein in human skeletal muscle that was associated with a significant loss of muscle mass. The beginning of significant changes to muscle physiology in our model of aging was observed at 18 months, with a significant loss of muscle mass and increase in the expression of NOX enzyme subunits, however this was not matched by an increase in O_2^- production that can be explained by the increase in SOD1 activity at this time. Further to this however, we cannot ignore the potential increase in H_2O_2 that can result from increased O_2^- dismutation. While this result is interesting in itself, we demonstrated a marked

increase in O_2^- levels in skeletal muscle at 24 months. OS develops from an accumulation of ROS such as O_2^- that was once thought to primarily contribute to oxidative damage in cells and tissues. However, it is now recognised that ROS, particularly O_2^- and H_2O_2, and the systems that generate them, are involved in complex signaling pathways, necessary for normal cellular function (Valko et al. 2007). ROS act as central mediators in redox-sensitive signalling pathways and therefore, when produced inappropriately, can significantly alter cellular function, which can indeed have crucial effects (Hancock, Desikan & Neill 2001; Rhee 2006). OS can cause severe damage by altering various types of gene expression and therefore significant alterations to normal cellular function (Bedard & Krause 2007; Navarro, Lopez-Cepero & del Pino 2001). Increases in cellular ROS can be a product of increased metabolic and oxidase activity, but can also result from a depression in antioxidant defences (Macdonald, Galley & Webster 2003; Scandalios 2005). The increase in NOX subunit expression in skeletal muscle, observed at 24 months, would appear to contribute to the increase in O_2^- levels. In addition to this however, we observed a significant decrease in SOD1 and SOD2 activity, despite an increase in their gene expression. The decrease in active SOD1 and SOD2 function would indeed contribute to the pro-oxidative shift that we observed in skeletal muscle at 24 months. The inability of the antioxidant system to compensate for a rise in ROS, or even normal ROS production, has been shown during aging (Esposito et al. 1999; Hollander et al. 2000; Pansarasa et al. 2002; Sundaram & Panneerselvam 2006) and our results further support this. Therefore this study demonstrates the complexity of oxidative changes in skeletal muscle during aging and points towards a dysfunction in antioxidant capacity, following significant muscle degradation.

3.4.5 Antioxidant Enzymes in Aging Sarcopenia

Antioxidant enzymes are valuable indicators of ROS production as well as changes in cellular redox signaling and evidence of cell functionality (Ji 2007; Landis & Tower 2005; Liu 2006). This study is indicative of a functional O_2^- dismutation at 18 months that appears to decline at 24 months. Furthermore, the increase in skeletal muscle SOD1 activity, observed at 18 months, suggests the response to an increase in O_2^- production that may be NOX-dependent, and our results further suggest successful elimination of O_2^- to avoid accumulation. However, the successful dismutation of O_2^- indicates the generation of H_2O_2 that may contribute to oxidative damage, but is also likely to contribute to complex redox-sensitive signaling (Rhee 2006; Stone & Yang 2006) in skeletal muscle growth pathways that contribute to the loss of muscle mass (Mansouri et al. 2006). Further to this, despite the increase in skeletal muscle O_2^-, observed at 24 months, and the increase in SOD1 and SOD2 gene expression, we observed a functional decline in SOD1 and SOD2 antioxidant enzymes. The function of these antioxidants is primarily for the protection against the potentially harmful effects of O_2^-, but are also important regulators of cellular redox-sensitive signaling pathways (Landis & Tower 2005). The decrease in SOD1 and SOD2 activity at 24 months is significant for skeletal muscle physiology, as we found an increase in O_2^-. In contrast with skeletal muscle at 18 months, these results suggest a decrease in H_2O_2 at 24 months, which also demonstrates the importance of antioxidant enzymes as regulators of cellular ROS. Furthermore, of particular interest was the preservation of muscle mass at 24 months, despite the increase in O_2^-, when compared to 18 months. This study indeed questions the role of NOX enzymes and O_2^- in aging skeletal muscle and the role of antioxidant enzymes.

The importance of these antioxidant enzymes in aging became particularly evident with the development of the SOD knockout animal models. Muller et al (2006) demonstrated an age-dependent loss of muscle mass in mice lacking SOD1 as well as a significant decrease in their average lifespan. However, the effect of mice lacking SOD2 is much more severe, with a significant increase in oxidative damage and shortened lifespan (Melov et al. 1999). Furthermore, Sun et al (2002) investigated the effects of SOD1 and SOD2 overexpression in Drosphila that demonstrated a decrease in cumulative oxidative damage and increased metabolic potential, with an increase in lifespan of up to 37% and 75% respectively (Sun et al. 2002). Further to this however, a model of SOD1 overexpression shows significant decline in lifespan with severe neurodegeneration and associated skeletal muscle wasting (Harraz et al. 2008). These altered SOD models demonstrate a crucial role for SOD in survival and oxidative-damage and are therefore of great importance during aging.

This study demonstrated a functional decline in skeletal muscle SOD1 and SOD2 at 24 months, when compared to 18 months. SOD2 is the only known scavanger of O_2^- in the mitochondrial matrix and is therefore a vital protector of the mitochondria from oxidative damage (Mansouri et al. 2006). If the antioxidant defense system is insufficient in detoxifying ROS from the mitochondria, an increase in mitochondrial DNA damage can result (Esposito et al. 1999). It is well documented that the mitochondria undergo considerable damage over the lifespan that significantly compromises function (Conley, Marcinek & Villarin 2007; Kent-Braun & Ng 2000; Mecocci et al. 1999). In response to this, studies have shown an increase in SOD2 mRNA levels and activity, reflecting increases in mitochondrial O_2^- and therefore the potential for oxidative damage (Judge et al., 2005; Gianni et al., 2004). Interestingly,

a contrasting study by Rasmussen et al (2003) did not demonstrate any changes in respiratory chain and ATP synthesis activity or the presence of mitochondrial oxidative damage in aged skeletal muscle. Similarly, Gianni et al (2004) demonstrated skeletal muscle oxidative damage, with no reduction in ETC activity. The increase in skeletal muscle SOD2 expression, observed at 24 months, is indicative of a need for O_2^- dismutation that is further supported by the increase in skeletal muscle O_2^-. Furthermore, the decrease in SOD2 activity and increase in O_2^- levels in skeletal muscle at this time appears to indicate a dysfunction in SOD2, despite potential signaling responses. Interestingly though, the dysfunctional SOD2 in skeletal muscle at 24 months, does not appear to contribute to age-associated muscle wasting or atrophy, but would certainly alter redox-sensitive signaling within the cellular system.

In addition to the changes observed in the SOD enzymes in skeletal muscle at 24 months, this study demonstrated a significant increase in GPx expression, suggestive of an increase in H_2O_2 production and potential scavenging as was verified by an increase at the time (Brigelius-Flohe 2006; Vives-Bauza, Starkov & Garcia-Arumi 2007). With the increase in SOD1 and SOD2 expression at 24 months, it is not surprising that GPx expression is also increased. H_2O_2 is an important signaling molecule that is implicated in a number of important growth regulating pathways (Rhee 2006; Stone & Yang 2006), however with the decrease in active SOD1 and SOD2 O_2^- dismutation at 24 months, it would be expected that the production of H_2O_2 would also decrease. In contrast, no changes were observed in the gene expression of the antioxidant enzymes catalase and GPx, for the dismutation of skeletal muscle H_2O_2, at 18 months. Collectively, these results indicate once again, the differential cellular changes in skeletal muscle during aging that appear to converge on the

production of H_2O_2, through changes in SOD, that would indeed have important consequences in complex redox-sensitive signaling pathways.

3.4.6 Skeletal Muscle Hypertrophy in Aging Sarcopenia

In addition to investigating muscle atrophy pathways in the loss of skeletal muscle, it is of equal importance to consider the pathways of muscle hypertrophy (Glass 2005; Latres et al. 2005). Interestingly, aging skeletal muscle has also been associated with a reduced capacity for hypertrophy (Blough & Linderman 2000; Haddad & Adams 2006; Parkington et al. 2004; Welle et al. 2002). IGF-1 promotes cell growth via the downstream activation of Akt/P13K hypertrophy pathway (Costelli et al. 2005; Lai et al. 2004; Stitt et al. 2004) (Appendix 2). Akt stimulates the expression or activation of key regulatory proteins such as Mammalian Target Of Rapamycin (mTOR) and 70-kDa ribosomal protein S6 kinase (p70S6K) involved in translation and protein synthesis (Lai et al. 2004; Stitt et al. 2004) (Appendix 2). A study by Welle et al (2002) in normal aging skeletal muscle revealed a significant decrease in IGF-1 gene expression and demonstrated a 43% lower serum IGF-1 concentration in aged humans (67-75 yr old) in a further study (Welle et al. 2003). Haddad and Adams (2006) demonstrated reduced muscle hypertrophy related signaling via components downstream of IGF-1 receptors, in 30 month old rats, while Parkington et al (2004), demonstrated a decrease in hypertrophy related protein kinases; mTOR and p70S6K in 30 month old rats compared to 6 months, following short bout contractile activity, suggestive of a reduced capacity for hypertrophy in aged animals. Further to these studies, Blough and Linderman (2000) demonstrated a significant loss of muscle mass as well as the capacity to undergo hypertrophy, in response to mechanical load, in 36 month old rats. Similarly, Funai et al (2006) demonstrated age-associated attenuation

of the anabolic response to muscle stimulation that may contribute to the limited capacity of hypertrophy in aged mice. The significance of IGF-1 in skeletal muscle is that it is an important regulator of many downstream growth pathways, and these studies suggest a decrease in this important growth factor that may also be involved in the changes that were observed in our aging muscle.

A decline in skeletal muscles ability to undergo hypertrophy has been associated with a heightened immune response. A study by Toth et al (2005) demonstrated a significant decrease in muscle protein synthesis during aging and increased circulating concentrations of several markers of immune activation. Further to this, Cuthbertson et al (2005) found an increase in cytokine levels and NF-κB expression in elderly muscle that appeared to be related to the inhibition of hypertrophy related protein kinases, such as mTOR and p70S6K. This particular study gave important evidence for an inflammatory mediated inhibition of the major mTOR/p70S6K skeletal muscle hypertrophy pathway during aging, via NF-κB activation. Further to this, NOX enzymes have been found to be stimulated under inflammatory conditions, and evidence for NOX-dependent ROS activation of NF-κB exists. It is therefore important to consider the possibility of inflammatory mediated NOX/ROS/NF-κB activation in aging skeletal muscle that may act through the suppression of hypertrophy, rather than an increase in atrophy, or may be a result of both processes. Furthermore, this study has recognised the importance of further research to understand these complex systems and their contribution to cellular changes in the development of sarcopenia.

The increase in NOX-dependent O_2^- generation, as a signaling molecule involved in atrophy and/or the suppression of hypertrophy in the development of sarcopenia, at 18 months, has been discussed. However, the pattern of NOX and antioxidant enzyme systems in skeletal muscle at 24 months, revealed interesting and differential changes that may have resulted in response to prior skeletal muscle insult. In addition to the changes in NOX enzyme subunits and antioxidant systems, this study demonstrated a significant increase in O_2^-, with no further loss in muscle mass, compared to 18 months. While the differential changes in NOX enzyme subunits are interesting in themselves, the decrease in skeletal muscle SOD1 and SOD2 activity at 24 months is indicative of a decrease in H_2O_2 production, despite the increase in GPx expression. Collectively, this study is suggestive of differences in H_2O_2 signaling in aging skeletal muscle that may explain the progressive degenerative nature of this age-associated condition of sarcopenia. That is, while an increase in H_2O_2 signaling may be involved in skeletal muscle atrophy at 18 months, the decrease in H_2O_2 signaling may result in skeletal muscle maintenance or potential balance in atrophy and hypertrophy pathways, thus maintaining muscle homeostasis. In particular, it is suggested, from the current knowledge of H_2O_2 signaling, that an increase in H_2O_2 may stimulate NF-κB activation and atrophy related gene expression, or the suppression of hypertrophy related pathways at 18 months, contributing to the loss of muscle mass. Therefore, it is possible that the decrease in H_2O_2 signaling potential in skeletal muscle at 24 months, may be a regulated process for the suppression of skeletal muscle atrophy or hypertrophy/regeneration pathways.

The role of IGF-1 in muscle growth pathways has been mentioned in this study and we have eluded to its involvement in redox-sensitive pathways in aging skeletal muscle that may contribute to the loss of skeletal muscle mass at 18 months. It is also important to note that IGF-1 plays a crucial role in muscle homeostasis and regeneration, promoting cell proliferation and differentiation, in hypertrophic processes and repair (Costelli et al. 2006; Musaro et al. 2001). This was demonstrated by Musaro et al (2001) with a transgenic model of active IGF-1 that induces functional myocyte hypertrophy. Interestingly, this model appeared to escape age-related muscle atrophy suggesting an important role for IGF-1 in the preservation of muscle and skeletal muscle regenerative capacity. Furthermore, Reynolds et al (2004) reported an increase in the hypertrophy related Akt/mTOR signaling that attenuated age-related decline in protein synthesis. Furthermore, Lai et al (2004) demonstrated an increase in muscle mass of up to 3 fold greater, in Akt transgenic mice Akt(Tg) that express a dramatic increase in Akt protein. Interestingly, the suggested downstream activation of p70S6K protein kinase was also shown to be activated, giving further evidence for the Akt/mTOR/p70S6K important hypertrophy pathway.

In addition, Stitt et al (2004) found that activation of the IGF-1/P13K/Akt pathway induces hypertrophy, but can also suppresses atrophy. Specifically, this pathway has been suggested to block the expression of atrophy related Atrogin-1 and MuRF-1, through the suppression of Forkhead Box O (FOXO) transcription factors (Stitt et al. 2004) (Appendix 2). Interestingly, Edstrom et al (2006) demonstrated a significant decrease in Atrogin-1 and MuRF-1 gene expression in skeletal muscle of old rats (30months), and suggested IGF-1/Akt mediated inactivation of FOXO4 was involved in the suppression of these atrophy related genes. Furthermore, the activation of this

pathway has been shown to induce hypertrophy of regenerating muscle (Stitt et al. 2004) and may therefore be activated in skeletal muscle at 24 months. Further to this, Abid et al (2007) demonstrated Akt and p38 MAPK activation, via NOX-derived ROS signaling in endothelial cells, therefore suggesting a role for NOX generated ROS in muscle hypertrophy. In addition, Mofarrahi et al (2008) demonstrated that inhibition of NOX2 and NOX4 attenuates the P13K/Akt/NF-κB hypertrophy pathway in skeletal muscle precursor cells, further demonstrating the importance of the NOX enzyme systems in promoting muscle hypertrophy. Therefore, evidence suggests a central mediating role for NOX-dependent O_2^- generation, not only in promoting skeletal muscle atrophy and suppression of hypertrophy, but also appears to play an important role in promoting skeletal muscle hypertrophy and/or suppression of atrophy, for regeneration. This central mediating role for NOX in skeletal muscle growth pathways could explain the progressive degenerative response in aging sarcopenia that has also been demonstrated in this study. It is evident however from these studies that further research of this system is required to better understand these complex pathways.

3.4.7 Conclusion

This study further supports age-associated loss of skeletal muscle mass, indicative of sarcopenia, and the involvement of NOX enzymes in this system during aging. The increase in the NOX2 and NOX4 enzyme systems, observed at 18 months when skeletal muscle loss was first established, indicates the potential contribution of these O_2^- generating systems in sarcopenia. While oxidative responses seem regulated well enough at 18 months by SOD enzymes, the consequential production of H_2O_2 may be the key factor to the loss of skeletal muscle mass at this time. Further to this, the

potential increase in H_2O_2 by SOD-dependent O_2^- conversion may indicate important signaling involvement in pathways of skeletal muscle atrophy, at 18 months. However, the maintenance of skeletal muscle at 24 months that demonstrated differential changes in the NOX2 enzyme, questions the role of NOX in this system. In addition, the potential dysfunction or lack of compensation by the antioxidant systems that most likely lead to the accumulation of O_2^-, may also be NOX-dependent. While this increase in O_2^- has potential for harm in the tissue, we cannot ignore the potential contribution that these changes would have in redox-sensitive intracellular signaling pathways that may also result from a decrease in H_2O_2 signaling, at 24 months. This comparative change in H_2O_2 signaling, in skeletal muscle at 18 and 24 months, despite no further loss of skeletal muscle mass, is indicative of NOX involvement in skeletal muscle atrophy and hypertrophy pathways. Therefore, while the changes in NOX and antioxidant enzyme systems appear to contribute to muscle atrophy at 18 months, the differential changes at 24 months are suggestive of a compensatory response in the skeletal muscle atrophy/hypertrophy processes, in response to insult, in order to maintain muscle integrity. However, despite evidence that suggest redox-sensitive NF-κB involvement in muscle wasting, the pathways of skeletal muscle atrophy and hypertrophy, at least in this study, appear to be independent of transcription factor NF-κB, but indeed suggests further investigation into the role of this redox-sensitive transcription factor, in sarcopenia.

Chapter 4 Cancer-Induced Cachexia Study 1

4.1 Introduction

Although cachexia develops in a number of pathological conditions including Cardiovascular Disease (CVD) (Filippatos, Anker & Kremastinos 2005), AIDS (Baronzio et al. 1999) and COPD (Farber & Mannix 2000), the loss of muscle mass presented in cancer patients has been reported to develop at a faster rate than any known cachectic condition (Giordano et al. 2003). The progressive loss of lean body tissue is characterised by a significant decrease in muscle mass that develops as a result of an imbalance in protein synthesis and protein degradation (Giordano et al. 2003; Hasselgren & Fischer 2001; Lecker et al. 2004; Tisdale 2001). Indeed, it is estimated that up to 50% of cancer patients develop skeletal muscle cachexia, suffering a significant loss of up to 30% of their original body weight (Tijerina 2004; Tisdale 2005). While cachexia develops in a wide range of pathologies, the cause of this severe muscle wasting condition, in response to several pathological conditions, remains relatively undefined. Interestingly, research has shown that weight loss associated with cancer-induced cachexia is not accounted for by a decrease in dietary intake, but rather a specific inflammatory catabolic response (Busquets et al. 2005; Diffee et al. 2002; Monitto et al. 2001). Cachexia has been suggested to involve proinflammatory cytokines and various proteolytic pathways, shown to alter muscle protein metabolism, favouring protein degeneration over protein synthesis (Argiles, Moore-Carrasco, Busquets et al. 2003; Diffee et al. 2002). Most of the accelerated loss of protein has been attributed to Ub-proteasome pathway activation (Lecker et al.

2004; Smith & Tisdale 2003; van Royen et al. 2000; Whitehouse & Tisdale 2002). However, in both human and animal studies of cancer-induced cachexia, evidence suggests the presence of oxidized proteins (Gomes-Marcondes et al. 2003), suggestive of a role for ROS and oxidative damage to skeletal muscle proteins.

4.1.1 Oxidative Stress (OS) in Cancer-Induced Cachexia

OS may play a potentially important role in the development of cancer-induced cachexia (Moylan & Reid 2007). The high metabolic nature of skeletal muscle, due to its high energy demand, makes it particularly vulnerable to protein catabolism (Giordano et al. 2003; Smith & Reid 2006). ROS, including O_2^-, H_2O_2 and $OH^\bullet$ are catabolic factors that are generated in response to metabolic activity (Gomes-Marcondes & Tisdale 2002; Whitehouse et al. 2001), however an increase in ROS that exceeds normal tissue levels can lead to uncontrollable accumulation and OS. Further to this, ROS within cells are recognised as important second messengers in intracellular signaling pathways (Valko et al. 2007) that can ultimately alter various types of gene expression and therefore cellular functioning (Bedard & Krause 2007). ROS such as O_2^- and H_2O_2, mediate important physiological responses including cell proliferation, differentiation and migration (Hancock, Desikan & Neill 2001; Scandalios 2005), and may therefore be directly involved in the catabolic response to cancer or in cellular signaling, influencing important growth pathways in skeletal muscle.

4.1.2 A Role for the NOX Enzymes in Cancer-Induced Cachexia

Indeed, OS has been implicated in progressive tissue damage in a number of tissues such a, cardiac muscle (Li et al. 2002), kidney (Geiszt et al. 2000), spinal cord tissue (Vaziri et al. 2004), vascular smooth muscle (Drummond et al. 2003), and skeletal muscle (Avanzo et al. 2001; Javesghani et al. 2002; Mansouri et al. 2006), and experimental evidence suggests a role for O_2^-, generated by NOX enzymes, in the damage of these tissues. Experimental evidence has already established a role for NOX generated O_2^- in the development of cardiovascular dysfunction and disease (Bengtsson et al. 2003; Filippatos, Anker & Kremastinos 2005; Griendling, Sorescu & Ushio-Fukai 2000; Grieve et al. 2006). Equally in skeletal muscle, NOX enzymes generate O_2^- under normal rest conditions that can rise significantly in response to physical activity and disease (Bejma & Ji 1999; Ji, Gomez-Cabrera & Vina 2006; Li & Shah, A. 2003). Thus, it was apparent that NOX enzymes may have a role in pathological conditions related to skeletal muscle.

NOX enzymes were the first identified enzyme systems to generate O_2^- not as a by-product, but as a primary enzyme function (Bedard & Krause 2007). The expression of NOX subunits in skeletal muscle has been recognised (Cheng et al. 2001; Hidalgo et al. 2006; Mofarrahi et al. 2008), with the expression of both NOX2 and NOX4 (Cheng et al. 2001; Mofarrahi et al. 2008) and associated NOX subunits (Javesghani et al. 2002), although its function in this particular cell system remains undefined. NOX enzymes have been suggested to generate O_2^- in skeletal muscle tissue as a result of cellular metabolism, through studies that show elevated levels of NOX activity and O_2^- production during physical activity (Bejma & Ji 1999). However, evidence for the role of NOX generated O_2^- in interactive signaling pathways that

mediate cellular processes including cell growth and proliferation, is of interest in this system (Brar et al. 2002; Mofarrahi et al. 2008; Petry et al. 2006). The abundance of evidence for NOX generated ROS in disease, further suggests its potential role as the primary oxidative system in the catabolic condition of cancer-induced cachexia.

4.1.3 Antioxidant Enzymes in Cancer-Induced Cachexia

ROS have also been implicated in cancer-induced cachexia through antioxidant-induced attenuation (Mantovani et al. 2004; Mantovani et al. 2003). Antioxidants are important enzymes for the protection against harmful ROS (Xia et al. 2003) and these studies further suggest a role for ROS in cancer-induced cachexia. The role of SOD is important for its initiation of the cascade of generating ROS from O_2^- dismutation to H_2O_2, and therefore regulates GPx and catalase scavenging (Cejudo-Marin et al. 2004; Zelko, Mariani & Folz 2002). It is important to note however, that O_2^- and H_2O_2 can give rise to the highly reactive $OH^\bullet$ (Landis & Tower 2005; Scandalios 2005). Consequently, an overproduction of ROS can lead to oxidative imbalance, which can overwhelm the cell causing oxidative damage and alterations in cellular redox-sensitive signaling (Blokhina, Virolainen & Fagerstedt 2003; Ji 2007). In addition, a dysfunction in endogenous antioxidant systems have been shown, particularly in response to disease (Barreiro et al. 2005; Mayne 2003). Therefore, due to the importance of tight regulation from endogenous antioxidants, to avoid ROS accumulation and oxidative damage, as well as alterations in cellular redox signaling (Valko et al. 2006), it is important to investigate these primary antioxidant enzyme systems in cancer-induced cachexia.

The aim of this preliminary study was to determine whether the NOX2 enzyme system, known to be expressed in skeletal muscle, plays a role in the development of cancer-induced cachexia, for its potential contribution to tissue OS. Therefore, this study sought to determine changes in gene expression profiles of the NOX2 enzyme subunits; NOX2, $p22^{phox}$, $p67^{phox}$, $p47^{phox}$ and $p40^{phox}$, and the primary O_2^- dismutating antioxidants, SOD1, SOD2 and SOD3, as well as total SOD activity, in the quadriceps muscle of mice bearing the MAC16-induced cachexia.

4.2 Methods

4.2.1 Animal Model of Cancer-Induced Cachexia

Mice were obtained from the inbred Aston University colony of female NMRI mice fed *ad libitum* with standard chow and water. Fragments of the MAC16 tumour maintained in mice within the colony, that established weight loss, were implanted subcutaneously into the flank as previously described (Beck & Tisdale 1987). Body weight was recorded in the MAC16-induced mice and weight matched controls. Tumour size was measured in the MAC16-induced mice daily by means of callipers. Cachectic weight loss was evident 9-12 days post implantation and mice were sacrificed at day 25 at varying degrees of weight loss. Quadriceps muscles were collected from MAC16-induced and control mice and immediately snap frozen in liquid nitrogen and stored at -80ºC for later use.

4.2.2 Reverse Transcription-Real-Time PCR

RNA was extracted from frozen quadriceps muscle using Tri Reagent (Molecular Research Centre) according to the manufacturer's protocol. Total RNA concentration was determined spectrophotometrically at 260/280nm. Prior to RT all RNA samples were DNase treated (Promega, Australia) and then first-strand cDNA was generated from 1µg RNA using AMV RT (Promega, Australia). The cDNA was stored at -20°C for subsequent analysis. Primer sequences used were the same as those used previously (Furukawa et al. 2004; Park et al. 2005) (Table 2.1). Real-time PCR was performed using Roche Light Cycler detection system with PCR reactions performed using LightCycler FastStart DNA Master SYBR Green I kit (Roche, USA). Briefly, a

real-time PCR mix of 10× LightCycler FastStart Enzyme and Reaction Mix SYBR Green I, forward and reverse primers (3μM), $MgCl_2$ concentration optimised for each primer pair (1μM – 5μM) and cDNA (12ng) was run for 40 cycles of PCR in a total volume of 20μl. To compensate for variations in input RNA amounts and efficiency of reverse transcription, 18s mRNA was quantified and all results were normalised to these values. Fluorescent emission data were captured and mRNA levels were analysed using the critical threshold (C_T) value (Schmittgen et al. 2000). The $2^{\Delta}C_T$ was calculated by subtracting the ${}^{\Delta}C_T$ for 18s ribosomal mRNA from the ${}^{\Delta}C_T$ for the gene of interest and divided by the ${}^{\Delta}C_T$ of a control sample in every PCR run, to control for any run to run variation. The relative expression of the gene of interest was calculated using the expression $2^{\Delta}C_T$ and reported as arbitrary units.

4.2.3 Superoxide Dismutase (SOD) Activity Assay

Frozen muscle aliquots (100mg) were placed in 10ml of ice-cold potassium phosphate buffer (100mM) with BSA (pH 7.4). Muscle aliquots in buffer were homogenised using a glass on glass homogeniser and centrifuged at 400xg for 10 minutes at 4ºC, to remove insoluble connective tissue and the supernatant was collected for SOD analysis. SOD activity was measured by spectrophotometric assay based on epinephrine autoxidation, described previously (Sun & Zigman 1978). Muscle homogenates were added to cuvettes containing epinephrine (30mM, pH 1.3) and $NaHCO_3$ buffer (pH 10.2) with EDTA and the change in absorbance at 320nm was measured over 3 minutes at 25ºC. The amount of activity was calculated and standardised for protein using the Bradford method (Bio-Rad).

4.2.4 Statistical Analysis

Statistical analysis was performed using SPSS statistical package (version 15.0). Results are expressed as mean ± SEM. Differences were determined by independent t-test and results were considered statistically significant if *p*-values were equal to or <0.05.

4.3 Results

4.3.1 Reverse Transcription-Real-Time PCR

4.3.1.1 NOX Subunit Gene Expression

NOX2 enzyme subunit gene expression was measured from the quadriceps muscle of the MAC16 model of cancer-induced cachexia and compared with the non-cachectic controls. Results are expressed as units of gene expression for each of the subunits of the NOX2 enzyme system. Increases in mRNA expression of the cytosolic subunits, $p67^{phox}$ (p=0.025) and $p40^{phox}$ (p=0.043) were observed in cachectic skeletal muscle compared to controls (Figure 4.3 & 4.5). The remaining subunits of the NOX2 enzyme, $p47^{phox}$ (Figure 4.4) and $p22^{phox}$ (Figure 4.2) showed no change in expression, whereas NOX2 mRNA expression was lower in cachectic skeletal muscle compared to controls (p=0.046) (Figure 4.1).

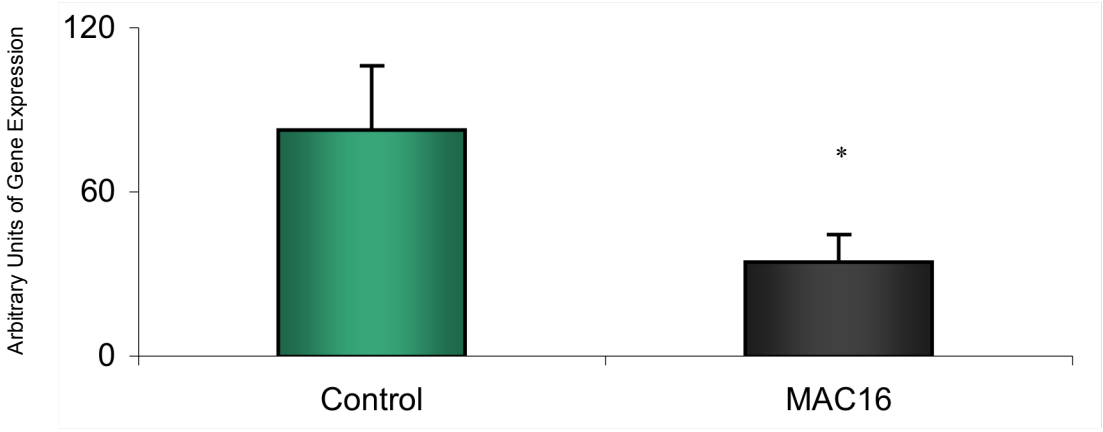


Figure 4.1 The mRNA levels of NOX2 in cachectic (MAC16) and control skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by *.

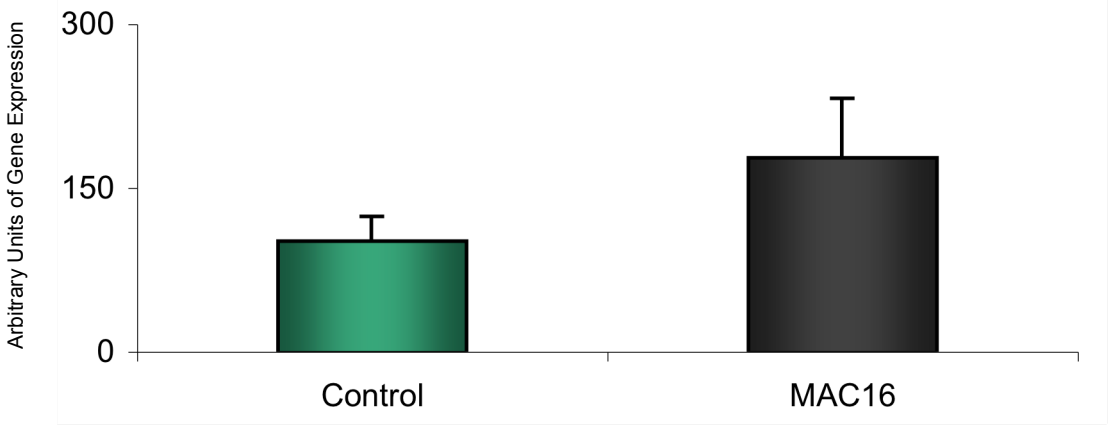


Figure 4.2 The mRNA levels of $p22^{phox}$ in cachectic (MAC16) and control skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by *.

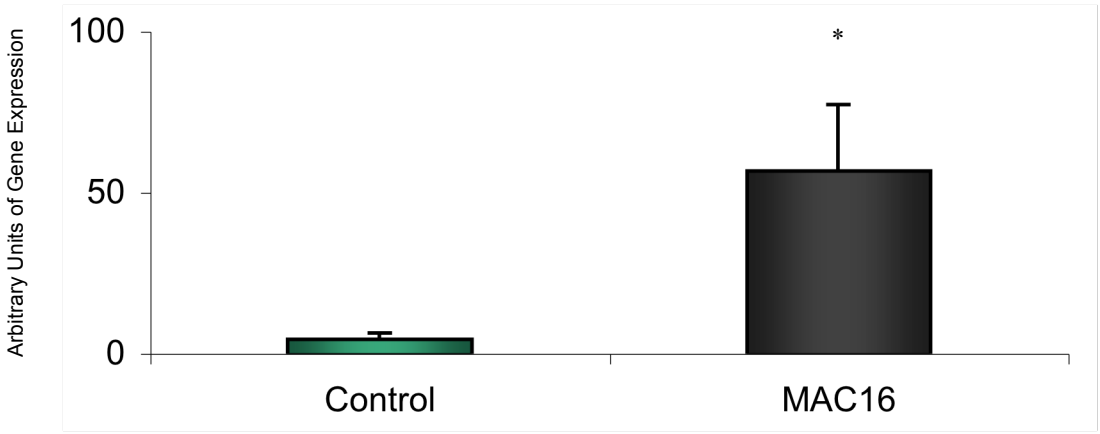


Figure 4.3 The mRNA levels of p67^{phox} in cachectic (MAC16) and control skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by *.

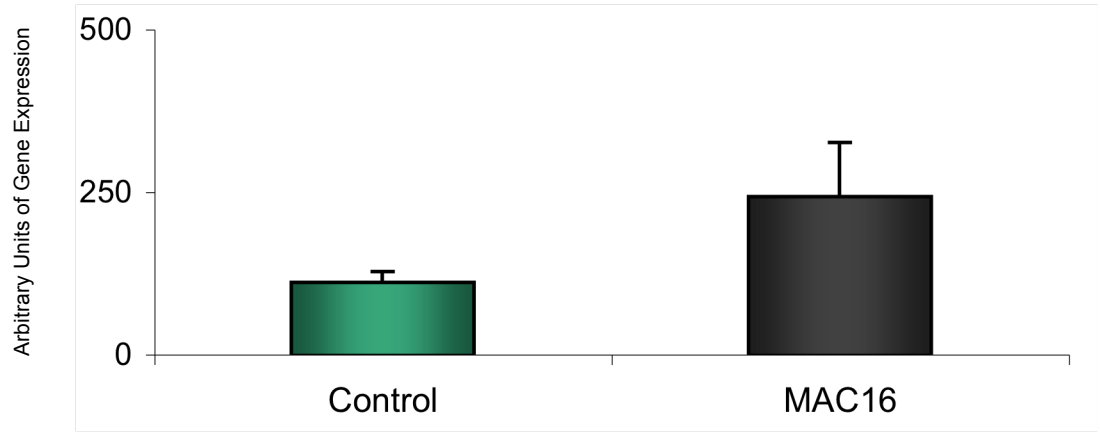


Figure 4.4 The mRNA levels of p47^{phox} in cachectic (MAC16) and control skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by *.

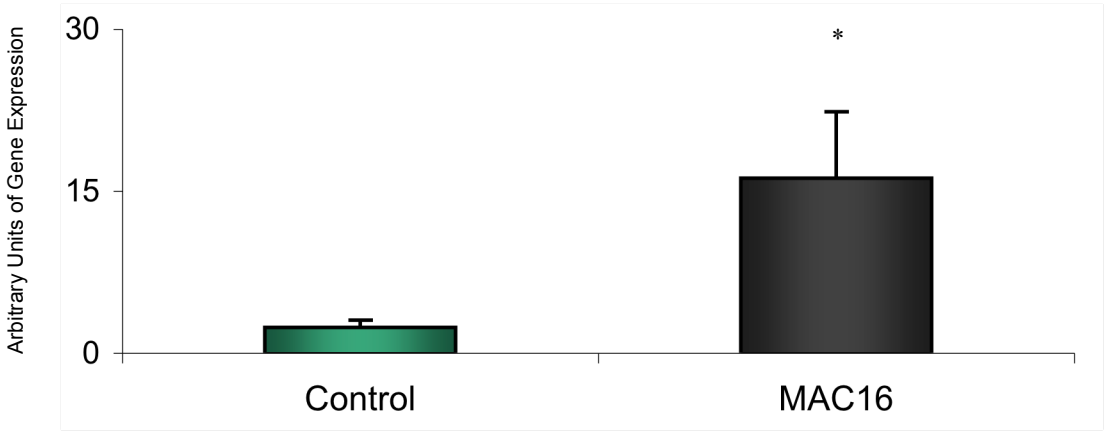


Figure 4.5 The mRNA levels of p40^{phox} in cachectic (MAC16) and control skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences ($P<0.05$) between groups are indicated by *.

4.3.1.2 Superoxide Dismutase (SOD) Gene Expression

The mRNA expression of SOD1 was higher in cachectic skeletal muscle compared to controls (p=0.005) (Figure 4.6). However, no change was observed in the mRNA expression of the SOD isoforms, SOD2 and SOD3 in skeletal muscle from the cachectic and control mice (Figure 4.7 & 4.8).

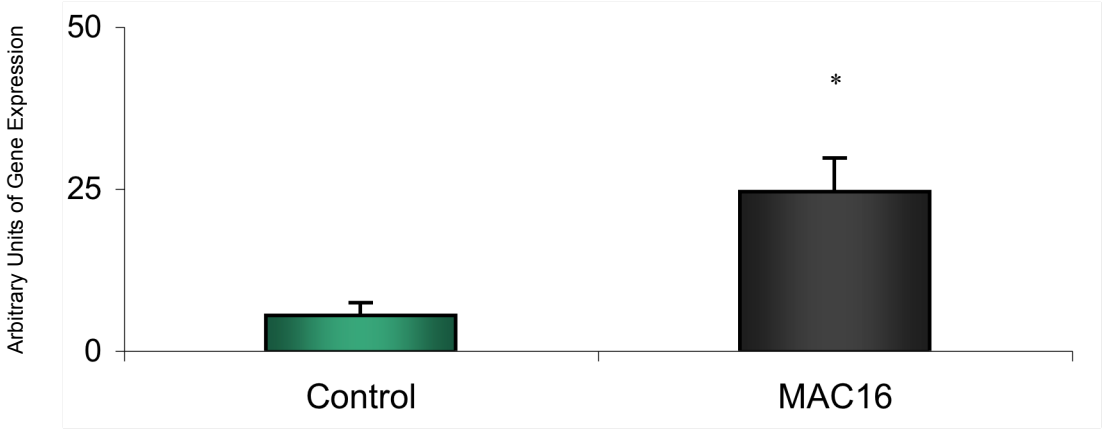


Figure 4.6 The mRNA levels of SOD1 in cachectic (MAC16) and control skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by *.

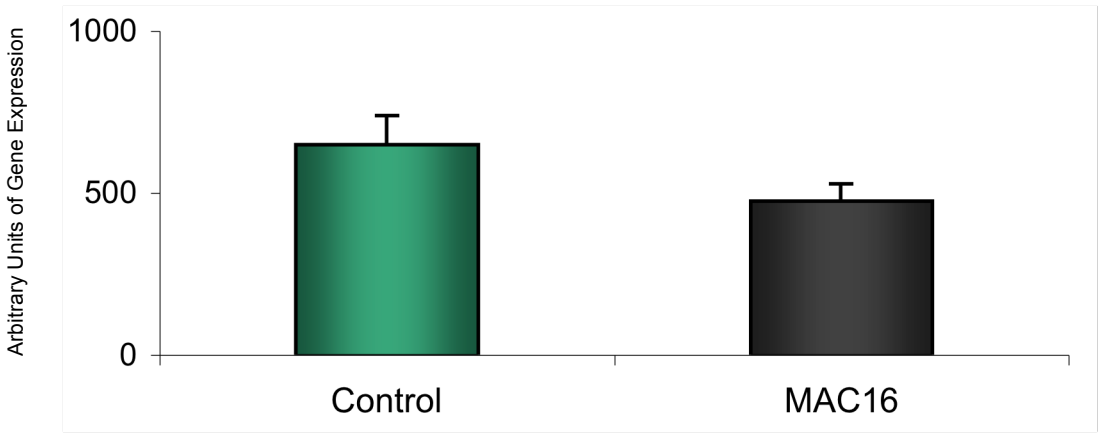


Figure 4.7 The mRNA levels of SOD2 in cachectic (MAC16) and control skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by *.

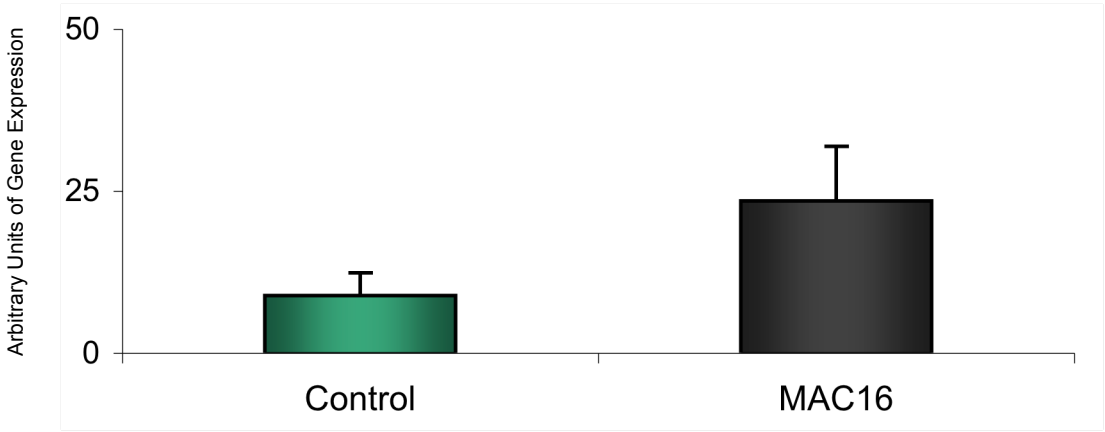


Figure 4.8 The mRNA levels of SOD3 in cachectic (MAC16) and control skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by *.

4.3.2 Superoxide Dismutase (SOD) Activity Assay

Total SOD activity was measured via epinephrine oxidation to determine the levels of O_2^- dismutation. No change in total SOD activity was observed in skeletal muscle from cachectic mice compared to controls (Figure 4.9).

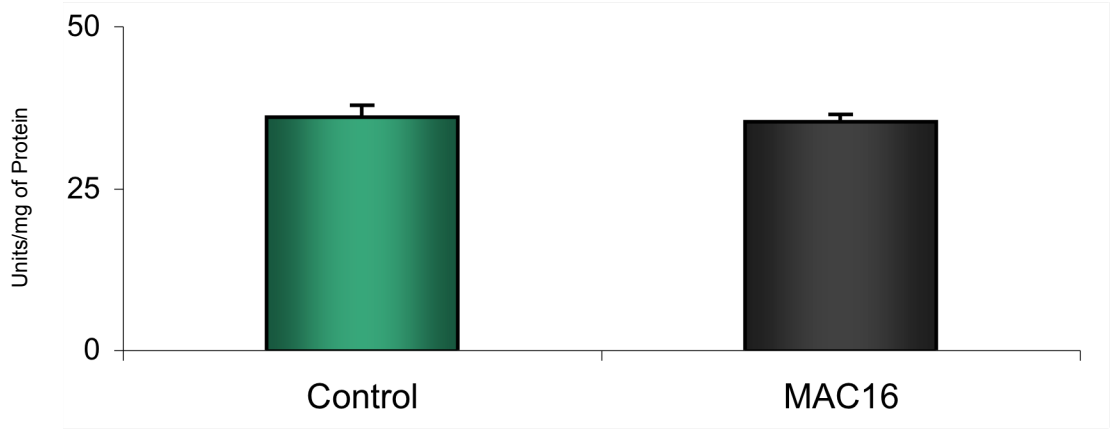


Figure 4.9 Total SOD activity in cachectic (MAC16) and control skeletal muscle, expressed as units per mg of protein. The values represent the mean ± SEM. Statistically significant differences ($P<0.05$) between groups are indicated by *.

4.4 Discussion

Cancer-induced cachexia is a debilitating condition related to the chronic inflammatory state, induced by the cancer. The inflammatory response results in elevated levels of a number of inflammatory cytokines, most noteworthy being IL-6 (Cahlin et al. 2000; Ebrahimi et al. 2004; Sturlan et al. 2002). However, it appears unlikely that these inflammatory markers alone are responsible for the complex mechanism of skeletal muscle wasting in cancer-induced cachexia as most cancer patients experience a significant change in their inflammatory cytokine profile. Rather, it appears that an unregulated catabolic factor, such as O_2^-, may be the direct downstream regulator of skeletal muscle wasting in cancer-induced cachectic patients (Russell, Eley & Tisdale 2007). Yet what remains to be understood is the origin of ROS they may play a significant role in the development of cancer-induced cachexia and the relative contribution of NOX in this process.

4.4.1 A Role for the NOX Enzymes in Cancer-Induced Cachexia

The NOX2 enzyme is the most well-established of the NOX enzyme systems and has been identified in a number of different tissues, including cardiac and vascular smooth muscle (Gorlach et al. 2000; Johar et al. 2006). However, it has only been in the last six years that NOX has been characterised in skeletal muscle tissue. Javesghani et al (2002) demonstrated the expression of all the subunits of the NOX2 enzyme in both hindlimb and respiratory muscle and the localisation of the subunits to the outer membranes of skeletal muscle. Further to these investigations, Hidalgo et al (2006) confirmed the presence of NOX2 in the external membrane system of skeletal muscle, but specifically isolated its presence to the transverse tubule network of the muscle. In

rat L6 myotubes, NOX activity has been shown to increase markedly in response to Ang II administration, leading researchers to speculate on the involvement of NOX in insulin resistance (Wei et al. 2006). Our investigations also confirm the gene expression of NOX2 enzyme subunits within mouse hindlimb skeletal muscle, in both control and cachectic mice. Specifically, elevations in the gene expression of the cytosolic subunits of the NOX2 enzyme, $p67^{phox}$ and $p40^{phox}$ were demonstrated in cachectic muscle, compared to controls, while interestingly, a decrease in the membrane component NOX2 was observed. Thus, while research of the NOX2 enzyme system in skeletal muscle is still limited, there is increasing evidence emerging of its role in regulated and unregulated ROS production.

The present study observed changes in gene expression of the regulatory and catalytic NOX2 enzyme subunits, suggesting possible changes in the state of the NOX2 enzyme system. Particularly interesting was the up-regulation of two of the three cytosolic components. In phagocytes, $p47^{phox}$ is responsible for transporting the cytosolic subunits, $p67^{phox}$ and $p40^{phox}$, to the membrane during oxidase activation. $p40^{phox}$ is suggested to be the stabilising component, responsible for the resting state of the oxidase and therefore, once activated, allows $p47^{phox}$ to initiate translocation of the cytosolic components to the membrane (Babior 2002; DeLeo & Quinn 1996; Pithon-Curi et al. 2002). Once assembled, the cytosolic component $p67^{phox}$ directly interacts with the membrane component NOX2, activating the enzyme and generating O_2^- (El-Benna et al. 2005; Takeya & Sumimoto 2003). Changes in the NOX2 enzyme subunit expression has previously been shown to be indicative of NOX enzyme activation (Javesghani et al. 2002; Welch et al. 2005; Zalba et al. 2000), accompanied by an increase in O_2^- production (Javesghani et al. 2002; Kitiyakara et al. 2003). The

upregulation of the cytosolic subunits in the present study therefore implies a potential increase in oxidase activation and O_2^- production in cachectic skeletal muscle.

Independent regulation of the subunits of the NOX2 enzyme system is not an uncommon finding in response to induced pathological conditions. A number of studies in both skeletal muscle and other cellular systems have demonstrated a disproportional change in the expression of the subunits of NOX2 (Javesghani et al. 2002; Kitiyakara et al. 2003; Paravicini et al. 2004). Indeed, it would appear that the importance of the membrane unit NOX2, and potentially $p22^{phox}$ seems of lesser importance, as previous studies have shown it not to correlate to change in the activity of the enzyme. Rather it is the cytosolic units that appear to be of greatest importance in the regulation and initiation of enzyme assembly and activation and therefore the relative production of O_2^- (Clark & Valente 2004; Javesghani et al. 2002)

4.4.2 Skeletal Muscle Atrophy in Cancer-Induced Cachexia

The muscle wasting observed in cancer-induced cachexia represents an uncontrolled increase in the proteolytic activity of the Ub-proteasome pathway (Wyke & Tisdale 2005). Researchers have determined that NF-κB has a pivotal role in the excessive stimulation of the Ub-proteasome pathway within a number of pathological skeletal muscle conditions (Hasselgren & Fischer 2001; Lecker et al. 2004). NF-κB is a highly regulated nuclear transcription factor that may be both inhibited and activated by a number of events. NF-κB is of primary importance in many tissues, as it is known to regulate the expression of over 150 genes that include antioxidant enzymes,

cytokines, metabolic enzymes, antigen presenting receptors, and the oxidative enzyme NOX (Allen & Tresini 2000; Pahl 1999). Most recently, evidence has emerged that ROS regulates the activation of NF-κB (Brar et al. 2002; Kamata et al. 2002). Specifically, it has been shown that IKK, the regulator of NF-κB activation, is itself activated by the dismuted product of O_2^- i.e. H_2O_2. Of equal interest is evidence that suggests NF-κB regulates the NOX enzyme system (Gauss et al. 2007). Furthermore, investigations in macrophage/monocytes have shown that TNF-α activation of NF-κB leads to increased expression and activity of the NOX enzyme system (Clark & Valente 2004). Combined with the knowledge that ROS activates NF-κB, the interaction and understanding of TNF-α and other cytokines, NF-κB and the NOX enzyme system, appears of significance to our overall understanding of skeletal muscle wasting conditions.

4.4.3 Antioxidant Enzymes in Cancer-Induced Cachexia

In addition to the changes in the subunits of the NOX2 enzyme system, an increase was observed in the gene expression of SOD1 in cachectic skeletal muscle. However, no change was observed in the gene expression of SOD2 and SOD3. Interestingly, there was no change in total SOD activity in cachectic skeletal muscle, compared to controls. The increase in the cytosolic form of SOD, as compared with mitochondrial or extracellular SOD, suggests a potential role for O_2^- producing NOX enzyme system within the intracellular compartment. To date, the NOX enzyme system has only been identified in the t-tubule and extracellular membranes of skeletal muscle (Hidalgo et al. 2006). However, it seems not surprising that the NOX enzyme system, may reside within the intracellular compartment. With a potential increase in O_2^- production and an additional increase in SOD1 expression, this study further supports

the accumulation of OS through O_2^- and H_2O_2 within cachectic skeletal muscle. The role of SOD is also yet to be elucidated, but its contribution to redox-sensitive NF-κB activation cannot be overlooked.

4.4.4 Conclusion

In conclusion, this study investigated the potential involvement of the NOX2 and primary antioxidant enzyme systems that are suggested to contribute to skeletal muscle wasting in cancer-induced cachexia. This study supports a role for the NOX2 enzyme system and potential accumulation of O_2^- and H_2O_2 in the development of cancer-induced cachexia. In further support of this, despite the increase in SOD1 gene expression, indicative of an increase in O_2^-, total SOD activity did not respond to compensate for this potential increase. O_2^- and H_2O_2 may act as direct catabolic contributors to oxidative changes and potential activation of NF-κB that has been shown to influence important skeletal muscle growth pathways and therefore contributing to skeletal muscle wasting. However, as this study did not investigate these systems further this proposed pathway of skeletal muscle wasting remains speculative. Our study therefore indicates a need for further research of the role of the NOX2 enzyme and its potential contribution to OS in the development of skeletal muscle wasting, in cancer-induced cachexia.

Chapter 5 Cancer-Induced Cachexia Study 2

5.1 Introduction

The findings of the previous study suggest a role for the NOX2 enzyme system in the development of cancer-induced cachectic. To extend the previous investigation, the current study sought to investigate the NOX2 enzyme and SOD antioxidant systems, but to also investigate further a role for the additionally expressed NOX4 enzyme system and antioxidant enzymes in skeletal muscle of MAC16-induced cachectic mice. The expression of the NOX enzyme in skeletal muscle has been recognised (Cheng et al. 2001; Hidalgo et al. 2006; Javesghani et al. 2002; Mofarrahi et al. 2008), with the expression of both NOX2 and NOX4 (Cheng et al. 2001; Mofarrahi et al. 2008) and associated subunits (Javesghani et al. 2002), although their function in the skeletal muscle system is relatively unknown. In addition, the recent discovery of NOX4 localisation on intracellular membranes (Geiszt 2006; Krause 2007), gives evidence for its potential involvement in intracellular O_2^- production that may be a key contributor to OS and intracellular changes in cancer-induced cachexia.

5.1.1 Antioxidant Enzymes in Cancer-Induced Cachexia

Results from the previous study indicated changes in the SOD antioxidant system for the elimination of O_2^-. This finding, lead us to investigate the additional antioxidant enzyme systems in skeletal muscle to further understand the potential oxidative contributors involved in cancer-induced cachectic. The role of SOD is for the

conversion of O_2^- to H_2O_2, whereas the antioxidant enzymes GPx and catalase are important scavengers of H_2O_2, and therefore these antioxidant enzymes contribute to cellular oxidative homeostasis (Landis & Tower 2005; Scandalios 2005). The combination of these antioxidant enzymes, play an important role in regulating cellular oxidative systems and redox-sensitive signaling pathways (Ji 2007). It is therefore important to recognise the roles that these individual antioxidants play and their contribution to oxidative changes in cancer-induced cachexia. Furthermore, the well-recognised signaling role for O_2^- and H_2O_2, and the redox-sensitive transcription factor NF-κB involvement in skeletal muscle growth pathways (Cai et al. 2004b; Clark & Valente 2004; Glass 2005; Guttridge 2004), indicate a role for NF-κB in cancer-induced cachexia.

5.1.2 Animal Model of Cancer-Induced Cachexia

Further to this, our preliminary investigation into the role of the NOX2 enzyme system in cancer-induced cachexia, utilized a well-established murine model of cancer-induced cachexia. The MAC16 model has been established in both the NMRI breed of mice (Whitehouse et al. 2001) and the immunosuppressed BALB/c *nu/nu* mouse (Monitto et al. 2001), which have both demonstrated cancer-induced cachectic tumour growth and loss of skeletal muscle mass. In addition to the MAC16-induced cachectic model, the colon cancer cell line utilises a similar MAC13 cell line of tumour growth in the mouse that establishes a non-cachectic phenotype. This model therefore allows for an additional comparison to the control mouse, between two similar tumour-bearing mouse models, with a cachectic (MAC16) and non-cachectic (MAC13) phenotype.

5.1.3 A Role for the NOX Enzymes in Cancer-Induced Cachexia

ROS have been implicated in cachexia and skeletal muscle wasting due to the presence of protein oxidation in human patients (Mecocci et al. 1999; Pansarasa et al. 1999) and experimental models (Barreiro et al. 2005; Sundaram & Panneerselvam 2006). The well-established involvement of ROS as a contributor to direct cellular oxidative damage and the recently recognised role for ROS in redox-sensitive signaling pathways has been suggested to be involved in the progression of cancer-induced cachexia (Russell, Eley & Tisdale 2007). The strong link between cancer-induced cachexia and proteolytic pathways such as the Ub-proteasome proteolytic pathway (Lecker, et al. 2004; Lorite et al. 2001; Whitehouse & Tisdale 2003; Wyke & Tisdale 2005), gives evidence for the potential pathway of muscle atrophy that may involve ROS (Busquets et al. 2005; Gomes-Marcondes & Tisdale 2002; Russell, Eley & Tisdale 2007). The Ub ligases, Atrogin-1 and MuRF-1 are atrophy related genes, suggested to play a key role in skeletal muscle atrophy and the development of cachexia (Cai et al. 2004a; Clavel et al. 2006; Lecker et al. 2004). Recently, studies have linked the changes in these genes to transcription factor activation, of which ROS are involved in (Clark & Valente 2004; Kamata et al. 2002; Ungvari et al. 2007; Valko et al. 2006). Furthermore, a role for transcription factor NF-κB has been demonstrated in cancer-induced cachexia (Busquets et al. 2004; Whitehouse & Tisdale 2003; Wyke & Tisdale 2005) and NF-κB inhibition has been shown to slow the progression of cachexia (Cai et al. 2004b; Langen & Schols 2007).

Circulating and intracellular cytokines have been implicated in the development of cancer-induced cachexia (Argiles, Moore-Carrasco, Busquets et al. 2003; Figueras et al. 2005; Fortunati et al. 2007; Giordano et al. 2003) and ROS appear to function as intracellular second messengers for TNF-α-induced transcription factor NF-κB activation in skeletal muscle (Clark & Valente 2004; Gauss et al. 2007; True, Rahman & Malik 2000). In particular, NF-κB has been suggested to influence the expression of genes involved in the Ub-proteolytic pathway, such as MuRF-1 and Atrogin-1, inducing atrophy and cachexia (Cai et al. 2004b). Furthermore, TNF-α has been proposed to stimulate NOX generating ROS (Chenevier-Gobeaux et al. 2006; Frey et al. 2002) that in turn activate NF-κB (Clark & Valente 2004), giving some evidence for TNF-α/NOX/ROS/NF-κB pathway and proteolysis in cancer-induced cachexia. It is also evident that tumour derived factors such as PIF, contribute to muscle protein breakdown in cancer-induced cachexia (Smith & Tisdale 2003; Wang et al. 2003). NF-κB has also been suggested to increase the expression of the atrophy related Ub genes, downstream of PIF and Ang II (Eley & Tisdale 2007). The induction of muscle degradation, characteristic of cancer-induced cachexia, via PIF (Whitehouse & Tisdale 2002; Wyke, Smith & Tisdale 2003) and Ang II (Russell, Sanders & Tisdale 2006; Sanders, Russell & Tisdale 2005), has been established. However, a recent study by Russell et al (2008) proposed a cachectic pathway of NF-κB-dependent Ub gene expression, downstream of NOX generated ROS, establishing a potential role for NOX in mediating skeletal muscle atrophy through the Ub-proteasome proteolytic pathway. The induction of cachexia by tumour-derived factors such as TNF-α and PIF, has also been established. Furthermore, the induction of muscle catabolism and atrophy, demonstrated in cancer-induced cachexia by the Ub-proteasome proteolytic pathway, is well recognised. What is yet to be elucidated however, are the central

mediators of this complex system, in response to tumour growth, and the development of cancer-induced cachexia.

Therefore, this study aims to investigate the O_2^- generating NOX and antioxidant enzyme systems in a well-established animal model of cancer-induced cachexia. In particular, this study aims to determine changes in the NOX2 and NOX4 enzyme systems and associated NOX enzyme subunits; NOX2, NOX4, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac1, the primary antioxidants for O_2^- and H_2O_2 elimination; SOD1, SOD2, SOD3, catalase and GPx, as well as O_2^- levels, in the skeletal muscle of cancer-induced cachectic mice (MAC16) compared to cancer-induced non-cachectic (MAC13) and control mice. Further to these oxidative associated measures, we aim to investigate the expression of the transcription factor NF-κB, proposed to play a key role in the proteolytic pathway through ROS.

5.2 Methods

5.2.1 Animal Model of Cancer-Induced Cachexia

All animal procedures carried out in this study were approved by, the Victoria University AEEC (AEETH 07/05). The MAC model was used for this study, utilising the two histologically similar colon cancer cell lines, MAC16 and MAC13 donated from Professor Michael J. Tisdale (Pharmaceutical Sciences Research Institute, Aston University, Birmingham, UK). Cells were grown in culture to 80% confluency and injected into BALB/c *nu/nu* mice (approximately 1 x 10^6 cells). BALB/c *nu/nu* mice were used in this study as they have previously been shown to establish tumour growth and cancer-induced cachexia, once injected with the MAC cell lines (Monitto et al. 2001). Female BALB/c *nu/nu* mice (n=46) were purchased from ARC, Western Australia, throughout the study. Mice were assigned to three groups based on the cell line injected including; MAC16-induced, MAC13-induced, and control mice that were not induced with cancer. As has previously been described (Hussey et al. 2000; Whitehouse et al. 2001), donor mice were used to establish and maintain a tumour line, which consistently produced cancer (MAC13) and cancer-induced cachexia (MAC16). MAC13-induced donor mice that established solid tumour growth by approximately day 9-12 and maintained body weight thereafter were considered for transplantation into MAC13 recipient mice. Similarly, MAC16-induced donor mice that established solid tumour growth by approximately day 9-12 with significant weight loss of up to 15-25% thereafter, were considered for transplantation into MAC16 recipient mice. Tumour fragments grown and maintained in donor mice were dissected and re-implanted subcutaneously into the flank of recipient mice. All mice used in this study were maintained under controlled environmental conditions, 12

hour light/dark cycle, 21 ± 2°C, 30% humidity, in conventional cages with *ad libitum* access to standard chow and water throughout the course of the study. Mice were monitored daily and body weight and tumour size was recorded. Cachectic weight loss was evident at approximately 9-12 days post implantation and animals were anaesthetised for tissue collection if weight loss exceeded 25% of original body weight or before tumour growth exceeded 1000 mm^3 (Hussey et al. 2000). Recipient MAC13 and MAC16 mice as well as controls were anaesthetised using pentobarbital sodium (70mg/kg) and skeletal muscle tissue was collected, weighed and immediately snap frozen in liquid nitrogen and stored at -80°C for later use.

5.2.2 Reverse Transcription-Real-Time PCR

RNA was extracted from frozen quadriceps muscle using Tri Reagent (Molecular Research Centre, USA), according to the manufacturer's protocol. Total RNA concentration was determined spectrophotometrically at 260/280nm. Prior to RT all RNA samples were DNase treated (Promega, Australia) and then first-strand cDNA was generated from 1µg RNA using AMV RT (Promega, Australia). The cDNA was stored at -20°C for subsequent analysis. Pre-designed TaqMan Gene Expression Assays (Applied Biosystems, USA) were used containing specific primers and probes for the genes of interest (Table 2.2). Real-time PCR was performed using Applied Biosystems 7500 detection system and PCR reactions were performed using TaqMan Gene Expression Master Mix (Applied Biosystems, USA). Briefly, a real-time PCR mix of 50% TaqMan Gene Expression Master Mix (Applied Biosystems, USA) and 0.5% TaqMan Gene Expression Assay Mix (20x), and cDNA, optimised to specific Gene Expression Assays, was run for 40 cycles of PCR in a total volume of 25µl. To compensate for variations in input RNA amounts and efficiency of reverse

transcription, GAPDH mRNA was quantified and all results were normalised to these values. Fluorescent emission data were captured and mRNA levels were analysed using the C_T value (Schmittgen et al. 2000). The $2^{\Delta}C_T$ was calculated by subtracting the ${}^{\Delta}C_T$ for GAPDH mRNA from the ${}^{\Delta}C_T$ for the gene of interest. The relative expression of the gene of interest was calculated using the expression $2\ {}^{\Delta}C_T$ and reported as arbitrary units.

5.2.3 Superoxide Dismutase (SOD) Activity Assay

Frozen muscle pieces (approximately 100mg) were placed in ice-cold HEPES buffer (20mM) containing; EGTA (1mM), mannitol (210mM), and sucrose (70mM) and adjusted to a pH of 7.2 (10ml/g). Muscle aliquots were homogenised in buffer, using a glass on glass homogeniser, and centrifuged at 1,500xg for 5 minutes at 4ºC to remove insoluble connective tissue. To separate the cytosolic and mitochondrial fractions, the supernatant was centrifuged at 10,000xg for 5 minutes at 4ºC and the resulting supernatant, containing the cytosolic fraction was collected for SOD1 enzyme analysis. The remaining pellet containing the mitochondrial fraction was resuspended and homogenised in ice-cold HEPES buffer (20mM) for SOD2 enzyme analysis. SOD1 and SOD2 activity was measured in cytosolic and mitochondrial muscle fractions respectively, by spectrophotometric assay with the use of a commercially available kit (706002 Superoxide Dismutase Assay Kit; Cayman Scientific). The amount of activity was calculated and standardised for protein using the Bradford method (Bio-Rad, USA).

5.2.4 *Detection of O_2^- by DHE Fluorescence Staining*

Skeletal muscle O_2^- was measured using O_2^- sensitive DHE dye. Cell permeable DHE reacts with O_2^-, converting DHE into ethidium fluorescence (Serrander et al. 2007). DHE (5μM) was applied to quadriceps cross-sections (5μm) and incubated in a light protected oven at 37°C for 30 min. The sections were washed with PBS to remove excess DHE and fluorescence was assessed by way of fluorescence microscopy (Axiocam HBO 50/AC, Zeiss, Germany). The muscle was analysed in three sections to obtain measurements from the whole tissue. Ethidium fluorescence density was detection from the whole section with MCID imaging software (Imaging Research Inc, Australia) and expressed as arbitrary units of fluorescence (Azumi et al. 2002; Miller et al. 2002; Williams & Allen 2007)

5.2.5 *Statistical Analysis*

Statistical analysis was performed using SPSS statistical package (version 15.0). Results are expressed as mean ± SEM. Differences were determined by one-way ANOVA with Tukey HSD as posthoc to determine significant differences between groups and results were considered statistically significant if *p*-values were equal to or <0.05.

5.3 Results

5.3.1 Animal Model of Cancer-Induced Cachexia

The Female BALB/c *nu/nu* mice that were used in this study were implanted with the MAC13 cell line that had previously developed a tumour in the same mouse model at approximately 9-12 days post implantation and where the animal did not lose weight. Female BALB/c *nu/nu* mice implanted with the MAC16 cell line that developed a tumour at approximately 9-12 days post implantation and lost at least 15% -25% of their original body weight were used in this study. Animal body weight and skeletal muscle weights were recorded at tissue collection and evaluated as a measure of body mass and skeletal muscle cachexia for all mice. Skeletal muscle weights were significantly lower in MAC16-induced mice compared to MAC13-induced (p=0.003) and control mice (p=0.022) (Figure 5.1), while no change in skeletal muscle weights were observed in the MAC13-induced mice, when compared to controls (Figure 5.1). Animal food intake was recorded throughout the course of the study and there were no significant differences in food intake between the groups (Figure 5.2).

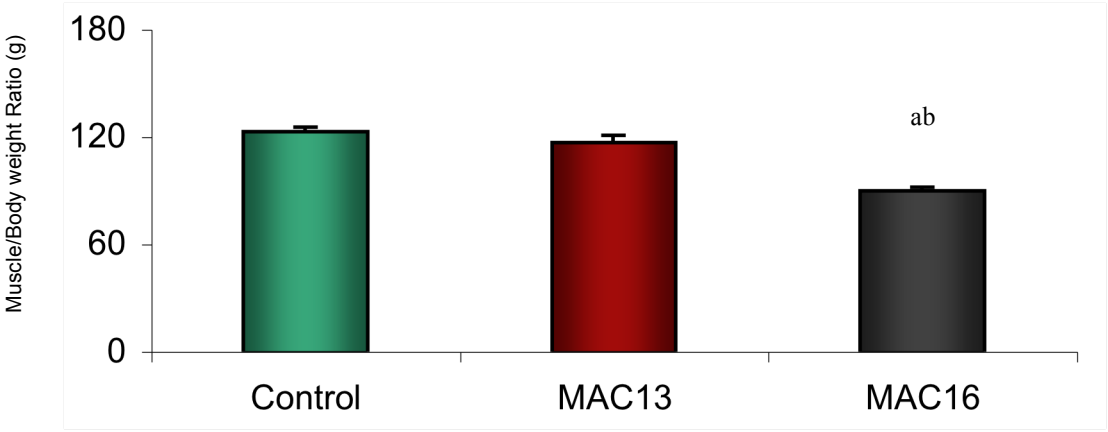


Figure 5.1 Quadriceps muscle weights in cancer-induced cachectic (MAC16), cancer-induced non-cachectic (MAC13) and control skeletal muscle. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters, where Control = a, MAC13 = b and MAC16 = c.

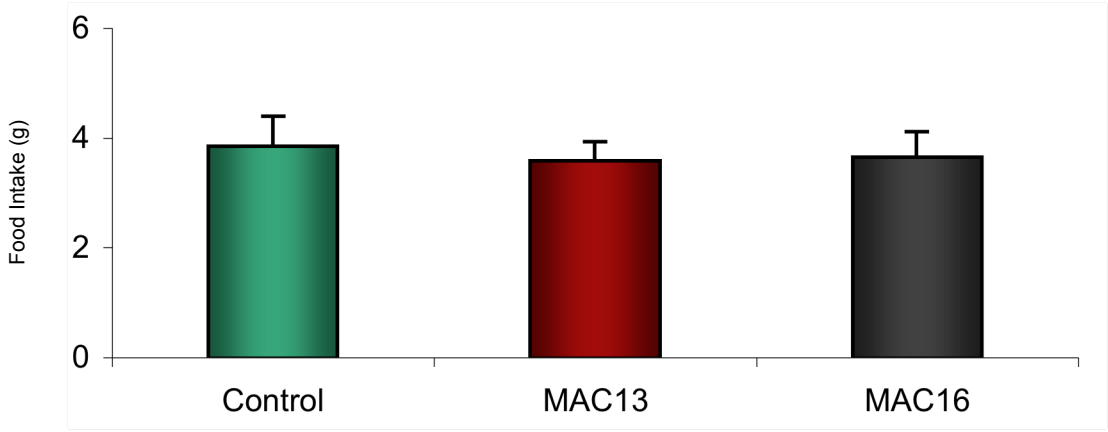


Figure 5.2 Food intake for cancer-induced cachectic (MAC16), cancer-induced non-cachectic (MAC13) and control skeletal muscle. Food intake was measured per box of four animals in each box. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters, where Control = a, MAC13 = b and MAC16 = c.

5.3.2 Reverse Transcription-Real-Time PCR

5.3.2.1 NOX Subunit Gene Expression

NOX subunit gene expression was measured in the quadriceps muscle of MAC13 and MAC16-induced mice and controls. Results are expressed as units of gene expression for each of the NOX subunits, NOX2, NOX4, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac1. The mRNA expression of NOX2 was significantly lower in the skeletal muscle of MAC16-induced mice (p=0.031) compared to MAC13-induced mice (Figure 5.3). However, no change was observed in NOX2 mRNA expression in skeletal muscle from MAC13-induced and MAC16-induced mice compared to controls (Figure 5.3). Similarly, the mRNA expression of $p40^{phox}$ was significantly lower in the skeletal muscle of MAC16-induced mice compared to the MAC13-induced (p<0.001) and control mice (p=0.009) (Figure 5.8). However, no change was observed in the mRNA expression of the cytosolic subunit $p40^{phox}$ in skeletal muscle from control mice compared to MAC13-induced mice (Figure 5.8). Interestingly, an increase in the mRNA expression of $p67^{phox}$ was observed in skeletal muscle from MAC13-induced compared to control mice (p=0.004) (Figure 5.6), and a significant decrease in mRNA expression of $p67^{phox}$ was observed in skeletal muscle from MAC16-induced mice compared to MAC13-induced (p=0.002) and control mice (p=0.003) (Figure 5.6). No change was observed in the mRNA expression of the additional NOX subunits, NOX4 (Figure 5.4), $p22^{phox}$ (Figure 5.5) and $p47^{phox}$ (Figure 5.7) or Rac1 (Figure 5.8).

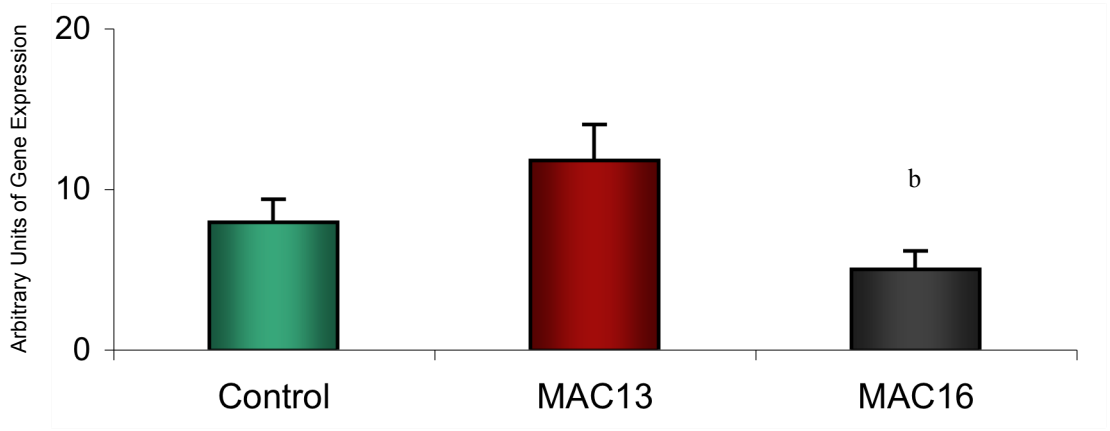


Figure 5.3 The mRNA levels of NOX2 in cancer-induced cachectic (MAC16), cancer-induced non-cachectic (MAC13) and control skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters where Control = a, MAC13 = b and MAC16 = c.

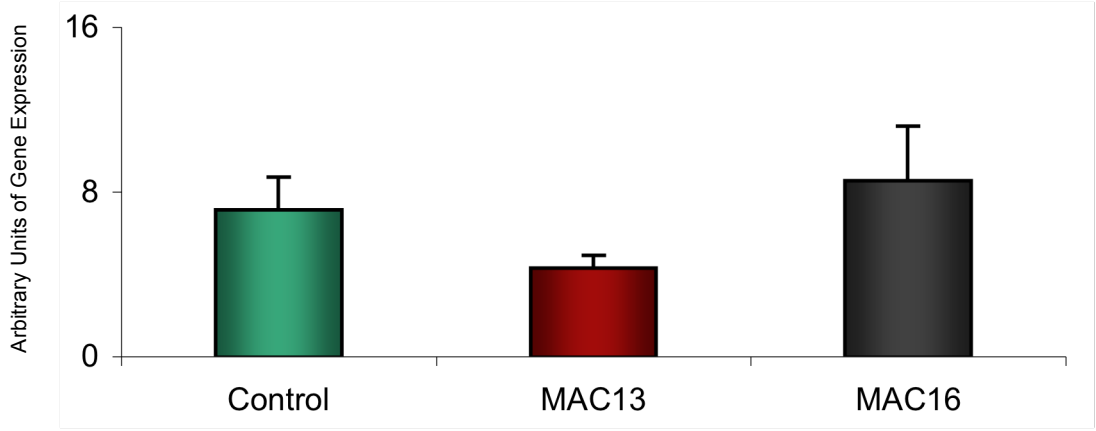


Figure 5.4 The mRNA levels of NOX4 in cancer-induced cachectic (MAC16), cancer-induced non-cachectic (MAC13) and control skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters where Control = a, MAC13 = b and MAC16 = c.

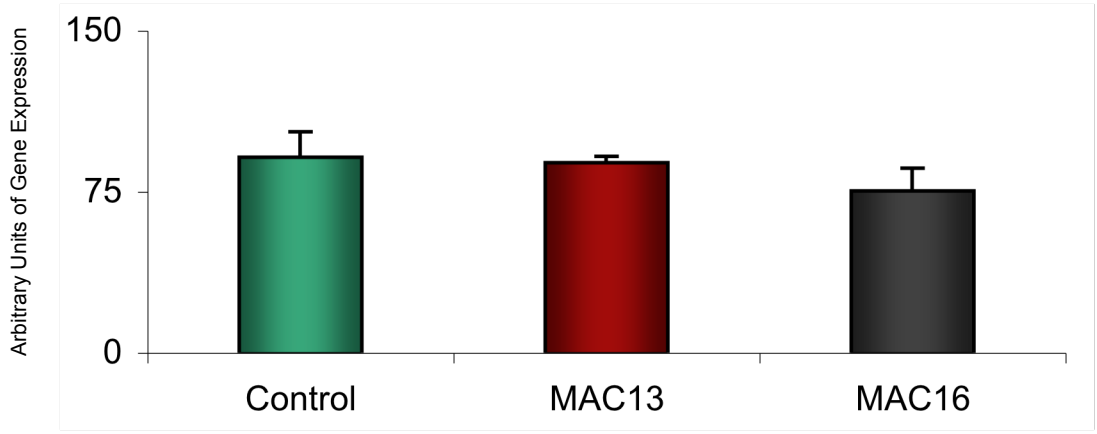


Figure 5.5 The mRNA levels of p22^{phox} in cancer-induced cachectic (MAC16), cancer-induced non-cachectic (MAC13) and control skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters where Control = a, MAC13 = b and MAC16 = c.

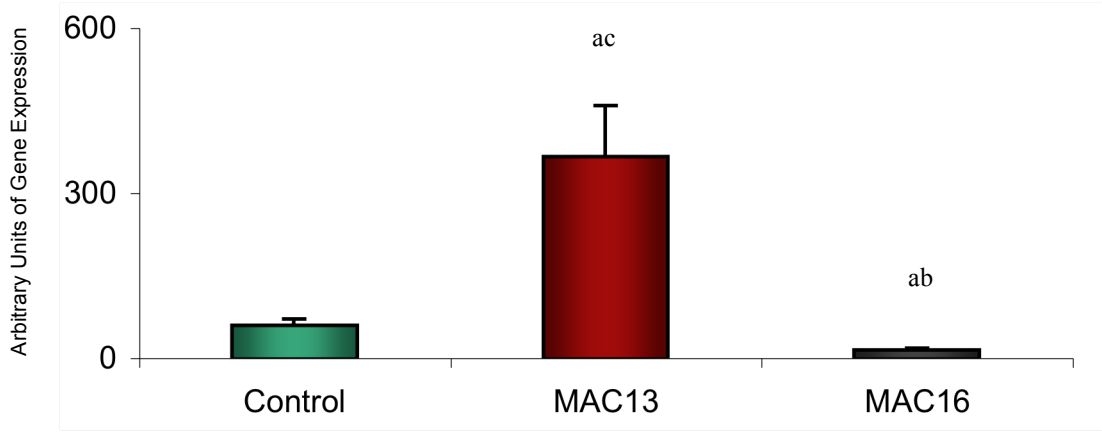


Figure 5.6 The mRNA levels of p67^{phox} in cancer-induced cachectic (MAC16), cancer-induced non-cachectic (MAC13) and control skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters where Control = a, MAC13 = b and MAC16 = c.

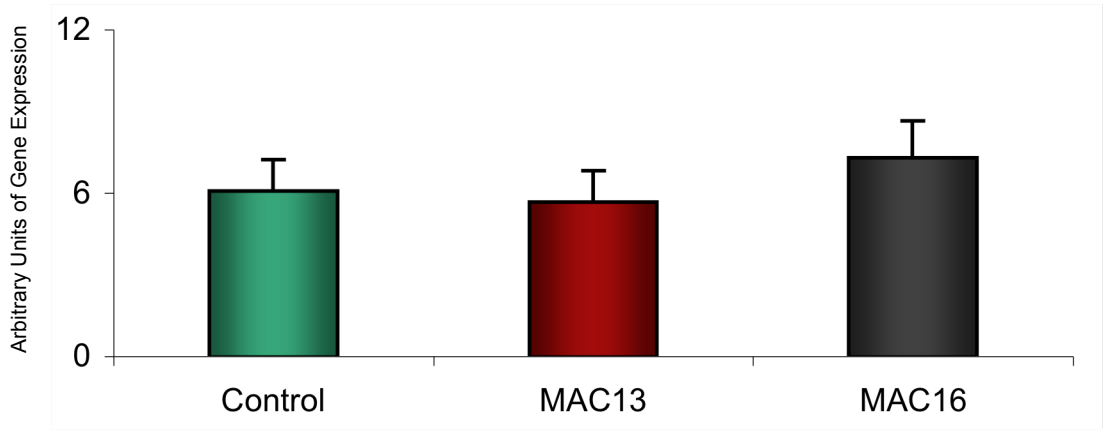


Figure 5.7 The mRNA levels of p47^{phox} in cancer-induced cachectic (MAC16), cancer-induced non-cachectic (MAC13) and control skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters where Control = a, MAC13 = b and MAC16 = c.

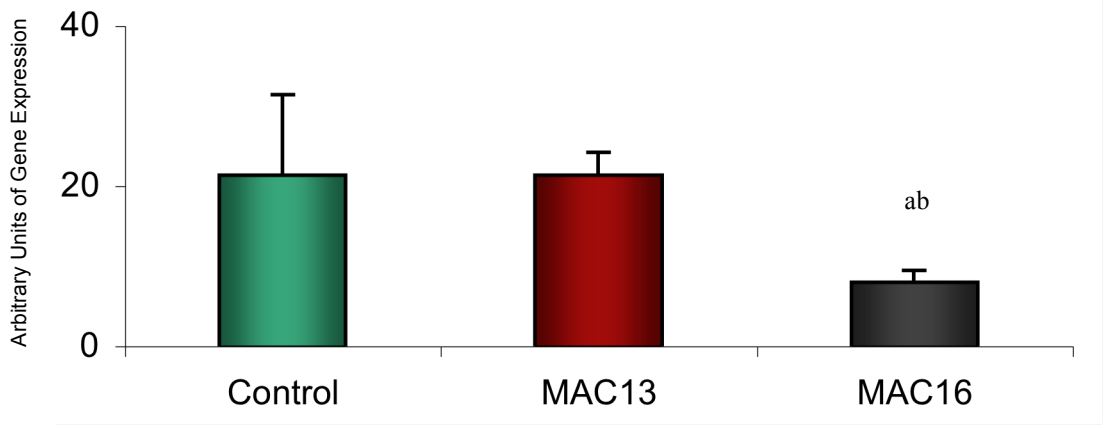


Figure 5.8 The mRNA levels of p40^{phox} in cancer-induced cachectic (MAC16), cancer-induced non-cachectic (MAC13) and control skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters where Control = a, MAC13 = b and MAC16 = c.

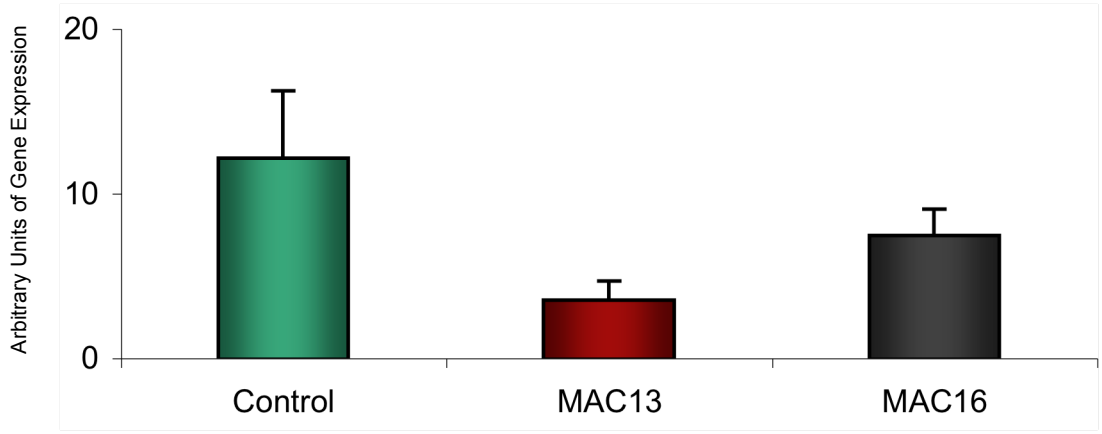


Figure 5.9 The mRNA levels of Rac1 in cancer-induced cachectic (MAC16), cancer-induced non-cachectic (MAC13) and control skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences ($P<0.05$) between groups are indicated by lower case letters where Control = a, MAC13 = b and MAC16 = c.

5.3.2.2 Antioxidant Enzyme Gene Expression

Antioxidant enzyme gene expression was measured in the quadriceps muscle of MAC13 and MAC16-induced mice, and control mice. Results are expressed as units of gene expression for each of the SOD isoforms, SOD1, SOD2 and SOD3, and GPx and catalase. The mRNA expression levels of SOD1 and SOD2 were lower in the skeletal muscle of MAC16-induced mice compared to MAC13-induced (p=0.007) (p<0.001) and control mice (p=0.001) (p=0.003) (Figure 5.10 & 5.11), while no change was observed in the mRNA expression of SOD1 and SOD2 in the skeletal muscle of MAC13-induced mice compared to controls (Figure 5.10 & 5.11). Although we observed an increase in SOD3 mRNA expression in the skeletal muscle of MAC16-induced mice compared to controls (p=0.006), SOD3 mRNA expression was significantly increased in the skeletal muscle of MAC13-induced mice compared to MAC16-induced mice (p=0.043) and controls (p<0.001) (Figure 5.12). In addition, mRNA expression of GPx was significantly lower in skeletal muscle from MAC16-induced mice compared to MAC13-induced (p<0.001) and control mice (p=0.001) (Figure 5.13), while there was no change in mRNA expression of GPx in skeletal muscle from the MAC13-induced and control mice (Figure 5.13). However, no change was observed in the gene expression of catalase in skeletal muscle between groups (Figure 5.14).

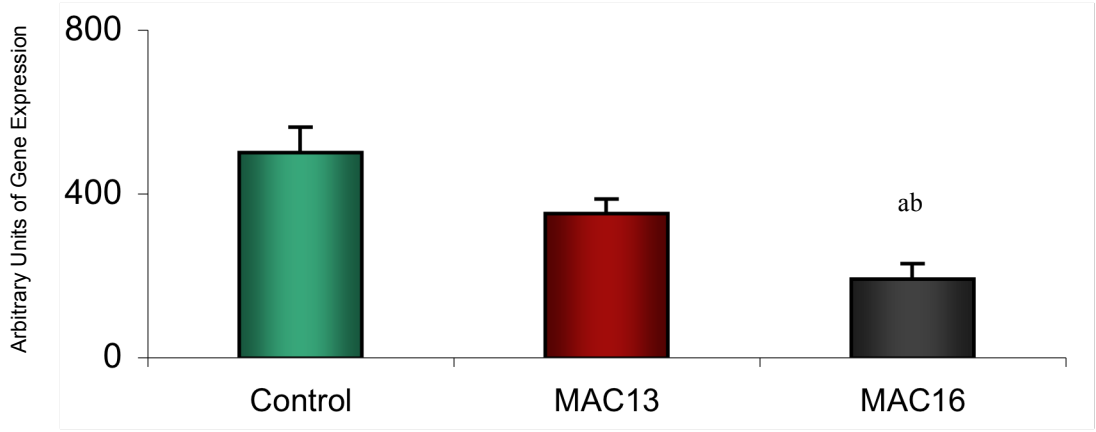


Figure 5.10 The mRNA levels of SOD1 in cancer-induced cachectic (MAC16), cancer-induced non-cachectic (MAC13) and control skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters where Control = a, MAC13 = b and MAC16 = c.

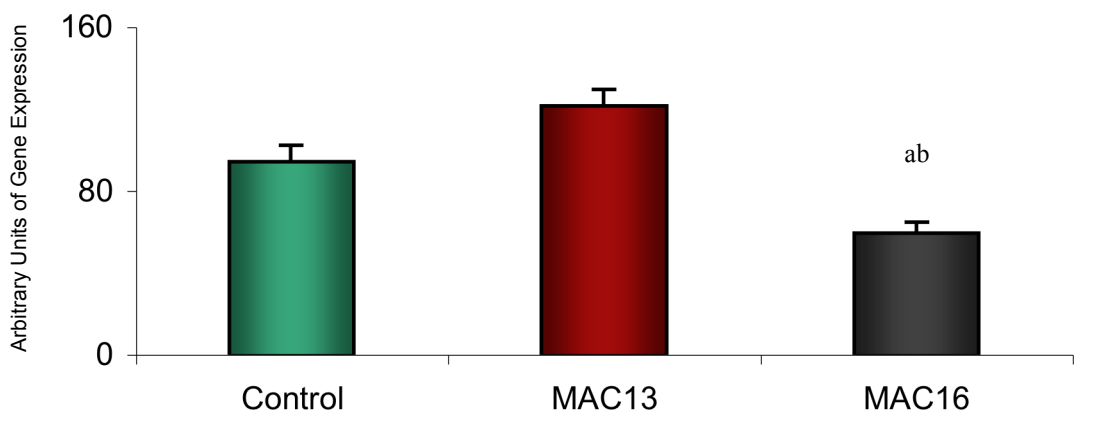


Figure 5.11 The mRNA levels of SOD2 in cancer-induced cachectic (MAC16), cancer-induced non-cachectic (MAC13) and control skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters where Control = a, MAC13 = b and MAC16 = c.

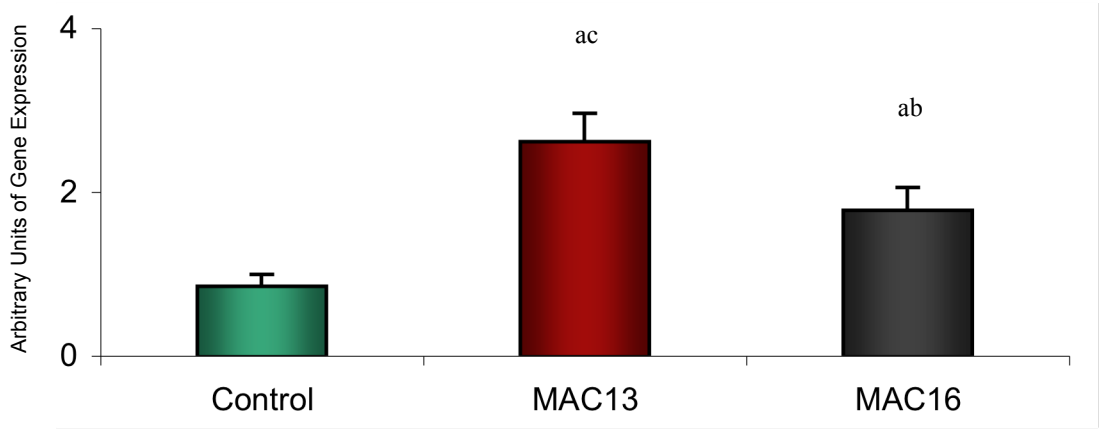


Figure 5.12 The mRNA levels of SOD3 in cancer-induced cachectic (MAC16), cancer-induced non-cachectic (MAC13) and control skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters where Control = a, MAC13 = b and MAC16 = c.

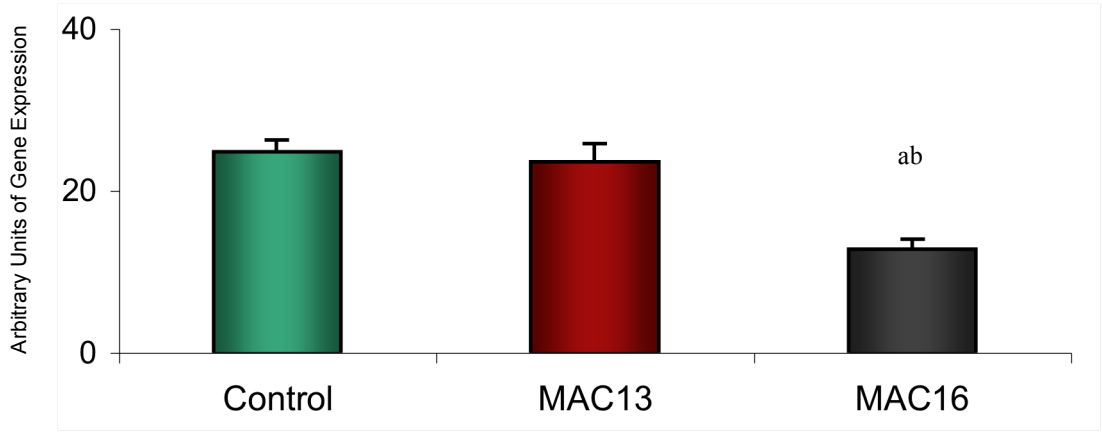


Figure 5.13 The mRNA levels of GPx in cancer-induced cachectic (MAC16), cancer-induced non-cachectic (MAC13) and control skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters where Control = a, MAC13 = b and MAC16 = c.

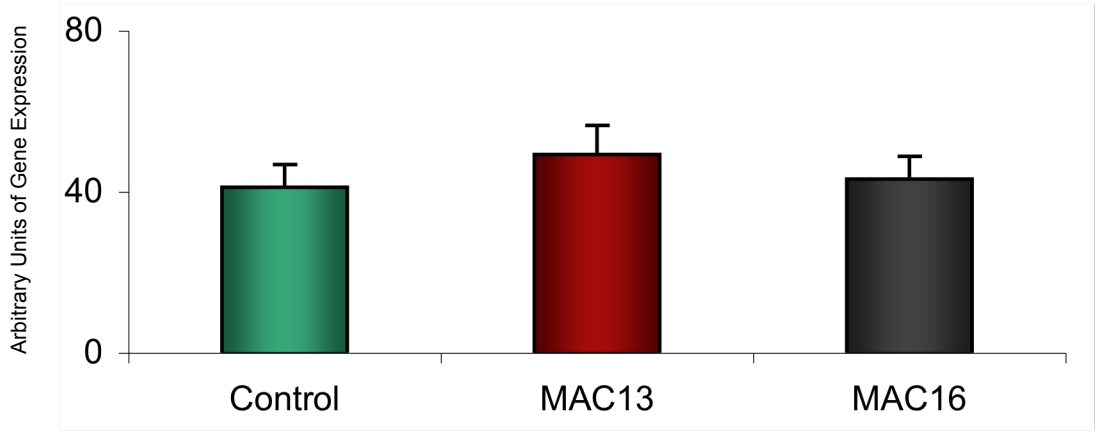


Figure 5.14 The mRNA levels of catalase in cancer-induced cachectic (MAC16), cancer-induced non-cachectic (MAC13) and control skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences ($P<0.05$) between groups are indicated by lower case letters where Control = a, MAC13 = b and MAC16 = c.

5.3.2.3 NF-κB Gene Expression

The mRNA expression of NF-κB was lower in skeletal muscle from MAC13 (p<0.001) and MAC16 (p<0.001)-induced mice compared to controls (Figure 5.15). However, no difference was observed in the mRNA expression of NF-κB in skeletal muscle from the cancer-induced mice, MAC13 & MAC16 (Figure 5.15).

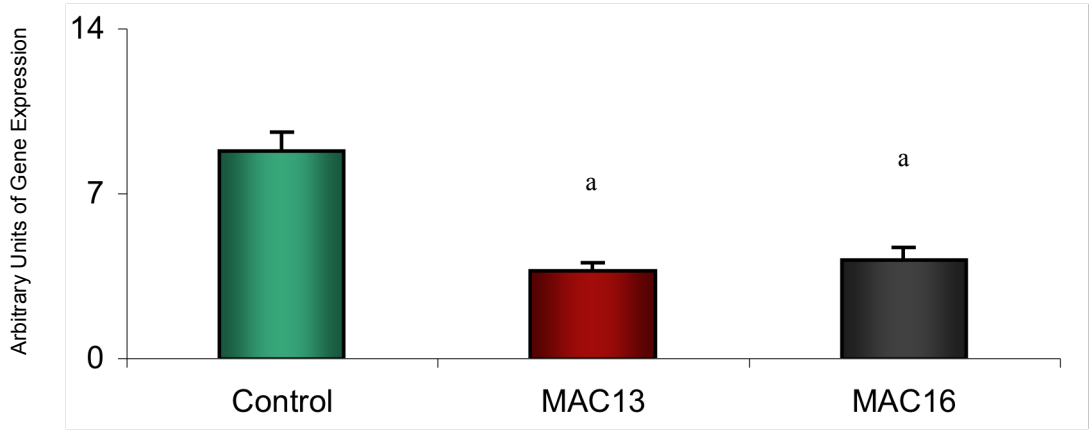


Figure 5.15 The mRNA levels of NF-κB in cancer-induced cachectic (MAC16), cancer-induced non-cachectic (MAC13) and control skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters where Control = a, MAC13 = b and MAC16 = c.

5.3.3 Superoxide Dismutase (SOD) Activity Assay

SOD activity was examined in cytosolic and mitochondrial skeletal muscle fractions as a measure of SOD1 and SOD2 activity. SOD1 activity was significantly lower in skeletal muscle from MAC16-induced mice compared to the MAC13-induced (p<0.001) and control mice (p=0.008) (Figure 5.16), while no change was observed in SOD1 activity in skeletal muscle from MAC13-induced mice compared to controls (Figure 5.16). Interestingly, our results indicated a decline in SOD2 activity in the MAC13 (p=0.001) and MAC16 (p<0.001)-induced mice compared to controls (Figure 5.17), while no differences were observed in SOD2 activity between the cancer-induced mice, MAC13 and MAC16 (Figure 5.17).

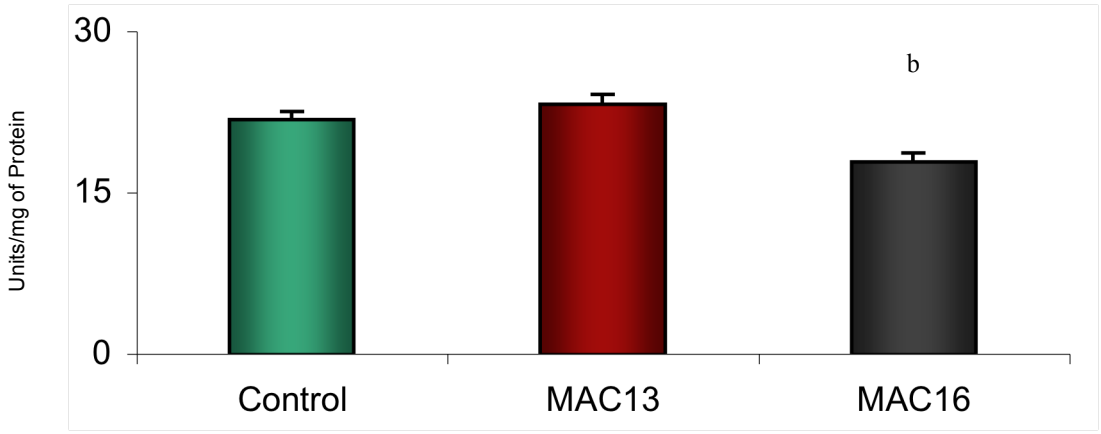


Figure 5.16 SOD1 activity in cancer-induced cachectic (MAC16), cancer-induced non-cachectic (MAC13) and control skeletal muscle, expressed as units per mg of protein. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters where Control = a, MAC13 = b and MAC16 = c.

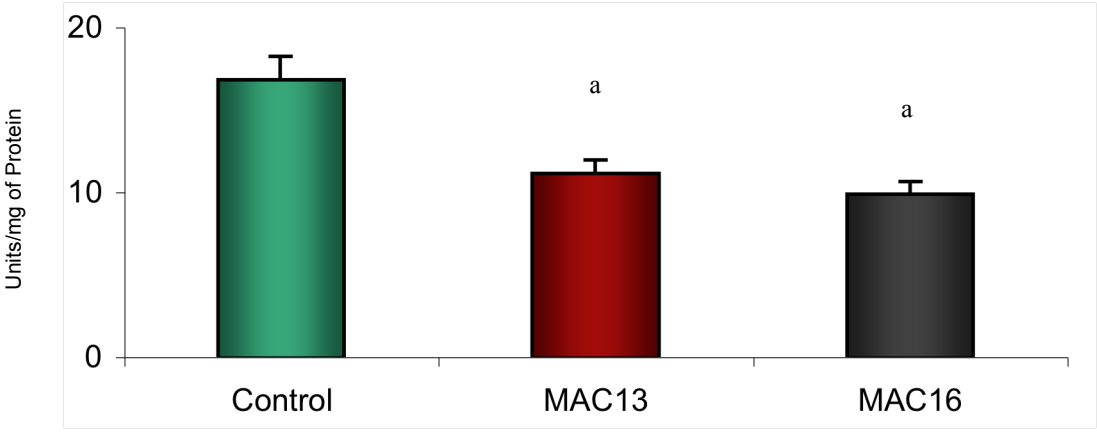


Figure 5.17 SOD2 activity in cancer-induced cachectic (MAC16), cancer-induced non-cachectic (MAC13) and control skeletal muscle, expressed as units per mg of protein. The values represent the mean ± SEM. Statistically significant differences ($P<0.05$) between groups are indicated by lower case letters where Control = a, MAC13 = b and MAC16 = c.

5.3.4 Detection of O_2^- by DHE Fluorescence Staining

Levels of O_2^- were detected in skeletal muscle sections by O_2^- sensitive DHE staining. Increased levels of O_2^- were detected in skeletal muscle from MAC16-induced mice, when compared to MAC13-induced and control mice (p=0.001) (Figure 5.18 & 5.19). No change was observed in O_2^- levels in the skeletal muscle from MAC13-induced mice compared to controls (Figure 5.18 & 5.19).

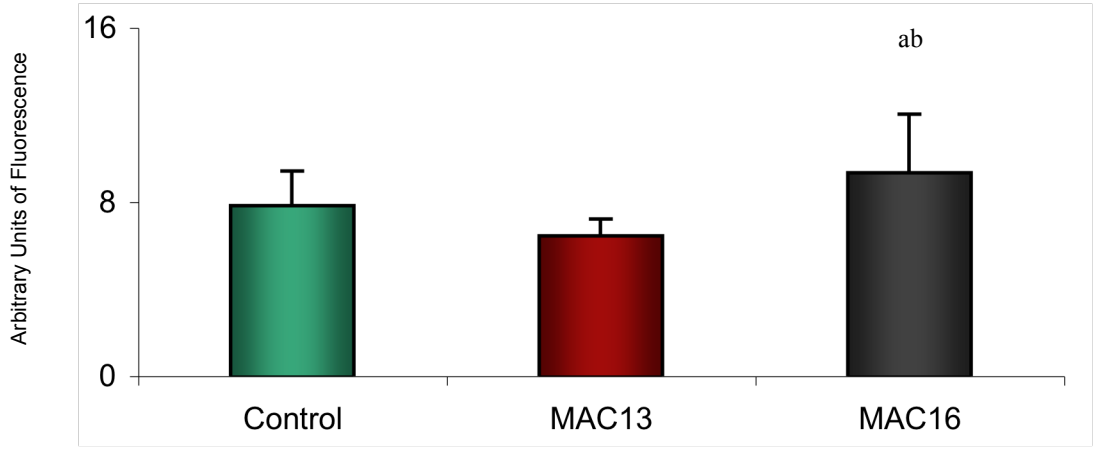


Figure 5.18 O_2^- levels in cancer-induced cachectic (MAC16), cancer-induced non-cachectic (MAC13) and control skeletal muscle, by histological DHE examination, expressed as arbitrary units of fluorescence. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by lower case letters, where Control = a, MAC13 = b and MAC16 = c.

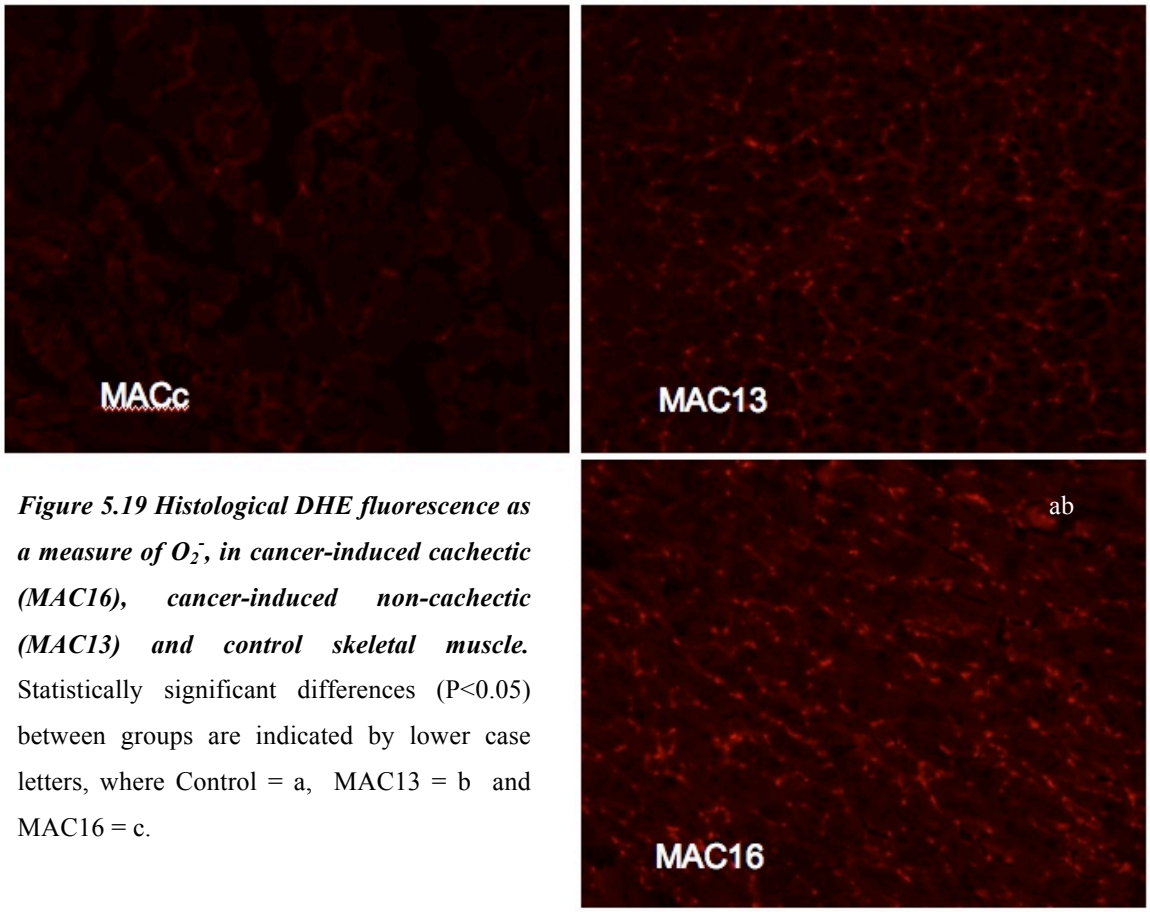


Figure 5.19 Histological DHE fluorescence as a measure of O_2^-, in cancer-induced cachectic (MAC16), cancer-induced non-cachectic (MAC13) and control skeletal muscle. Statistically significant differences ($P<0.05$) between groups are indicated by lower case letters, where Control = a, MAC13 = b and MAC16 = c.

5.4 Discussion

Cancer induces changes in skeletal muscle that causes an imbalance in protein synthesis and degradation, resulting in the loss of muscle protein and function (Argiles, Moore-Carrasco, Fuster et al. 2003; Giordano et al. 2003). Interestingly, not all cancer patients develop this secondary condition and it is this phenomena that has made the development of cancer-induced cachexia relatively undefined (Diffee et al. 2002; Giordano et al. 2003; Tijerina 2004). In order to further investigate this complex condition, we investigated a model that utilises two similar MAC models, only one of which induces the secondary muscle wasting condition of cancer-induced cachexia (MAC16). We observed a significant decline in the body weights (15-25%) and muscle mass of MAC16-induced mice, demonstrating the development of cachexia after MAC16 tumour cell induction, and this was not mimicked in the MAC13-induced mice. Interestingly, these physical changes to skeletal muscle were mirrored by significant cellular oxidative changes, in response to MAC16 induction and cachectic development. While this study observed a significant increase in O_2^- levels, in cancer-induced cachectic skeletal muscle, the rate limiting NOX2 enzyme subunit and regulatory and catalytic subunits; $p40^{phox}$ and $p67^{phox}$, for NOX2, were significantly decreased. These results would suggest that while there is an increase in O_2^- in cachectic skeletal muscle, NOX is not the primary source. However further to this, we demonstrated a significant decrease in the three SOD isoforms; SOD1, SOD2 and SOD3 and GPx expression, with a decline in SOD2 activity in cancer-induced cachectic skeletal muscle. Therefore the decline in antioxidant potential would appear to be a significant contributor to OS in cachectic skeletal muscle. Interestingly though, we observed a significant increase in the catalytic NOX2 enzyme subunit

p67^{phox}, and SOD3 gene expression, in skeletal muscle from MAC13-induced non-cachectic mice, compared to cachectic and control muscle. These results are therefore suggestive of a potential increase in NOX generated O_2^- production into the extracellular space within the skeletal muscle of cancer-induced non-cachectic mice. Together with the changes observed in cachectic skeletal muscle, these results suggest that cachexia is associated with a decrease in the NOX2 enzyme, and therefore questions the role of this NOX enzyme system in the cancer-induced cachectic condition.

Consistent with other studies (Barreiro et al. 2005; Gomes-Marcondes & Tisdale 2002; Sukhanov et al. 2007), we found a marked increase in O_2^- levels in cachectic skeletal muscle. These results suggest O_2^- accumulation and presence of OS with potential for oxidative damage by O_2^-, in cachectic skeletal muscle, despite the downregulation of the O_2^- producing NOX2 enzyme system. However, we demonstrated significant changes in the NOX2 enzyme system at the gene level that cannot be overlooked. The marked decrease in the NOX enzyme subunits and antioxidant enzymes in cachectic skeletal muscle and comparative upregulation in non-cachectic skeletal muscle is of interest in this study. In addition to the potential accumulation of oxidative damage that may directly contribute to the cachectic condition, the downregulation of NOX subunits and antioxidant enzymes is undoubtedly involved in the complex redox signaling that may lead to the development of cancer-induced cachexia. Furthermore, we observed MAC tumour-induced changes in the skeletal muscle of MAC16 and MAC13-induced mice. The decline in SOD2 activity observed in both the cachectic and non-cachectic skeletal muscle may indicate MAC tumour-induced changes to cellular metabolism and

mitochondrial function. Transcription factor NF-κB gene expression in MAC-induced skeletal muscle was also found to decrease. NF-κB is a complex that responds to and regulates a number of intracellular pathways. It is therefore difficult to ascertain its direct role in the important cellular changes during cancer, but suggest no direct attributable change in the development of cancer-induced cachexia.

5.4.1 Muscle Mass as an Indicator of Cancer-Induced Cachexia

Cachexia is the loss of lean body tissue and significant loss of skeletal muscle mass, which has been demonstrated in a number of animal models. However, the MAC model is unique in its induction of cachectic and non-cachectic tumours (Monitto et al. 2001; Whitehouse & Tisdale 2002). The MAC16 model of cancer-induced cachexia has been developed in murine models including the NMRI mouse (Whitehouse et al. 2001) and BALB/c *nu/nu* (Monitto et al. 2001). These studies have demonstrated various degrees of weight loss as a result of MAC16 induction and indicated protein degradation with significant weight loss. Smith and Tisdale (1993) observed progressive weight loss that was associated with protein degradation in MAC16-induced NMRI mice, with various degrees of weight loss greater than 10%. The present study established significant weight loss between 15-25% in MAC16-induced mice that was associated with a significant decline in skeletal muscle mass. Cancer-induced cachexia has been demonstrated as the fastest developing secondary condition compared to any other known wasting conditions. Lecker et al (2004) observed a 14% loss of muscle weight with a 63% increase in protein degradation in tumour bearing (Yoshida hepatoma) mice, establishing the highest degradation compared to similar muscle wasting conditions. These conditions included; fasting

(14% muscle weight loss and 49% protein degradation), chronic renal failure (29% muscle weight loss and 57% protein degradation) and diabetes (20% muscle weight loss and 40% protein degradation). In addition, cancer-induced cachexia has been suggested to develop as a result of calorie restriction, however this study like many others (Baracos 2000; Busquets et al. 2005; Monitto et al. 2001) did not observe any changes in food intake in MAC-induced mice. These results therefore suggest a more complex catabolic mediated protein degradation in the development of cancer-induced cachexia.

5.4.2 A Role of the NOX Enzymes in Cancer-Induced Cachexia

The knowledge that the NOX enzyme systems function primarily to produce O_2^- (Bedard & Krause 2007; Lambeth et al. 2000), which act as catabolic mediators in a variety of cells and tissues, recognises NOX generated O_2^- as more than a by-product of cellular metabolism, but rather a product of a regulated response to stimuli for a physiological purpose. Recently, ROS have been proposed as potential oxidative mediators in the development of cancer-induced cachexia (Barreiro et al. 2005; Fortunati et al. 2007; Gomes-Marcondes & Tisdale 2002), however the source of ROS in this process remains to be elucidated. To our knowledge, our studies are the first to investigate the NOX enzyme family in cancer-induced cachectic skeletal muscle. In this investigation, we have determined a significant depression in the regulatory subunit $p40^{phox}$, and the catalytic subunits NOX2 and $p67^{phox}$, of the NOX2 enzyme system. These subunits function to recruit the cytosolic subunits to the membrane, necessary for NOX2 enzyme assembly and activation (Babior 2002; El-Benna et al. 2005). Interestingly though, the NOX2 enzyme has consistently been

shown to increase in degenerative conditions (Harraz et al. 2008; Salles et al. 2005; Williams & Allen 2007), generating ROS and contributing to oxidative tissue damage, and we have previously shown an increase in the NOX2 enzyme subunits in the skeletal muscle of MAC16-induced NMRI cachectic mice. Therefore, this study questions the role of NOX in the skeletal muscle system and response to stimuli that may differ in the MAC16-induced Balb/c *nu nu* cachectic model. While this is interesting in itself and requires further investigation, these results suggest alternative roles for the NOX2 enzyme system in skeletal muscle and responses to cancer development.

This study demonstrated a significant decrease in NOX2 gene expression in cachectic skeletal muscle. NOX2 is a necessary component of NOX2 enzyme activation as it is the key catalytic subunit responsible for the reduction of molecular oxygen to O_2^- (Takeya & Sumimoto 2003). In addition, we also observed a decrease in the regulatory NOX2 enzyme subunit $p40^{phox}$ in cachectic skeletal muscle, necessary for cytosolic subunit regulation and translocation to the membrane. This regulatory role is important for the enzyme as it functions to stabilise the cytosolic subunits at rest and initiates their recruitment to the membrane upon appropriate stimulation (Babior, B. 2002; DeLeo & Quinn 1996). This was made evident through the inhibition of $p40^{phox}$ phosphorylation, a process that was found to inhibit NOX2 enzyme activation (Chen et al. 2007; Matute et al. 2005), through stabilising the cytosolic subunits and eliminating translocation to the membrane. In the NOX2 enzyme, one of the most important interactions between subunits is the one between $p40^{phox}$ and $p67^{phox}$, as their interaction directly results in recruitment to the membrane, for enzyme activation (Kuribayashi et al. 2002; Matute et al. 2005; Ueyama et al. 2007). We also

found a significant decrease in p67^{phox} gene expression in cachectic skeletal muscle, compared to the non-cachectic MAC13 and control muscle. The NOX2 enzyme subunit p67^{phox} is the cytosolic catalytic component that binds to NOX2, following translocation to the membrane and it is the interaction between p67^{phox} and NOX2 that activates the enzyme and generates O_2^- (Takeya & Sumimoto 2003). The downregulation of these important regulatory and catalytic subunits, in cachectic skeletal muscle, provides evidence for a depression in NOX2 activity potential, but also suggests its involvement in complex redox signaling that may contribute to the development of cancer-induced cachexia. NOX2 in particular, is regulated at the gene level, through redox-sensitive stimulators such as cytokines, hormones, growth factors, and ROS themselves (Colston et al. 2005; Frey et al. 2002; Gauss et al. 2007; Lambeth, Kawahara & Diebold 2007). Furthermore, the enzyme itself is regulated by phosphorylation of one or more of the subunits when appropriately stimulated. Interestingly, Ang II has been shown to induce the expression of p40^{phox} (Touyz et al. 2002) and p67^{phox} (Cifuentes et al. 2000) in vascular smooth muscle. Furthermore, Ang II has been well established to induce the cachectic response in skeletal muscle cells (Eley & Tisdale 2007; Russell, Sanders & Tisdale 2006; Sukhanov et al. 2007) and has been suggested to act through NOX-dependent ROS signaling (Russell, Eley & Tisdale 2007; Wei et al. 2006). This study however, does not support the Ang II-induced expression of these NOX subunits and NOX-dependent ROS signaling in cancer-induced cachectic skeletal muscle.

Further to the changes observed in cachectic skeletal muscle, this study demonstrated a significant increase in p67^{phox} in non-cachectic skeletal muscle, compared to control and cachectic muscle. These results suggest an increase in NOX2 activity potential in

non-cachectic skeletal muscle following cancer induction, further suggesting an important role for the NOX2 enzyme in non-cachectic tumour-bearing mice. The upregulation of the important catalytic subunit could suggest a protective role for NOX2 in skeletal muscle of tumour-bearing mice. Abid et al (2007) demonstrated a role for NOX-dependent ROS signaling in hypertrophy related activation of protein kinases, Akt and p38 MAPK in endothelial cells. Interestingly, Mofarrahi et al (2008) inhibited NOX2 and NOX4, demonstrating attenuation of the P13K/Akt/NF-κB hypertrophy pathway in skeletal muscle precursor cells, further demonstrating an important role for these NOX enzymes in promoting skeletal muscle hypertrophy. Therefore, while our results do not support the NOX enzymes as the source of O_2^- in cancer-induced cachectic skeletal muscle, our results give evidence for changes in the NOX2 enzyme system and therefore potential changes in NOX-dependent ROS signaling in cancer-induced skeletal muscle. Furthermore, our results may suggest a protective role for the NOX2 enzyme in skeletal muscle in cancer pathology, through regulation of important skeletal muscle growth pathways, providing further evidence for an alternative role for the NOX2 enzyme system in skeletal muscle.

Despite the increase in the subunit $p67^{phox}$, that regulates the NOX2 enzyme and initiates its activation, skeletal muscle from MAC13-induced mice did not show an increase in O_2^-. However, further to these results, this study demonstrated an increase in antioxidant enzyme SOD3 gene expression in non-cachectic skeletal muscle. NOX2 is thought to reside to the plasma membrane where $p67^{phox}$ binds to form the active complex and generation of O_2^-. In phagocytes, the NOX2 enzyme generates O_2^- into the extracellular space and although the localisation of the NOX2 enzyme and direction of O_2^- production in skeletal muscle cells has not yet been confirmed,

we can only speculate that the NOX2 enzyme system may function in a similar manner. This could be further speculated by the increase in the expression of SOD3 observed in our non-cachectic skeletal muscle, as it is the antioxidant enzyme that actively dismutates O_2^- in the extracellular space (Zelko, Mariani & Folz 2002). What is of particular interest though, is the decrease in the NOX2 enzyme subunits in cachectic skeletal muscle, compared to an increase in the non-cachectic skeletal muscle. Despite these differences, this study supports a role for the NOX2 enzyme system in cancer-induced cachexia.

It could be suggested that H_2O_2 generated extracellularly, from the dismutation of NOX-dependent O_2^- production by SOD3, diffuses back into the cell, influencing important intracellular redox signaling pathways. H_2O_2 has been shown to activate hypertrophy pathways upstream of hypertrophy related kinases (P13K & p70S6K) in cardiomyocytes (Tu, Bahl & Chen 2002). A protective role for NOX-dependent ROS generation has been demonstrated by Bell et al (2005) and Bendall et al (2002), suggesting an important role for the NOX enzyme in cardiac remodeling and hypertrophy. Interestingly, Ushio-Fukai et al (1999) demonstrated a positive role for Ang II-induced NOX-dependent H_2O_2 production in modulating hypertrophy pathways in vascular smooth muscle cells. Similarly, Djordjevic et al (2005) demonstrated a role for NOX in remodeling, through redox-sensitive activation of mitogen activated hypertrophy pathways in pulmonary hypertension. It is therefore possible that the NOX2 enzyme plays a role in promoting muscle growth pathways in cancer-induced non-cachectic skeletal muscle that appears to be absent in cachectic skeletal muscle. As we cannot simply ignore the significant downregulation of the NOX2 enzyme subunits in cachectic skeletal muscle, we propose a more complex role

for NOX2 in a redox signaling pathway that suggests an alternative role for NOX in the development of skeletal muscle wasting pathologies.

5.4.3 Oxidative Stress (OS) in Cancer-Induced Cachexia

Despite the significant downregulation of NOX subunit expression, our results indicated an increase in O_2^-, in cachectic skeletal muscle. Furthermore, we found no change in O_2^- levels in non-cachectic skeletal muscle, indicating that OS is implicated in the cancer-induced cachectic condition. However, the source of O_2^- leading to the increase in OS in this study remains unknown. OS develops from an increase in ROS, particularly in the absence of a well functioning antioxidant enzyme system, to eliminate ROS accumulation in the cell (Macdonald, Galley & Webster 2003; McCord & Edeas 2005; Scandalios 2005; Wei & Lee 2002). OS has been implicated in skeletal muscle wasting conditions, particularly due to the marked attenuation following antioxidant induction (Hussey, Bibby & Tisdale 1996). The decrease in SOD gene expression and activity that we found in our cachectic skeletal muscle, is suggestive of a decrease in O_2^- production that may, at least in part, be due to a decrease in NOX generated O_2^- production. In further support of this, we observed a decrease in the H_2O_2 scavenging antioxidant enzyme, GPx. OS develops from an imbalance in ROS and antioxidants in favour of ROS and as our results are not indicative of an increase in antioxidant activity, but rather a decrease, the changes observed in these oxidative associated systems is suggestive of a decrease in the production of O_2^- and H_2O_2. However, we observed an increase in O_2^- in cachectic skeletal muscle and therefore the decrease in antioxidant activity indicates antioxidant dysfunction, which may be the key contributor to OS in cancer-induced cachexia. Therefore, the accumulation of O_2^-, observed in cachectic compared to non-cachectic skeletal muscle in this study, may not be due to an increase in its production, but

rather a decrease in its necessary conversion to H_2O_2 and therefore H_2O_2 signaling. These changes in ROS levels in the cell would indeed influence redox-sensitive pathways and thererfore normal cellular function that may contribute to the development of cancer-induced cachexia.

5.4.4 Skeletal Muscle Atrophy in Cancer-Induced Cachexia

Lecker et al (2004) suggested an important role for ROS in the induction of the Ub ligases, Atrogin-1 and MuRF-1 mRNA in tumour-bearing cachectic muscle. The Ub-proteasome proteolytic pathway is known to induce muscle atrophy (Clavel et al. 2006; Lecker et al. 2004; Stitt et al. 2004). MuRF-1 and Atrogin-1 both encode Ub ligases, proteins that bind and mediate ubiquitination of specific substrates (Stitt et al. 2004). These two genes have been found to be upregulated in skeletal muscle atrophy and expressed specifically in skeletal and cardiac muscle (Glass 2003). MuRF-1 and Atrogin-1 play an important role in the breakdown of myofibrillar proteins and are highly induced in atrophying muscles and cachectic conditions (Cai et al. 2004b; Clavel et al. 2006; Edstrom et al. 2006; Lecker et al. 2004; Sacheck et al. 2004). A study by Doucet et al (2007) demonstrated atrophy related Atrogin-1 and MuRF-1 upregulation in the quadriceps muscle of COPD patients suffering skeletal muscle atrophy, while, Li et al (2003) demonstrated ROS-induced protein catabolism in skeletal muscle through upregulation of the Ub conjugation system, including Atrogin-1 and MuRF-1. Furthermore, Van Royen et al (2000) demonstrated an increase in TNF-α that was involved in the activation of the Ub-dependent proteolysis during tumour growth. These studies suggest a strong relationship between Ub ligase, MuRF-1 and Atrogin-1, activation and the downstream effect of cytokine-induced ROS production. Specifically, TNF-α appears to activate the proteasome pathway,

through the production of ROS and downstream activation of MuRF-1 and Atrogin-1 (Supinski & Callahan 2007).

Further to this, TNF-α-induced ROS signaling of the Ub ligases requires transcription factor activation (Li & Reid 2000; True, Rahman & Malik 2000). Transcription factors are particularly sensitive to redox signaling and are therefore likely to be involved in regulating atrophy related genes in cancer-induced cachexia (Cai et al. 2004b; Reid & Li 2001a). Transcription factors AP-1 and NF-κB have been directly associated with growth regulation pathways (Glass 2005; Moore-Carrasco et al. 2006). Zhou et al (2003) demonstrated a role for transcription factor NF-κB in the development of colon-26 adenocarcinoma-induced cachexia. Wyke and Tisdale (2005) demonstrated NF-κB-dependent regulation of the Ub-proteasome proteolytic pathway and protein degradation in murine C2C12 myoblasts. These transcription factors become activated via redox signaling, in response to ROS, and function to transcribe genes involved in cell growth regulation pathways (Klaunig & Kamendulis 2004; Valko et al. 2006). Although ROS have been demonstrated to activate NF-κB in the absence of TNF-α, Li & Reid (2000) demonstrated TNF-α activation of NF-κB downstream of ROS, stimulating muscle wasting. In addition, Hunter & Kandarian (2004) demonstrated inhibition of skeletal muscle atrophy and decreased NF-κB activity with unloading in NF-κB1 knockout mice. However, NF-κB is a transcription factor activated by various stimulatory pathways and transcribes various genes and has been proposed to also be involved in skeletal muscle hypertrophy, as well as atrophy. Chen et al (2007) demonstrated an NF-κB-dependent pathway in myocardic hypertrophic growth and Gupta et al (2002) demonstrated a positive role for NF-κB in mediating hypertrophy in cardiomyocytes, as was also found by Purcell et al (2001). Furthermore, Kramer and Goodyear (2007) reported a role for NF-κB in preventing

skeletal muscle atrophy as a particularly important proinflammatory response for muscle regeneration post exercise. The significant downregulation of NF-κB mRNA in our cachectic skeletal muscle may therefore be involved in suppressing muscle hypertrophy. Furthermore, the transcription of NOX2 is dependent on NF-κB (Frey, Ushio-Fukai & Malik 2008; Gauss et al. 2007) and may explain the decrease in our cachectic skeletal muscle. Furthermore, H_2O_2 is suggested to directly signal the transcription factor activation of NF-κB (Clark & Valente 2004; Kamata et al. 2002; Muller, Rupec & Baeuerle 1997). We have suggested that H_2O_2 is potentially decreased as a result of the significant decrease in SOD activity in cachectic skeletal muscle. Therefore, our results do not support an increase in NF-κB activation, downstream of ROS and activation of the Ub-proteasome pathway, in the development of cancer-induced cachexia that has been previously described. However, we can only speculate, from the knowledge that NF-κB influences antioxidant gene expression, that the decline in this important process would certainly contribute to the decline in ROS scavenging and increase in OS that we observed in our cachectic skeletal muscle.

Interestingly, a study by Busquets et al (2001) was unable to revert muscle wasting in cachexia through NF-κB inhibition. Similarly, Costelli et al (2005) did not support NF-κB involvement in the development of Yoshida AH-130 hepatoma-induced cachexia, but rather suggests AP-1 transcription activation. Similarly, Moore-Carrasco et al (2006) demonstrated an important role for transcription factor AP-1 signaling cascade in the gastrocnemius muscle of Yoshida AH-130 hepatoma-induced cachectic rats, by specifically influencing the expression of Ub conjugate enzymes. We did not measure additional transcription factor gene expression, such as AP-1,

however evidence suggests its involvement in the induction of proteolytic pathways in cancer-induced cachexia that may be of significance to this study. Thus, the downregulation of NF-κB in our non-cachectic skeletal muscle, despite an upregulation of SOD3 and $p67^{phox}$ and no change in additional SOD isoforms and NOX enzyme subunits, may also indicate additional transcription factor involvement or the important compensatory H_2O_2 signaling.

5.4.5 Antioxidant Enzymes in Cancer-Induced Cachexia

Antioxidants are important enzymes for the protection against harmful ROS and function to maintain a balanced cellular oxidative environment (Zelko, Mariani & Folz 2002). this study demonstrated a significant decrease in the antioxidant enzyme isoforms SOD1, SOD2, and SOD3 and GPx gene expression and SOD1 activity levels in cachectic skeletal muscle that was also associated with a decrease in NOX subunit gene expression. Taken together, these results would suggest a decrease in the need for ROS protection and elimination, however the increase in O_2^- that we observed in cachectic skeletal muscle would suggest otherwise. Furthermore, ROS can cause damage to cellular proteins (Mecocci et al. 1999; Scandalios 2005) and may therefore have lead to the decrease in NOX2 enzyme subunits and antioxidant enzymes. Further to this, the decrease in antioxidant gene expression and activity that function as part of the cellular defence system, for the elimination of O_2^- and H_2O_2, may indicate antioxidant dysfunction in cachectic skeletal muscle. These results also suggest influential signaling involving NOX2 subunits and ROS-specific antioxidants. However, the signaling pathway(s) leading to the decrease in NOX2 subunits and antioxidant enzyme expression, in cachectic skeletal muscle, remains unknown. In

response to OS, cells activate the expression of a number of genes via transcription factor regulation, leading to modifications in the gene expression of important proteins, including antioxidant enzymes (Liu et al., 2006) and those involved in muscle protein synthesis and regeneration (Klaunig & Kamendulis, 2004). It is evident from this study that the depression of these important antioxidant systems, would contribute to the increase in OS, consequently leading to cellular oxidative damage as well as significant changes in redox-sensitive signaling pathways and the development of cancer-induced cachexia.

Interestingly, a decrease in SOD2 activity in the skeletal muscle from MAC16 and MAC13-induced mice was observed. SOD2 is the O_2^- dismutating antioxidant located in the mitochondria for oxidative protection (Mates & Sanchez-Jimenez 1999). This result suggests a decrease in O_2^- dismutation in skeletal muscle mitochondria of MAC-induced mice. The mitochondria is the primary source of O_2^- generation as it is the site of metabolic ATP production and is generally highly enriched with antioxidants (Valko et al. 2006; Vielhaber et al. 2000). Mitochondrial ROS production has been implicated to play a significant role in degenerative diseases associated with muscle atrophy, such as aging sarcopenia (Dirks et al. 2006) and neurodegeneration (Kostrominova et al. 2007). These studies suggest mitochondrial dysfunction as a major causative factor in this development. Furthermore, our results would suggest an additional source of O_2^- production in cancer-induced cachectic skeletal muscle that is likely to be from the mitochondrial ETC as it is not only the major O_2^- generating system in skeletal muscle, but is also known to be increased in cancer-induced cachectic patients (Tisdale 1999). An increase in O_2^- from this system would indeed require a functional SOD2 antioxidant enzyme. Therefore, we could

speculate that the increase in O_2^- may induce mitochondrial damage that would certainly compromise normal cellular function and skeletal muscle degeneration and therefore potential cause of the cachectic response to cancer induction. However, Barreiro et al (2005) observed no changes in the mitochondrial antioxidants in tumour-bearing rats, despite elevated levels of protein oxidation markers in cachectic skeletal muscle. It is evident from this study that MAC induction is associated with a significant decrease in SOD2 activity, however because of the increase in O_2^- levels in cachectic skeletal muscle, the depression of SOD2 may be the key contributor to the development of cancer-induced cachexia.

5.4.6 Tumour-Derived Factors in Cancer-Induced Cachexia

Our results suggest a role for O_2^- in the development of cancer-induced cachexia that would indeed have an affect on skeletal muscle redox-sensitive signaling pathways. In particular, as ROS are recognised as important signaling molecules, involved in hypertrophy/atrophy pathways (Jackman & Kandarian 2004; Sandri 2008), the role of O_2^- in these muscle growth pathways requires investigation. Also of significance to this study, is that not all cancer patients develop cachexia and different cancers are more likely to induce cachexia than others. Patients with pancreatic and stomach carcinomas have one of the highest incidence of cancer-induced cachexia, whereas breast cancer, leukemia and sarcomas have the lowest incidence (Giordano et al. 2003), further suggesting that different cancer types induce differential changes in skeletal muscle.

It is evident that tumours induce metabolic changes in the host, which often result in the secondary loss of skeletal muscle tissue in cancer-induced cachexia (approximately 50%) (Giordano et al. 2003). PIF was isolated from cachexia-inducing MAC16 tumours (Smith & Tisdale 2003) and has been found in the urine and tumours of patients with cancer-induced cachexia, but not in weight stable patients (Wigmore et al. 2000). PIF has been shown to stimulate protein breakdown in C2C12 myotubes (Whitehouse & Tisdale 2003) and to also induce weight loss when administered to non-tumour bearing mice (Lorite, Cariuk & Tisdale 1997). This tumour-derived glycoprotein is thought to induce muscle proteolysis through the Ub-proteasome pathway (Smith, Wyke & Tisdale 2004; Whitehouse & Tisdale 2003). Khal et al (2005) observed a 3-5 fold increase in the expression of key components of the Ub-proteasome pathway (C2 and C5) in the skeletal muscle of cancer patients, which were highly dependent on the extent of weight loss. In addition, Khal et al (2005) found a similar pattern of proteasome expression to weight loss, as has been previously found by Smith and Tisdale (1993) in serum levels of PIF, in the MAC16 cachectic model. These studies suggest a link between the tumour-producing factor PIF, and induction of the proteasome pathway, in the pathogenesis of cancer-induced cachexia. Recently, Russell et al (2007) suggested that PIF activates the Ub-proteasome pathway, downstream of ROS. Specifically, it was suggested that ROS generated by NOX contributes to cachexia through numerous proteolytic pathways by inducing proteolysis, through activation of NF-κB and the Ub-proteasome pathway (Russell, Eley & Tisdale 2007). Furthermore, Smith, Wyke and Tisdale (2004) suggested that PIF induces Protein Kinase C (PKC)-dependent IKK, which phosphorylates I-kB-alpha degradation, initiating NF-κB activation and nuclear binding, consequently influencing a number of genes, and these genes are likely to be

associated with the Ub-proteasome proteolytic pathway. Further to this, NOX enzymes activity has been demonstrated downstream of PKC (Chenevier-Gobeaux et al. 2006; Frey et al. 2002; Grandvaux, Elsen & Vignais 2001). Furthermore, Inoguchi et al (2000) demonstrated PKC-dependent activation of NOX generated ROS in both vascular smooth muscle and endothelial cells. In addition, PKC has been shown to regulate TNF-α activation of NOX in endothelial cells (Frey et al. 2002).

Although we did not measure PIF in this study, we can only speculate from the well-recognised MAC16 model that PIF is produced by the MAC16 tumour and contributes to proteolysis and cancer-induced cachexia in our MAC16 model. PIF has been linked with increased proteolysis by inducing NF-κB expression in cultured muscle cells (Argiles, Busquets & Lopez-Soriano 2005), potentially through redox signaling (Russell, Eley & Tisdale 2007). However, we demonstrated a decrease in the NOX2 enzyme subunits as well as NF-κB in cachectic skeletal muscle, which does not support the PIF-induced NOX/ROS/NF-κB pathway of Ub related proteolysis. We have however proposed that the increase in O_2^- and decrease in O_2^- dismutation in our model would result in a consequential decrease in H_2O_2. Therefore, H_2O_2 may be an important central mediator of this pathway, maintaining gene transcription through NF-κB activation that is decreased in cachexia. While we demonstrated significant muscle atrophy in MAC16-induced cachectic skeletal muscle compared to MAC13-induced non-cachectic and control muscle, yet do not support the typical PIF-induced proteolytic pathway and induction of atrophy, we propose that skeletal muscles capacity to regenerate is substantially compromised in our model of cancer-induced cachexia. It is evident from many degenerative studies that regeneration, following oxidative insult, is an important process in maintaining

cellular function and overall tissue composition. Langen et al (2006) demonstrated impaired muscle regeneration capacity in the skeletal muscle of COPD patients that was associated with a significant increase in TNF-α mRNA expression. TNF-α-induced muscle atrophy has been previously suggested to depend on NF-κB activation (Jackman & Kandarian 2004). However, the Langen study (2006) showed that impaired muscle regeneration was a result of decreased MyoD synthesis that was independent of NF-κB activation.

5.4.7 Skeletal Muscle Hypertrophy in Cancer-Induced Cachexia

Monitto et al (2004) investigated the differences in the gene expression profiles of the MAC13 and MAC16 cell lines, in order to understand further, the complex interactions and potential pathways that lead to the development of cancer-induced cachexia after MAC induction. Interestingly, a marked increase in IGF binding protein-4 expression has been demonstrated in the MAC16 cells, but not the MAC13 cells. IGF binding protein-4 has been shown to inhibit the binding of IGF to the IGF receptor (Monitto et al. 2004). IGF-1 is a key component of the major hypertrophy pathway in skeletal muscle (Appendix 2). IGF-1 induces skeletal muscle hypertrophy by activating the IGF-1 receptor, which initiates signaling pathways involved in muscle regeneration and protein synthetic pathways (Barton et al. 2002; Song et al. 2005). Decreased levels of IGF-1 have been proposed to play a key role in the induction of muscle wasting conditions, including cancer-induced cachexia. Simons et al (1999) found decreased levels of IGF-1 in lung cancer patients, attributing its involvement to the development of cancer-induced cachexia. Sanders et al (2005) inhibited Ang II-induced Ub-proteasome-dependent degradation in skeletal muscle myotubes through IGF-1 treatment, suggesting a role for IGF-1 in cancer-induced

cachexia. IGF-1 promotes cell survival through hypertrophy pathways and signals the depression of the atrophy-inducing Ub-proteasome proteolytic pathway in skeletal muscle (Song et al. 2005). IGF-1 stimulates intracellular kinases such as PI3K and PKB/Akt that form the major growth pathway that play a prominent role in increasing protein synthesis, associated with muscle hypertrophy (Kandarian & Jackman 2006; Stitt et al. 2004). Interestingly, Stitt et al (2004) demonstrated a regulatory role for the IGF-1/PI3K/Akt skeletal muscle hypertrophy pathway in suppressing atrophy, through inhibition of atrophy related MuRF-1 and Atrogin-1. Likewise, the suppression of the hypertrophy related IGF-1/P13K/Akt pathway, can lead to skeletal muscle atrophy (Latres et al. 2005; Stitt et al. 2004). Specifically, suppression of IGF-1/P13K/Akt pathway has been shown to increase the transcription of MuRF-1 and Atrogin-1 and induce myofibrillar protein degradation (Sacheck et al. 2004). Costelli et al (2006) demonstrated a 50% decrease in IGF-1 mRNA in cachectic gastrocnemius muscle of AH-130 hepatoma bearing rats, which was associated with a significant upregulation of the atrophy related Atrogin-1 and MuRF-1. Leger et al (2006) demonstrated a decrease in Akt and increase in Atrogin-1 in severely atrophied skeletal muscle of ALS patients. It is therefore possible that our non-cachectic skeletal muscle has a functional or potentially stimulated IGF-1/P13K/Akt hypertrophy pathway that suppresses the expression of atrophy related genes and may further explain the downregulation of NF-κB. Furthermore, ROS have been associated with the IGF-1 hypertrophy pathway, with studies demonstrating a role for IGF-1 in the elimination of OS and potential damage. Pi et al (2007) demonstrated a protective role for IGF-1 in hypoxic cardiomyocytes, through the reduction of ROS associated damage. Similarly, Sukhanov et al (2007) demonstrated an increase in circulating IGF-1 levels that was associated with deceased vascular inflammation and OS in

ApoE-deficient mice. This would give further evidence for a functional IGF-1 system in non-cachectic skeletal muscle, due to the presence of a stable oxidative environment, compared to controls. Further to this, it has been proposed that ROS, involved in muscle atrophy pathways, may act to block the IGF-1/PI3K/Akt pathway when overproduced (Latres et al. 2005; Sandri 2008). Therefore it is possible that ROS generation signals the suppression of the IGF-1/P13K/Akt pathway, decreasing the ability of muscle to regenerate. This process is important in our cachectic skeletal muscle due to the potential oxidative tissue damage, directly associated with the increase in ROS and OS. Interestingly, Russell et al (2007) recently demonstrated an important role for ROS in cancer-induced cachexia, with marked attenuation following antioxidant administration, in PIF and Ang II-induced murine myotubes. The decrease in protein degradation and increase in protein synthesis was attributed to the depression of NOX generated ROS that appeared to also involve PI3K. Treatment with a highly selective inhibitor of PI3K attenuated NOX-dependent ROS production and total protein degradation. This particular study demonstrated that P13K, known for its role in the IGF-1/P13K/Akt hypertrophy pathway (Latres et al. 2005; Stitt et al. 2004), also plays a potentially important role in stimulating NOX-dependent ROS-induced atrophy. These findings may further explain the depression of NOX2 enzyme activation, through the decrease in IGF-1/P13K stimulus, in our cachectic skeletal muscle. Furthermore, these findings suggest an important link between atrophy and hypertrophy pathways in skeletal muscle, suggesting a dual regulatory role for P13K that may function to inhibit atrophy, when hypertrophy is suppressed. Therefore, in addition to the potential decrease in NOX associated protein degradation, there is some evidence to suggest a decline in muscle regeneration following oxidative tissue damage, in cancer-induced cachexia. Collectively, the results of these studies further

demonstrate the complex signaling that results in selective downstream signals, mediating a variety of cellular responses.

5.4.8 Conclusion

It is evident from cachectic studies that show attenuation following antioxidant administration that ROS plays an important role in the development of cachexia. While the exact mechanisms are poorly defined, experimental research and our studies in the NOX enzyme systems, in skeletal muscle, have indicated a number of important roles for ROS. In addition to the recognised role of ROS in oxidative tissue damage evidence suggests a significant role for ROS in mediating cellular growth pathways. While studies have implicated a role for ROS and associated oxidative damage as a major contributor in the pathogenesis of cachexia, and have suggested a role for NOX, this study is suggestive of an increase in O_2^- that does not appear to be the direct result of increased NOX enzyme activation. Although this would indicate an alternative source of O_2^- production in cachectic skeletal muscle, we cannot ignore the marked decrease in antioxidant compensation. However, it is also important to consider that although we demonstrated a decrease in the catalytic and regulatory subunits of the NOX2 enzyme system, a dysfunction in SOD activity would indeed compromise the dismutation of even normal cellular O_2^- production. The inability of SOD to actively dismutate O_2^- is therefore proposed to be a major contributing factor in the enhanced levels of O_2^- observed in cancer-induced cachectia. Furthermore, it is possible to speculate from this that the downregulation of the NOX2 enzyme system is a compensatory response to O_2^- accumulation, induced primarily by SOD antioxidant system dysfunction and is therefore a regulated response to O_2^- buildup in the cellular system. While a role exists for NF-κB in gene transcription regulation and

involvement in regulating skeletal muscle growth pathways, the induction of NF-κB does not appear to have a role in cancer-induced cachexia. While these results, along with the additional findings of this study indeed demonstrate complex changes in cachectic skeletal muscle, this multifactoral condition coupled with a multifunctional system, further demonstrates the complexity of skeletal muscle response(s) to cancer induction.

The changes that we observed in sarcopenic and cachectic skeletal muscle have appeared to converge on a common finding of antioxidant dysfunction or decreased compensation to oxidative changes. What we do not know however is whether this result is a regulated response in the cellular system or an important contributor to the changes observed in skeletal muscle physiology. Nonetheless, we have observed an increase in O_2^- and a decrease in SOD in sarcopenic and cachectic skeletal muscle that indeed indicates changes in oxidative homeostasis and potential for oxidative damage. It is therefore important to understand further the role that SOD plays in the skeletal muscle system and in conditions of muscle wasting.

Chapter 6 SOD1^{G93A} Study

6.1 Introduction

SOD1 is an important cytosolic (Zelko, Mariani & Folz 2002) and mitochondrial (Schon & Manfredi 2003) located enzyme that facilitates the conversion of O_2^- to H_2O_2. Dominant mutations in this enzyme have shown symptoms of neurodegeneration and onset of ALS (Harraz et al. 2008). The FALS, making up approximately 10% of ALS cases, of which 20% are associated with the dominant SOD1 mutations (Mahoney et al. 2006) and suffer a toxic 'gain-of-function' (Dobrowolny et al. 2005). Neurodegenerative studies have focused primarily on neuronal tissue to further understand the effects of SOD1 overexpression in the progression and development of ALS (Harraz et al. 2008; Jaarsma et al. 2000; Rohde et al. 2008; Watanabe et al. 2001). However, the progressive neurodegeneration that develops in ALS patients includes the degeneration of motor neurons as well as skeletal muscle that leads to debilitating paralysis (Dobrowolny et al. 2005) in the final stages of the disease. In addition, ALS has been proposed as a multisystem disorder of which skeletal muscle plays an important role in the pathogenesis of the disease (Mahoney et al. 2006), and therefore deserves greater attention.

There are a number of SOD1 mutant animal models that have been extensively used to investigate and further understand the ALS condition (Grieb 2004). The SOD1^{G93A} mouse model of ALS is the most widely used as it has been shown to give reproducible similarities to human ALS, as well as a relatively short lifespan

(Mahoney et al. 2006). SOD1 mutant transgenic mice carrying the common G93A mutation develop severe motor neuron disease, reminiscent of ALS (Schon & Manfredi 2003). In addition to the neurodegeneration, the SOD1^{G93A} also displays hindlimb weakness and atrophy (Brooks et al. 2004; Dobrowolny et al. 2008), beginning at about 100 days of age and progresses to complete paralysis and death within 130-140 days of age (Mahoney et al. 2006). Interestingly, this mutation does not appear to affect functional activity, but rather has been suggested to result in a 'gain-of-function' and therefore an increase in SOD1 mediated ROS generation (Dobrowolny et al. 2005; Kostrominova et al. 2007; Mahoney et al. 2006).

6.1.1 Oxidative Stress (OS) in SOD1 Overexpression

The important role of antioxidant enzymes in regulating both oxidative enzyme systems (Harraz et al. 2008) and cellular OS, indeed proposes a role for ROS in a system where SOD is altered. As has been mentioned previously, OS has been proposed to play a key role in various degenerative conditions in a number of tissue types (Barreiro et al. 2005; Maier & Chan 2002). An increase in OS and associated oxidative damage has been observed in neuronal tissue of ALS patients (Vielhaber et al. 2000) and in the SOD1^{G93A} mutant model (Dobrowolny et al. 2008; Harraz et al. 2008; Mahoney et al. 2006). Furthermore, reports of antioxidant supplementation have demonstrated clinical benefits in SOD1^{G93A} transgenic mice (Mahoney et al. 2006). The alteration of function in the SOD1 enzyme and the associated increase in ROS production still remains relatively undefined. However, it is proposed to develop due to the increase in SOD1 O_2^- dismutation (Harraz et al. 2008; Mahoney et al. 2006). Specifically, it has been reported that redox-dependent dissociation of SOD1 is

impaired in SOD1 mutant models, which leads to sustained activation and H_2O_2 generation (Harraz et al. 2008).

It is well known that ROS are generated in skeletal muscle as an essential by-product of cellular metabolism (McArdle et al. 2004; Vasilaki et al. 2006) and oxidative enzymes (Hidalgo et al. 2006). Due to the high metabolic activity and oxidative capacity of skeletal muscle, the antioxidant system is a crucial component for the maintenance of cellular oxidative homeostasis (Ji 2007). Alterations in this important protective system can lead to oxidative imbalance, and induce critical consequences to cellular structure and function (Blokhina, Virolainen & Fagerstedt 2003; Esposito et al. 1999; Johnson & Giulivi 2005; McCord & Edeas 2005). Therefore, ROS are potential key players in skeletal muscle degeneration, in a cellular environment where SOD1 function is significantly altered. Although ROS appear to be implicated in tissue degeneration and may play a key role in SOD1^{G93A} associated muscle degeneration, what remains to be elucidated are the changes in cellular oxidative systems as a result of SOD1 modification in skeletal muscle. Furthermore, investigating oxidative cellular systems most likely to be effected by changes in SOD, may give further insight into potential redox-sensitive cellular signaling pathways that lead to muscle atrophy and wasting conditions.

6.1.2 A Role for the NOX Enzymes in SOD1 Overexpression

In addition to gross atrophy and neurodegeneration, SOD1^{G93A} transgenic mice demonstrate severe mitochondrial dysfunction, hypermetabolism and total SOD upregulation (Mahoney et al. 2006), each of which are associated with elevated levels of ROS (Esposito et al. 1999; Giordano et al. 2003; Kowald, Lehrach & Klipp 2006).

It is well recognised that although the major source of ROS generation in skeletal muscle is the mitochondrial ETC (Fulle et al. 2004), other independent enzyme systems, such as the NOX, are also important sources of ROS generation (Valko et al. 2007). Interestingly, ROS have been shown to stimulate NOX activity and therefore the potential increase in mitochondrial dysfunction, metabolism and SOD1 activity are all likely contributors of an increase in ROS that may stimulate NOX activation and further ROS production (Colston et al. 2005). Furthermore, NOX has been shown to play a key role in various degenerative conditions (Griendling, Sorescu & Ushio-Fukai 2000; Sukhanov et al. 2007; Williams & Allen 2007) and has been associated with the development of neurodegeneration and ALS (Harraz et al. 2008; Marden et al. 2007; Wu et al. 2006). We were therefore interested in investigating this important ROS producing enzyme system, as an alternative source of ROS that may play a key role in the degeneration of skeletal muscle, in the SOD1^{G93A} mutant model.

6.1.3 Antioxidant Enzymes in SOD1 Overexpression

The primary function of SOD is to catalyze the conversion of two O_2^- molecules to molecular oxygen and H_2O_2 (Ermilova et al. 2005). This dismutation of O_2^- is important to avoid cumulative OS that skeletal muscle is particularly vulnerable to (Sundaram & Panneerselvam 2006). Therefore, a dysfunction in SOD would certainly result in critical consequences to the cell. The consequences of SOD antioxidant enzyme modifications have been demonstrated in studies investigating SOD knockout models. Muller et al (2006) demonstrated an age-dependent loss of muscle mass in mice lacking SOD1, as well as a significant decrease in their average lifespan. Furthermore, Sun et al (2002) investigated the effects of SOD1 and SOD2

overexpression in Drosphila that demonstrated a decrease in cumulative oxidative damage and increased metabolic potential, with an increased lifespan by up to 37% and 75% respectively (Sun et al. 2002). Further to this however, the mouse models of SOD1 overexpression present with severe neurodegeneration, skeletal muscle wasting and a significant decline in lifespan, similar to the human characterised ALS condition (Grieb 2004). These models demonstrate a crucial role for SOD1 shown through its significant contribution to morbidity and mortality.

SOD, not only has a crucial role in eliminating O_2^- accumulation, but also plays an important role in intracellular redox-sensitive signaling and regulation of oxidative associated systems (Blokhina, Virolainen & Fagerstedt 2003; Ji 2007). As ROS have been described as important mediators of redox-sensitive intracellular signaling, so too are the antioxidants that regulate them. It is well known that with the generation of intracellular O_2^-, SOD1 functions to dismutate O_2^- to H_2O_2 (Zelko, Mariani & Folz 2002). H_2O_2 in particular, has been shown to be involved in numerous signaling cascades and therefore its cellular regulation, via SOD, has the potential to influence a number of important cellular pathways (Rhee et al. 2005; Stone & Yang 2006; True, Rahman & Malik 2000). It is evident that the SOD1 mutant associated ALS has a change in function, whether that is a 'gain-of-function' or a dysfunctional SOD1, despite the overexpression (Dobrowolny et al. 2005; Maier & Chan 2002). The changes in SOD1 function would indeed have critical consequences to cellular function that is most certainly redox related. Therefore, it would be expected that the modification to SOD1 function would influence OS in most tissues of the $SOD1^{G93A}$ model, including skeletal muscle, and may also induce changes in ROS generating enzyme systems such NOX. With the current knowledge that NOX generated ROS

are involved in intracellular signaling pathways in a number of tissues as well as its implication in muscle growth pathways, the NOX enzymes are likely to play a key role in skeletal muscle pathology in the SOD1^{G93A} mutant model. Therefore, it is important to investigate this major ROS generating enzyme system and oxidative contribution(s), to further understand the changes in skeletal muscle that induce muscle wasting in this model.

6.2 Methods

6.2.1 Animal Model of SOD1 Overexpression

The SOD1^{G93A} transgenic model was used for this study. Transgenic mice carrying the G93A human SOD1 mutation and WT human SOD1^{G93A} transgene were obtained from Jacksons Laboratory (Bar Harbor, ME, USA) and bred at The Howard Florey Institute (Melbourne, Australia). Offspring were genotyped by PCR assay of DNA extracted from tail tissue and comparisons were made with mice with an overexpression of the WT human SOD1 gene. All mice used in this study were maintained under controlled environmental conditions; 12 hour light/dark cycle, 21 ± 2°C, 30% humidity, in conventional cages with *ad libitum* access to standard chow and water throughout the course of the study. Mice were monitored daily and staged for disease phenotype according to Turner et al (2003). SOD1^{G93A} mice, clinically scored for stage III disease progression, and SOD1^{WT} controls were anaesthetised using pentobarbital sodium (70mg/kg) and skeletal muscle tissue was collected and immediately snap frozen in liquid nitrogen and stored at -80°C for later use.

6.2.2 Reverse Transcription-Real-Time PCR

RNA was extracted from frozen quadriceps muscle using Tri Reagent (Molecular Research Centre, USA) according to the manufacturer's protocol. Total RNA concentration was determined spectrophotometrically at 260/280nm. Prior to RT all RNA samples were DNase treated (Promega, Australia) and then first-strand cDNA was generated from 1µg RNA using AMV RT (Promega, Australia). The cDNA was stored at -20°C for subsequent analysis. Pre-designed TaqMan Gene Expression

Assays (Applied Biosystems, USA) were used containing specific primers and probes for the genes of interest (Table 2.2). Real-time PCR was performed using Applied Biosystems 7500 detection system and PCR reactions were performed using TaqMan Gene Expression Master Mix (Applied Biosystems, USA). Briefly, a real-time PCR mix of 50% TaqMan Gene Expression Master Mix (Applied Biosystems, USA) and 0.5% TaqMan Gene Expression Assay Mix (20x) and cDNA, optimised to specific Gene Expression Assays, was run for 40 cycles of PCR in a total volume of 25µl. To compensate for variations in input RNA amounts and efficiency of reverse transcription, GAPDH mRNA was quantified and all results were normalised to these values. Fluorescent emission data were captured and mRNA levels were analysed using the C_T value (Schmittgen et al. 2000). The $2^{\Delta}C_T$ was calculated by subtracting the ${}^{\Delta}C_T$ for GAPDH mRNA from the ${}^{\Delta}C_T$ for the gene of interest. The relative expression of the gene of interest was calculated using the expression $2\ {}^{\Delta}C_T$ and reported as arbitrary units.

6.2.3 Detection of O_2^- by DHE Fluorescence Staining

Skeletal muscle O_2^- was measured using O_2^- sensitive DHE dye. Cell permeable DHE reacts with O_2^-, converting DHE into ethidium fluorescence (Serrander et al. 2007). DHE (5µM) was applied to quadriceps cross-sections (5µm) and incubated in a light protected oven at 37°C for 30 minutes. The sections were washed with PBS to remove excess DHE and fluorescence was assessed by way of fluorescence microscopy (Axiocam HBO 50/AC, Zeiss, Germany). The section was analysed in three sections to obtain measurements from the whole tissue. Ethidium fluorescence density was detected from the whole section with MCID imaging software (Imaging

Research Inc) and expressed as arbitrary units of fluorescence (Azumi et al. 2002; Miller et al. 2002; Williams & Allen 2007)

6.2.4 Statistical Analysis

Statistical analysis was performed using SPSS statistical package (version 15.0). Results are expressed as mean ± SEM. Differences were determined by independent t-test and results were considered statistically significant if *p*-values were equal to or <0.05.

6.3 Results

6.3.1 Reverse Transcription-Real-Time PCR

6.3.1.1 NOX Subunit Gene Expression

NOX subunit gene expression was measured in the quadriceps muscle of $SOD1^{G93A}$ and $SOD1^{WT}$ controls. Results are expressed as units of gene expression for each of the NOX subunis, NOX2, NOX4, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac1. The mRNA expression of the membrane subunit $p22^{phox}$ (p=0.036) (Figure 6.3) and cytosolic subunits $p67^{phox}$ (p<0.001) (Figure 6.4), $p47^{phox}$ (p=0.039) (Figure 6.5), and $p40^{phox}$ (p=0.011) (Figure 6.6) were significantly lower in skeletal muscle from $SOD1^{G93A}$ mice compared to $SOD1^{WT}$ controls. However, no change was observed in the mRNA expression of NOX2 (Figure 6.1), NOX4 (Figure 6.2) or Rac1 (Figure 6.7) in skeletal muscle quadriceps from $SOD1^{G93A}$ mice compared to $SOD1^{WT}$ controls.

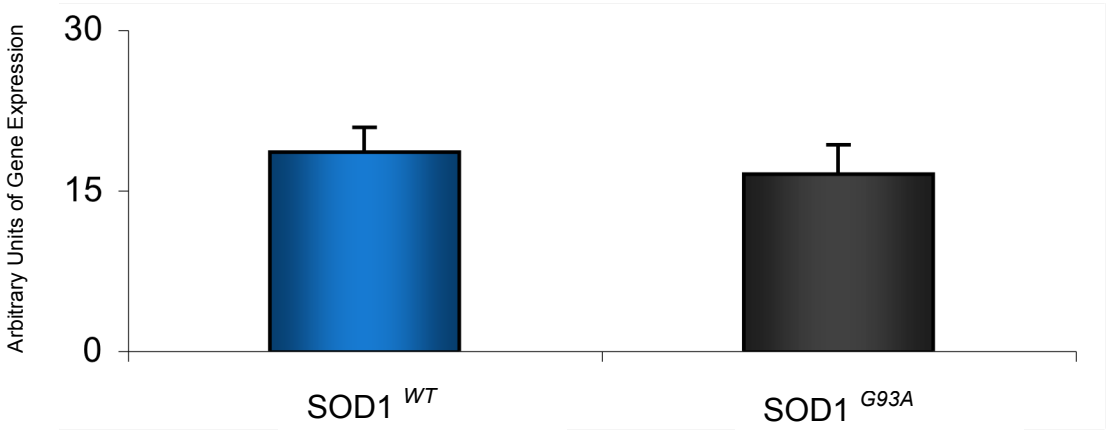


Figure 6.1 The mRNA levels of NOX2 in SOD1^{G93A} and SOD1^{WT} skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by *.

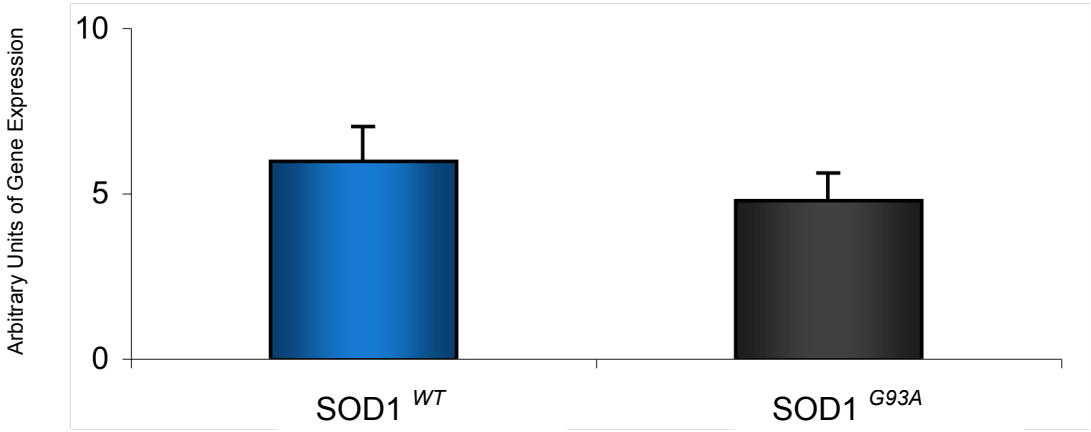


Figure 6.2 The mRNA levels of NOX4 in SOD1^{G93A} and SOD1^{WT} skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by *.

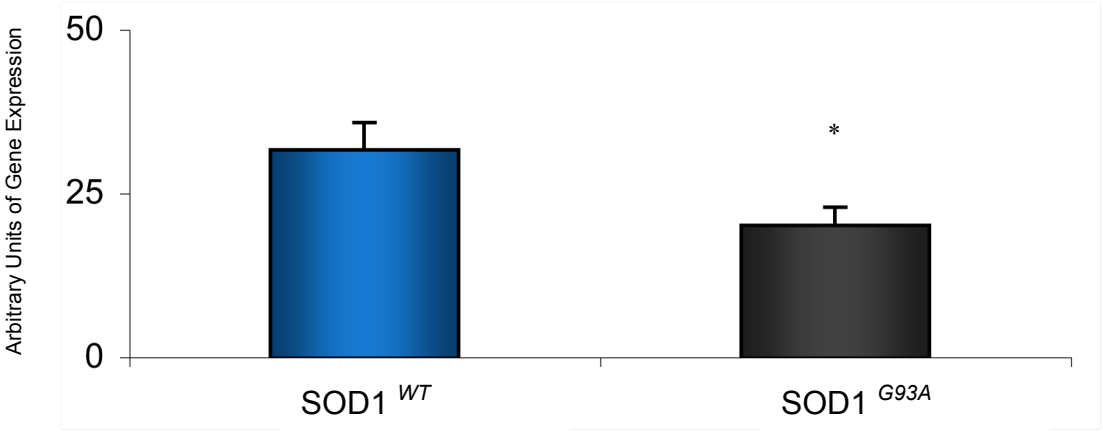


Figure 6.3 The mRNA levels of p22^{phox} in SOD1^{G93A} and SOD1^{WT} skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by *.

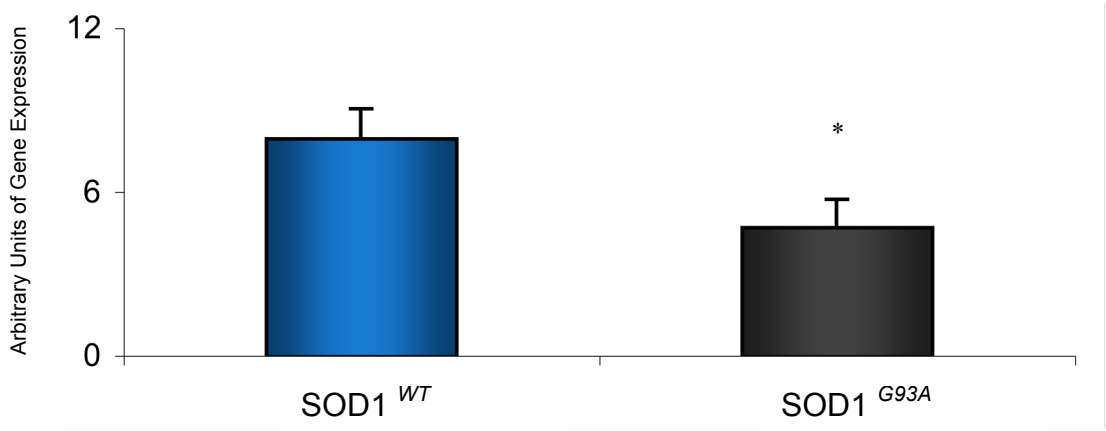


Figure 6.4 The mRNA levels of p67^{phox} in SOD1^{G93A} and SOD1^{WT} skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by *.

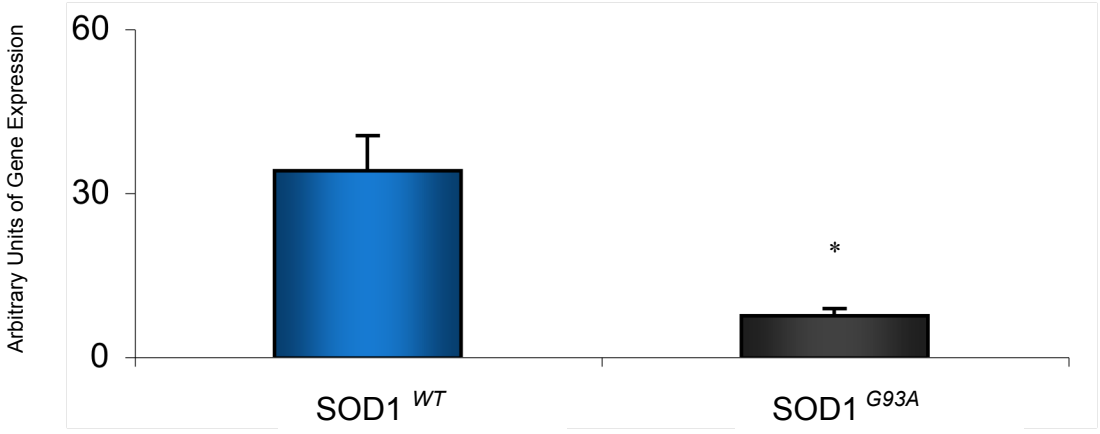


Figure 6.5 The mRNA levels of p47^{phox} in SOD1^{G93A} and SOD1^{WT} skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by *.

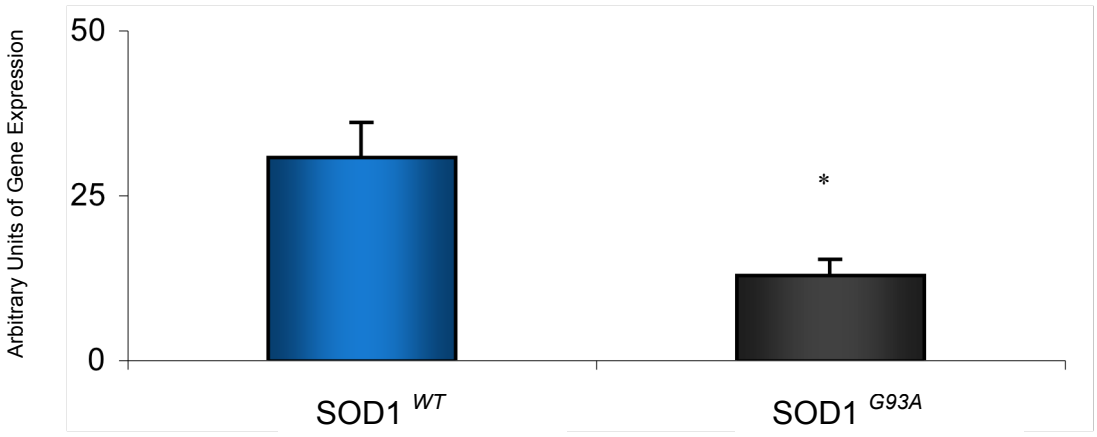


Figure 6.6 The mRNA levels of p40^{phox} in SOD1^{G93A} and SOD1^{WT} skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by *.

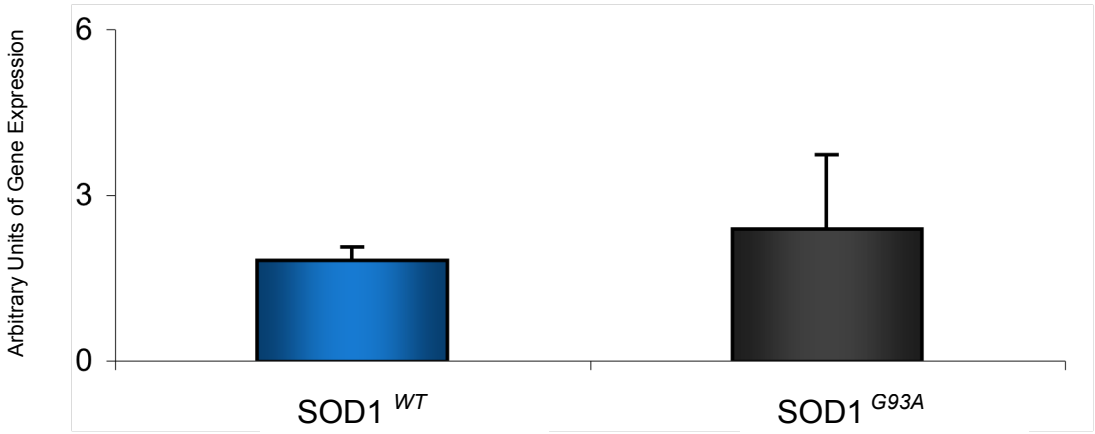


Figure 6.7 The mRNA levels of Rac1 in SOD1^{G93A} and SOD1^{WT} skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences ($P<0.05$) between groups are indicated by *.

6.3.1.2 Antioxidant Enzyme Gene Expression

Antioxidant enzyme gene expression was measured in the quadriceps muscle of SOD1^{G93A} and SOD1^{WT} control mice. Results are expressed as units of gene expression for each of the SOD isoforms, SOD1, SOD2 and SOD3, and GPx and catalase. SOD2 mRNA expression was significantly lower in the skeletal muscle of SOD1^{G93A} mice, compared to SOD1^{WT} controls (p=0.017) (Figure 6.9). However, no changes were observed in the mRNA expression of SOD1 (Figure 6.8) and SOD3 (Figure 6.10) in the skeletal muscle of SOD1^{G93A} mice compared to SOD1^{WT} controls. In addition, the mRNA expression of GPx (p=0.008) (Figure 6.11) and catalase (p=0.016) (Figure 6.12) were significantly higher in skeletal muscle from SOD1^{G93A} compared to SOD1^{WT} controls.

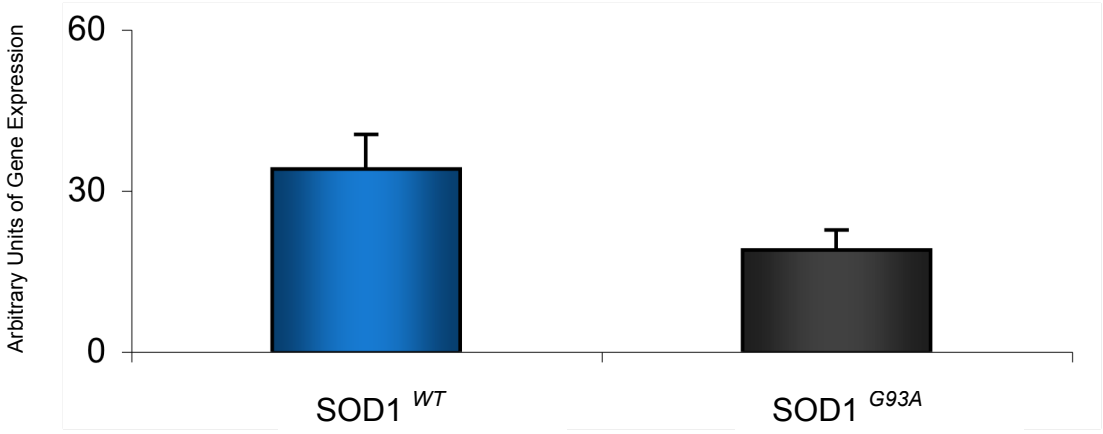


Figure 6.8 The mRNA levels of SOD1 in SOD1^{G93A} and SOD1^{WT} skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by *.

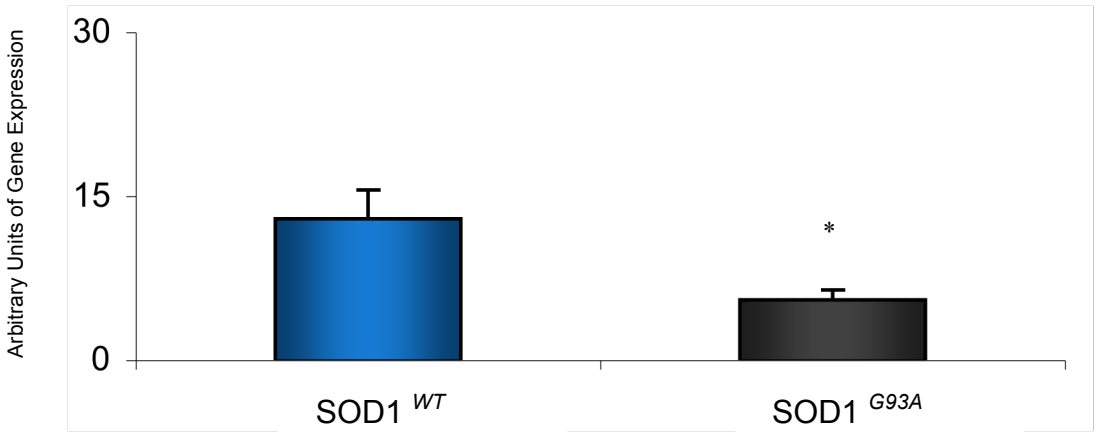


Figure 6.9 The mRNA levels of SOD2 in SOD1^{G93A} and SOD1^{WT} skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by *.

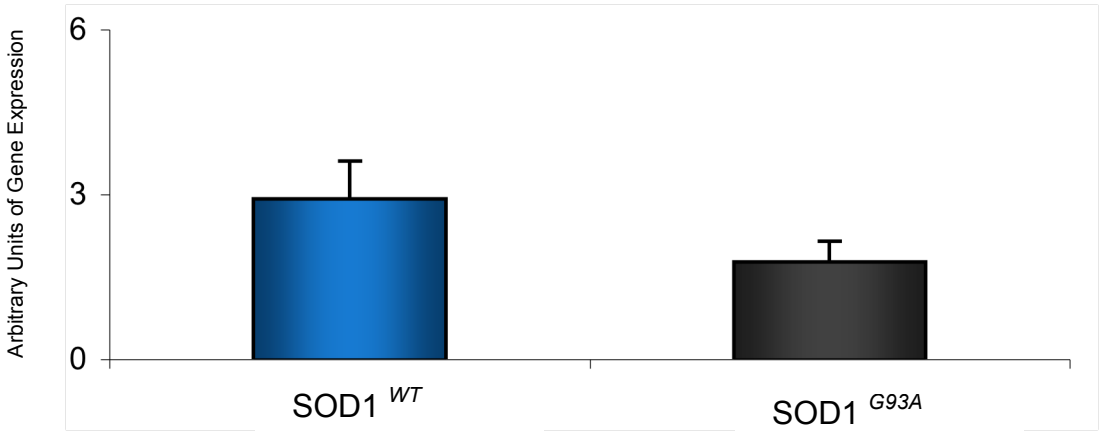


Figure 6.10 The mRNA levels of SOD3 in SOD1^{G93A} and SOD1^{WT} skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by *.

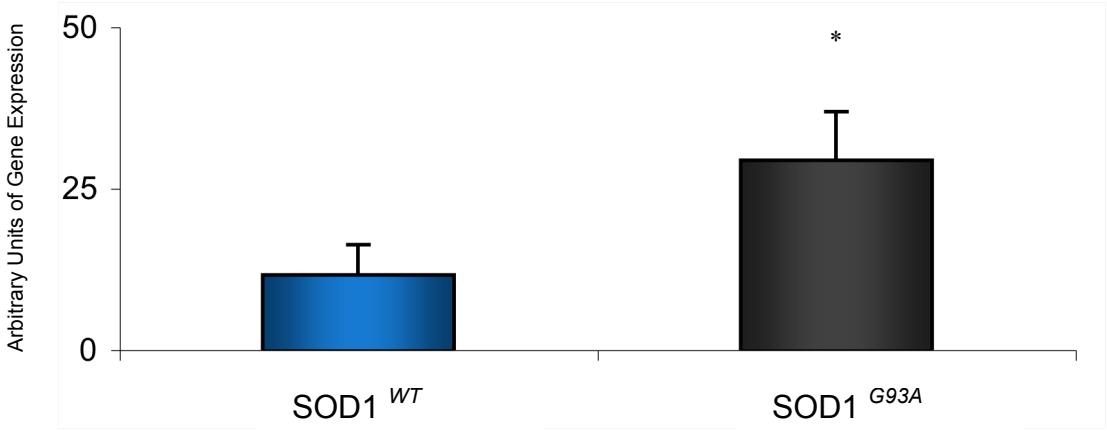


Figure 6.11 The mRNA levels of GPx in SOD1^{G93A} and SOD1^{WT} skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by *.

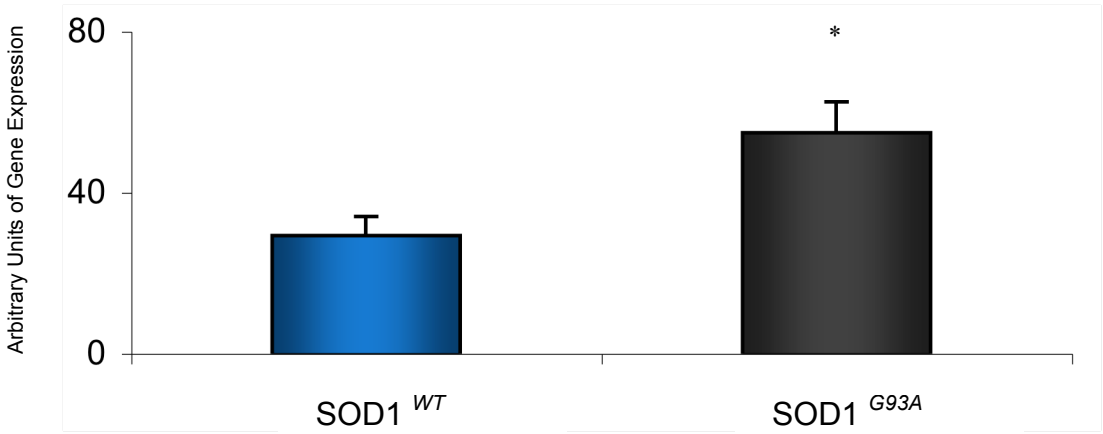


Figure 6.12 The mRNA levels of catalase in SOD1^{G93A} and SOD1^{WT} skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by *.

6.3.1.3 NF-κB Gene Expression

The mRNA expression of transcription factor NF-κB was significantly lower in skeletal muscle from SOD1^{G93A} compared to SOD1^{WT} controls (p=0.005) (Figure 6.13).

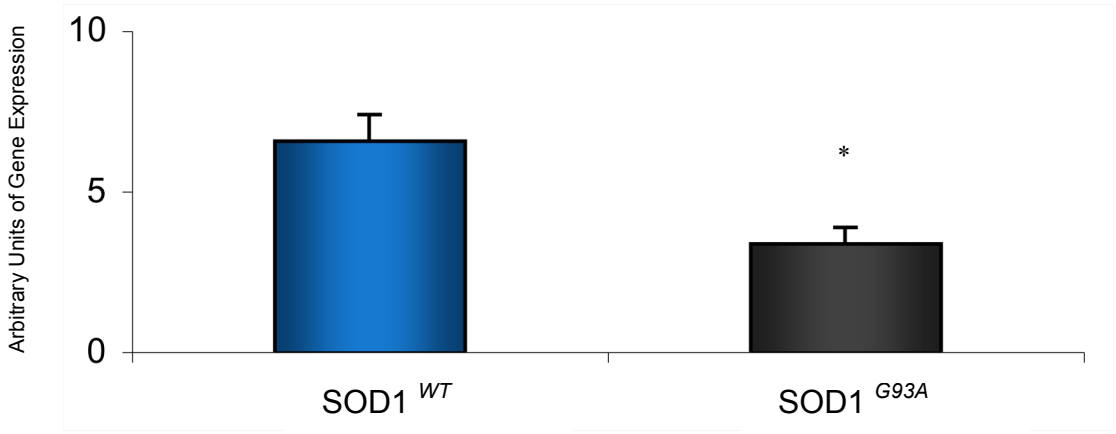


Figure 6.13 The mRNA levels of NF-κB in SOD1^{G93A} and SOD1^{WT} skeletal muscle, expressed as units of gene expression. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by *.

6.3.2 *Detection of O_2^- by DHE Fluorescence Staining*

Levels of O_2^- were detected in skeletal muscle cryosections by O_2^- sensitive DHE staining. Lower levels of O_2^- were detected throughout the skeletal muscle of $SOD1^{G93A}$ mice compared to $SOD1^{WT}$ controls (p=0.001) (Figure 6.14 & 6.15).

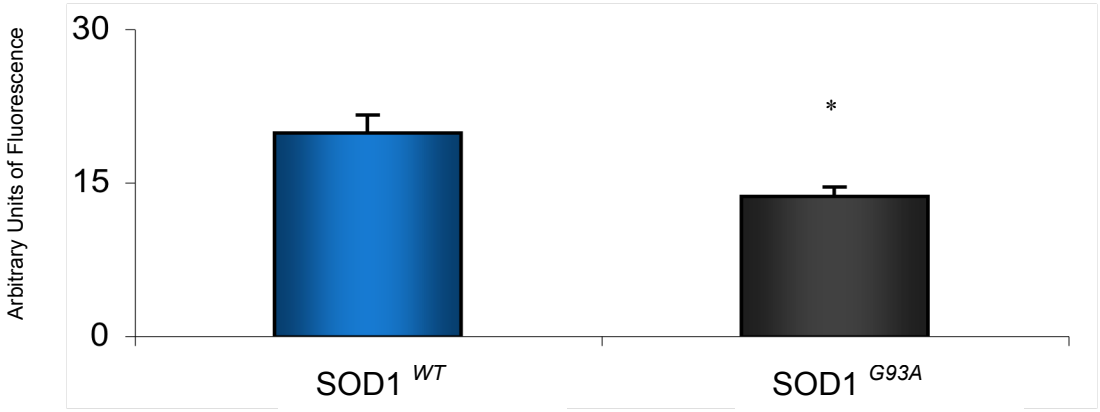


Figure 6.14 O_2^- levels in $SOD1^{G93A}$ and $SOD1^{WT}$ skeletal muscle, by histological DHE examination, expressed as arbitrary units of fluorescence. The values represent the mean ± SEM. Statistically significant differences (P<0.05) between groups are indicated by *

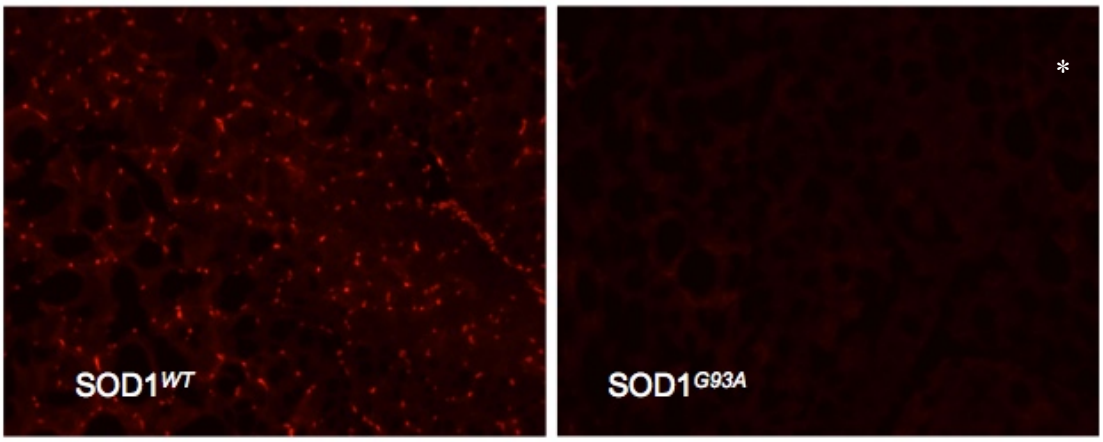


Figure 6.15 Histological DHE fluorescence detection, as a measure of O_2^-, in SOD1^{G93A} and SOD1^{WT} skeletal muscle. Statistically significant differences (P<0.05) between groups are indicated by *

6.4 Discussion

SOD1 is an important cytosolic antioxidant enzyme for the conversion of O_2^- to H_2O_2. SOD1 has been shown to be necessary for cell survival (Orr & Sohal 2003; Sun et al. 2002) and therefore mutations in this important antioxidant enzyme has critical consequences to cellular function (Landis & Tower 2005; Zelko, Mariani & Folz 2002). The $SOD1^{G93A}$ mutant model expresses ubiquitous SOD1 that results in degenerative motor neuron disease and severe muscle atrophy, characteristic of human ALS (Mahoney et al. 2006). We observed physical changes to skeletal muscle, with a decrease in physiological function that was mirrored by significant cellular oxidative changes, in the $SOD1^{G93A}$ mutant mouse. In particular, this study observed a significant decrease in the gene expression of NOX2 enzyme subunits, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and antioxidant enzyme SOD2 gene expression, as well as a decrease in O_2^-, in skeletal muscle from $SOD1^{G93A}$ mutant mice compared to $SOD1^{WT}$ controls. While the marked decrease in NOX enzyme subunits is an interesting finding, what was not surprising was the decrease observed in O_2^- in skeletal muscle from $SOD1^{G93A}$ mutant mice. The $SOD1^{G93A}$ model has been described as a model of SOD1 hyperactivity and therefore dismutation of O_2^-, consequently resulting in decreased cellular O_2^- levels. Further consequences of this however, would be an increase in the conversion of O_2^- to H_2O_2 and therefore increased levels of H_2O_2 in the cell.

In support of an increase in cellular H_2O_2 levels, a significant increase in the gene expression of the antioxidant enzymes, catalase and GPx was observed in skeletal muscle from the SOD1^{G93A} mutant mice, compared to SOD1^{WT} controls. The increase in the gene expression of the H_2O_2 scavenging antioxidants are suggestive of an increase in H_2O_2, although our results do not suggest NOX generated O_2^- production as a potential contributor. However, it would appear that NOX may be downregulated as a potential consequence of decreased ROS signaling. The increase in SOD1 conversion of O_2^- to H_2O_2 and increased scavenging of H_2O_2 by catalase and/or GPx, would indeed compromise redox-sensitive signaling pathways. In further support of this, we observed a marked decrease in NF-κB gene expression in the skeletal muscle of SOD1^{G93A} mutants. As NF-κB has been associated with an increase in NOX-dependent ROS signaling (Brar et al. 2002; Clark & Valente 2004), this result is not surprising. Furthermore, NF-κB has been suggested to regulate NOX subunit gene expression (Gauss et al. 2007) and therefore the decrease in NOX subunit expression in skeletal muscle from the SOD1^{G93A} mouse, could be explained by a decrease in transcription factor NF-κB regulation. Together with the changes observed in NOX2 enzyme subunits, these results suggest that ubiquitously expressed SOD1 is associated with a decline in skeletal muscle NOX activity potential, which may in part contribute to the decline in O_2^-. However, it would appear more likely to result in response to decreased redox signaling of gene transcription, through NF-κB. Therefore, this study questions the role of NOX generating O_2^- in skeletal muscle, in response to an over-expression of SOD1, in the SOD1^{G93A} model of ALS associated skeletal muscle atrophy.

6.4.1 Skeletal Muscle Wasting in a Model of SOD1 Overexpression

Transgenic mice expressing G93A develop progressive hindlimb weakness and muscle wasting between 4 and 6 months of age (Bruijn et al. 1997). Dobrowolny et al (2005) demonstrated motor neuronal degeneration, accompanied by severe muscle atrophy and complete paralysis, in $SOD1^{G93A}$ mice at about 123 days. Leclerc et al (2001) observed ALS disease onset in $SOD1^{G93A}$ mice at 70-80 days and hindlimb paralysis at 140-150 days. The $SOD1^{G93A}$ mutant model displays an overexpression and heightened activity levels of the important cytosolic O_2^- dismutating antioxidant enzyme SOD1, which induces neurodegeneration and hindlimb wasting to progressive paralysis (Bruijn et al. 1997). A study by Brooks et al (2004) observed significantly smaller hindlimb MRI volumes in $SOD1^{G93A}$ transgenic mice, with a 25% decline by 10 weeks and 36% decline by 15 weeks, when compared to $SOD1^{WT}$ mice. The $SOD1^{G93A}$ transgenic mice used in this study displayed the stage III phenotype of severe hindlimb paresis and abnormal gait, indicative of severe muscle atrophy.

It has been reported that the hindlimb paralysis that often develops in the later stages of the disease, may be a result of denervation. Nervous system innervation is critical for skeletal muscle growth and maintenance, and denervation is well known to cause muscle atrophy (Muller et al. 2007). As the communication between skeletal muscles and motor neurons is absolutely essential for muscle function (Chargé & Rudnicki 2003), denervation is indeed a potential factor in the changes observed in the skeletal muscle from our $SOD1^{G93A}$ mice. Interestingly though, it has been suggested that the

expression of mutant SOD1 in skeletal muscle causes muscle fiber degeneration, through a mechanism independent of motorneuron loss (Derave et al. 2003) and that changes in muscle and hindlimb wasting occurs well before motor neuron degeneration (Kostrominova et al. 2007). Similarly, Leclerc et al (2001) found an increase in OS, suggested as a consequence of enhanced SOD1 activity that affected limb muscles independently of motorneuron loss. This study suggests independent skeletal muscle dysfunction in SOD1^{G93A} mutants that could potentially contribute to the overall dysfunction and degeneration, associated with ALS. In addition, with the direct contact skeletal muscle has with motor neurons and changes that have been observed in skeletal muscle prior to motor neuron dropout, skeletal muscle has been implicated as a major contributor to ALS pathogenesis (Atkin et al. 2005; Mahoney et al. 2006). A study by Dobrowolny et al (2005) demonstrated significant attenuation of muscle atrophy in SOD1^{G93A}/mIgf-1 transgenic mice, overexpressing muscle specific growth factor IGF-1, but interestingly found a significant enhancement in motor neuron survival and delay in onset and disease progression, compared to SOD1^{G93A} transgenic mice. This study gives evidence for the changes observed in skeletal muscle in SOD1^{G93A} mutants, as a major contributor to the motorneuron degeneration and disease progression, rather than a result of motor neuron loss or denervation. Although we did not measure motorneuron loss in our model, we cannot ignore the significant changes observed in skeletal muscle cellular oxidative systems in the SOD1^{G93A} model that would certainly alter normal skeletal muscle function, but that may also contribute to overall disease pathogenesis.

6.4.2 A Role of the NOX Enzymes in SOD1 Overexpression

While the NOX enzyme has indeed been implicated in many progressive degenerative diseases, the enzyme system has also recently been suggested to play a role in the $SOD1^{G93A}$ model of ALS (Harraz et al. 2008; Wu et al. 2006). Interestingly, we found a marked decrease in the NOX2 enzyme subunits; $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ in the skeletal muscle of $SOD1^{G93A}$ mice, which function to regulate the assembly of the oxidase and catalyze the production of O_2^- (Takeya & Sumimoto 2003). It is therefore suggested from these results that NOX2 activation and production of O_2^- is decreased in $SOD1^{G93A}$ mutant skeletal muscle. This is a novel finding for NOX in degenerative disease, as studies have shown NOX enzyme involvement in ROS related tissue damage that has also been implicated in neurodegeneration (Marden et al. 2007; Patel et al. 2005; Wu et al. 2006), in response to SOD1 mutation. Wu et al (2006) demonstrated an upregulation of NOX and overproduction of ROS in the spinal cord of $SOD1^{G93A}$ mice, as well as delayed neurodegeneration as a result of NOX enzyme inactivation, proposing a key role for NOX-dependent ROS in promoting neurodegeneration. In addition, this study by Wu et al (2006) demonstrated in transgenic $SOD1^{G93A}$/$gp91^{phox}$- mice, a significant delay in the onset of end-stage paralysis, later than their transgenic $SOD1^{G93A}$/$gp91^{phox}$+ counterparts. However, evidence of a negative role for the NOX enzyme system in atrophied skeletal muscle in the $SOD1^{G93A}$ mouse, was not further supported by our study. Interestingly, Harraz et al (2008) proposed that hyperactivation of NOX-derived O_2^- is a consequence of SOD1 sustained activation, which leads to H_2O_2 accumulation and OS. Therefore, SOD1 has been proposed to play an indirect but important role in controlling the activity of the NOX enzyme (Harraz et al. 2008).

However, this study demonstrated a decrease in NOX subunit expression and O_2^-, suggestive of a decrease in NOX generated O_2^- production, in skeletal muscle from SOD1^{G93A} mice. Although this finding does not comply with the common theories of NOX as a primary O_2^- producing enzyme system in degenerative tissue damage, it may suggest an alternative role for NOX in the skeletal muscle system. With the current knowledge of the SOD1^{G93A} model and progressive nature of muscle degeneration, our results give further insight into the changes in skeletal muscle, when skeletal muscle degeneration and dysfunction is evident. Furthermore, changes to the NOX enzyme and subsequent ROS production, would indeed have significant consequences in the muscle, altering redox state and important redox-sensitive signaling pathways. Specifically, NOX-dependent ROS production has been associated with the important muscle growth pathways and has been shown to influence the expression of growth related genes, through transcription factor activation (Brar et al. 2002; Cai et al. 2004b). The decrease in the NOX enzyme subunits and O_2^- that we demonstrated in SOD1^{G93A} skeletal muscle would indeed alter these important muscle growth pathways and associated gene expression, through significant modifications to redox-signaling. The consequence of changes in muscle growth pathways would significantly alter the balance between muscle degradation and regeneration. As an increase in NOX enzyme activation and ROS production have been associated with muscle atrophy (Russell, Eley & Tisdale 2007), a decrease in NOX function may therefore result as an adaptive or protective response to muscle atrophy.

6.4.3 Oxidative Stress (OS) in SOD1 Overexpression

SOD1 plays a crucial role in the regulation of cellular ROS and therefore protection against oxidative cellular damage of redox-sensitive signaling (Valko et al. 2007). Therefore, ROS and cumulative OS have been proposed to play a role in the SOD1^{G93A} model of ALS (Muller et al. 2007). Specifically, OS is thought to develop by either diminished SOD1 scavenging activity, due to impaired function (Bruijn et al. 1997) and therefore O_2^- accumulation, or the result of a 'gain-of-function', and therefore elevated O_2^- dismutation and accumulation of cascading ROS, such as H_2O_2 and $OH^\bullet$ (Mahoney et al. 2006; Wu et al. 2006). Either way, OS and tissue damage is a common finding in degenerative tissue, associated with the SOD1 mutation, in the SOD1^{G93A} and human ALS patients. A recent study by Muller et al (2007) showed SOD1^{G93A}-induced muscle atrophy was accompanied by a 10-fold increase in mitochondrial ROS production. A recent study by Mohoney et al (2006) showed significant elevation in protein carbonyls in 95 day-old SOD1^{G93A} gastrocnemius muscle, accompanied by a substantial compensatory enzyme upregulation. Further supporting this theory, Leclerc et al (2001) demonstrated enhanced OH^- production in SOD1^{G93A} mice, attributing this particular ROS to the increase in OS. This increase in OH^-, in the SOD1^{G93A} mutant model, was also demonstrated in an earlier study by Wiedau-Pazos et al (1996). These later studies however do not support the 'gain-of-function' theory that has been associated with the SOD1 mutation, but rather suggest that the increase in SOD1 results in response to increased OS. However, OH^- is generated when there is an increase in O_2^- and H_2O_2 availability (Ji 2007), which our results do not support.

Our results do not support an increase in OS from O_2^- in the skeletal muscle of SOD1^{G93A} mutant mice. However, the decrease observed in skeletal muscle O_2^- levels in the SOD1^{G93A} mutant mice could be a direct result of increased SOD1 function and therefore conversion of O_2^- to H_2O_2. Although this result is indeed suggestive of an increase in H_2O_2, we also demonstrated an increase in the expression of the H_2O_2 antioxidant enzymes, catalase and GPx. Furthermore, the decrease in O_2^- that we found in the skeletal muscle of SOD1^{G93A} mice, is suggestive of a potentially important contribution by NOX, as we found a decrease in the gene expression of this major skeletal muscle ROS producing enzyme system, which may have contributed directly to the decrease in O_2^-. Collectively, our results suggest an alternative role for NOX and OS in skeletal muscle degeneration in the SOD1^{G93A} model. However, it is certainly possible that OS from other ROS, and ROS sources, could be present in this tissue and contribute to oxidative tissue damage and/or intracellular signaling that may be responsible for the skeletal muscle atrophy in the SOD1^{G93A} mutant mouse.

However, it is also important to note that we investigated these skeletal muscle oxidative measures in the SOD1^{G93A} mutant mouse during stage III, displaying severe hindlimb paresis or partial paralysis and abnormal gait. During this stage SOD1^{G93A} mice show severe muscle atrophy and loss of muscle mass, however these physical changes in skeletal muscle begin as early as 60-70 days (Muller et al. 2007). Interestingly, Mohoney et al (2006) observed significant protein oxidation in SOD1^{G93A} gastrocnemius muscle before the onset of clinical symptoms. Therefore, the changes that we observed in skeletal muscle from SOD1^{G93A} model during late stage III, may be indicative of more pronounced changes, in response to muscle damage during the earlier stages of pathogenesis. Therefore, we propose that the

changes in the NOX enzyme and O_2^- levels observed in skeletal muscle from SOD1^{G93A} mice, may be a compensatory response to prior oxidative insult by the mutation of SOD1.

6.4.4 Antioxidant Enzymes in SOD1 Overexpression

The antioxidant enzymes have been extensively studied in the SOD1^{G93A} model, demonstrating high expression of SOD1 in skeletal muscle and functional O_2^- dismutation (Leclerc et al. 2001; Mahoney et al. 2006). Due to the overexpression of SOD1 in this model, it has been proposed that SOD1 activity is substantially increased, resulting in the overproduction of H_2O_2 (Grieb 2004). Interestingly, Leclerc et al (2001) found an increase in total SOD activity in the muscle of transgenic SOD1^{G93A} mice during disease progression, however this was suggested as a response to an increase in OS, rather than a primary function in response to SOD1 mutation. Further to this study, Mohoney et al (2006) demonstrated a marked increase in catalase activity, indicative of an increase in H_2O_2, which was likely to be generated from SOD1 and SOD2 mediated dismutation of O_2^-. The significance of an increase in catalase activity suggests the production of high levels of H_2O_2 (Chelikani, Fita & Loewen 2004). In addition to this, we found an increase in catalase and GPx gene expression that further supports this process in SOD1^{G93A} muscle. The contrasting theory that SOD1 function is impaired in this model, would expect to show an increase in OS due to the accumulation of O_2^-, which was not observed in skeletal muscle from our SOD1^{G93A} mice. Furthermore, we cannot ignore the significant increase in catalase and GPx gene expression that was observed in skeletal muscle from SOD1^{G93A} mice, indicative of an increase in H_2O_2 generation.

Further to these results, a decrease in SOD2 mRNA levels were demonstrated in the skeletal muscle of SOD1^{G93A} mutant mice, when compared to SOD1^{WT} controls. Interestingly, Mohoney et al (2006) demonstrated an expected increase in SOD1 activity in the SOD1^{G93A} mutant skeletal muscle (approximately 95 days-old), but found an unexpected increase in SOD2 activity. Further to this, Muller et al (2007) showed enhanced mitochondrial ROS production that was strongly correlated with the extent of muscle atrophy, in SOD1^{G93A} mutant mice. These recent findings suggest an increase in ETC activity and production of O_2^-, despite previous reports of ETC dysfunction in models of ALS and ALS patients (Jung, Higgins & Xu 2002; Vielhaber et al. 2000). Similarly, Vielhaber et al (2000) demonstrated decreased levels of SOD2 in skeletal muscle with multiple mitochondrial abnormalities, which were suggested to result from oxygen radical damage. Interestingly though, Jung et al (2002) demonstrated a persistent decrease in skeletal muscle mitochondrial enzymes, beginning in the early stages of ALS, suggesting a role for mitochondrial damage in muscle atrophy associated with SOD1^{G93A} mutation. However, Mohoney et al (2006) suggested that the increase in skeletal muscle OS, observed in the SOD1^{G93A}, and an adaptive regulation of antioxidant enzymes, was in the absence of gross mitochondrial dysfunction. While, Echaniz-Laguna et al (2002) reported that mitochondrial dysfunction is not systemic in ALS and could be restricted to the central nervous system.

Despite contrasting studies, we cannot ignore the evidence of mitochondrial dysfunction in the SOD1^{G93A} model, from the decrease in SOD2 gene expression in the SOD1^{G93A} skeletal muscle that was observed. However, we must also consider the

localisation of SOD1. It is thought that in addition to its cytosolic location, SOD1 is also located in the intermembrane of the mitochondria, while SOD2 is localised in the mitochondrial matrix (Schon & Manfredi 2003; Vives-Bauza, Starkov & Garcia-Arumi 2007). It has been proposed that the toxic 'gain-of-function' of mutant SOD1, located in the mitochondrial intermembrane space, causes excessive generation of H_2O_2 (Derave et al. 2003), consequently causing mitochondrial oxidative damage. Further to this, mice lacking SOD2 have fatal consequences, with a significant increase in oxidative damage and shortened lifespan (Melov et al. 1999). Li et al (1995) demonstrated a survival rate of only up to 12 days after birth in SOD2-/- mice. Interestingly, Sun et al (2002) investigated the effects of SOD2 overexpression in Drosphila that demonstrated a decrease in cumulative oxidative damage and increased metabolic potential, with an increased lifespan by up to 75% (Sun et al. 2002). However, it is possible that the decrease in SOD2 expression that we observed is a compensatory response to the overexpression of SOD1 in the mitochondria. With the knowledge that H_2O_2 can diffuse freely through membranes (Rhee 2006), an increase in SOD1 conversion of O_2^- to H_2O_2 would result in increased intracellular H_2O_2 that our results support, by the increase in catalase and GPx expression in skeletal muscle from SOD1^{G93A} mice.

Although muscle atrophy, associated with the SOD1^{G93A} mutation, is indeed multifactoral, particularly in its progression of disease, this study demonstrates significant changes in the ROS generating NOX2 enzyme and O_2^-, in response to SOD1 modification. Specifically, we demonstrated a novel reduction in NOX activity potential, O_2^- and SOD2 gene expression, with an increase in catalase and GPx mRNA levels, in severely atrophied skeletal muscle from SOD1^{G93A} mice, in stage III

pathogenesis. Although these changes would indeed result in significant consequences, with the current knowledge that an increase in OS causes oxidative muscle damage, these changes during severe muscle atrophy may be an important adaptive or compensatory response. Therefore, the development of skeletal muscle atrophy in the SOD1^{G93A} model is likely to involve progressive changes in complex cellular oxidative signaling that particularly effects muscle growth pathways.

6.4.5 Skeletal Muscle Atrophy in SOD1 Overexpression

The SOD enzymes are not only important antioxidants for the dismutation of O_2^- and elimination of OS, but also play a key role in regulating oxidative enzymes and redox-sensitive signaling (Landis & Tower 2005). ROS have been described as central mediators of numerous signaling pathways and have been proposed to play a role in muscle growth pathways (Ji 2007; Valko et al. 2007). ROS have been proposed to mediate muscle atrophy, through the activation of transcription factor gene transcription regulation. In particular, NOX-dependent ROS generation has been suggested to regulate atrophy related genes such as MuRF-1 and Atrogin-1, upstream of transcription factor NF-κB activation (Cai et al. 2004b; Clark & Valente 2004). Interestingly, Leger et al (2006) demonstrated a significant increase in the atrophy related protein Atrogin-1 mRNA and protein content, which was associated with a decrease in the activity of the hypertrophy related kinase Akt, in the skeletal muscle of ALS patients. The Leger study (2006) suggests an increase in muscle atrophy, through induction of the Ub-proteasome pathway, and suppression of muscle hypertrophy in ALS patients. Kabashi et al (2004) demonstrated impaired 20S proteasome in the lumbar spinal motor neurons of SOD1^{G93A} transgenic mice, as an

early event and contribution to ALS pathogenesis. This impairment of the proteasome can also have significant consequences in skeletal muscle, and may indicate the involvement of the Ub-proteasome pathway. Furthermore, the 20S proteasome has been implicated in muscle degeneration as part of the Ub pathway (Lecker et al. 1999), which is associated with NOX-dependent ROS (Russell, Eley & Tisdale 2007). Therefore, it could be suggested that a decrease in NOX-dependent ROS, suppresses NF-κB activation and atrophy related gene expression. The downstream cascade of NOX/ROS/NF-κB was demonstrated in the skeletal muscle from SOD1^{G93A} mice in this study, with a decrease in NOX-dependent O_2^- potential and NF-κB gene expression.

In addition, we cannot ignore the consequences of the SOD1 overexpression and hyperactivity in skeletal muscle and its contribution to this intracellular growth pathway. An increase in SOD1-dependent H_2O_2 generation would indeed alter these redox-sensitive signaling pathways to stimulate NF-κB activation (Clark & Valente 2004; Kamata et al. 2002; Ungvari et al. 2007) and atrophy related gene expression (Kandarian & Jackman 2006; Lecker et al. 2004). In addition, H_2O_2 has been shown to further stimulate the activation of NOX (Gauss et al. 2007), which would further generate this cycle of excess NOX generated ROS production and muscle atrophy. However, decreased NOX subunit and NF-κB gene expression in skeletal muscle from our SOD1^{G93A} mice suggest a decrease in NOX generated ROS, H_2O_2 signaling and atrophy related gene expression. Our results are again suggestive of an adaptive response to prior muscle damage, potentially induced by this atrophic signaling cascade, induced by the SOD1 mutation.

It has been suggested that before the onset of disease in SOD1 mutant mice, there is an inflammatory response (Clement et al. 2003). Specifically, TNF-α, normally undetected in healthy mice, was found to accumulate in the spinal cord of SOD1^{G93A} mice in a study by Dobrowolny et al (2005). Interestingly, NOX activation is known to be associated with neuroinflammation. Quinn et al (2004) demonstrated a critical role for NOX-mediated neurotoxicity and extracellular ROS production in mediating microglial proinflammatory signaling pathways. In addition, Wu et al (2006) demonstrated a clear association between ALS and the NOX enzyme system in spinal cord microgliosis. Specifically, it was proposed that NOX-dependent ROS damage proteins including IGF-1, consequently causing oxidative modifications and hinders IGF-1/Akt survival pathway in motor neurons (Wu et al. 2006). Interestingly, Dobrowolny et al (2005) demonstrated a reduction in spinal cord inflammation in SOD1^{G93A}, with local IGF-1 expression, which maintained muscle integrity by inducing the cellular regeneration pathway with significant delays in the onset and progression of disease. Furthermore, Harraz et al (2008) suggested that NOX-dependent OS is a secondary event to late stage inflammation in ALS. It is therefore possible that the NOX enzyme system induces muscle atrophy downstream of inflammatory signaling, inhibiting the hypertrophic pathway in the skeletal muscle of SOD1^{G93A} mice and that the decrease in NOX activity potential that we observed in the skeletal muscle of SOD1^{G93A} mice, may therefore be an adaptive response to prior damage.

6.4.6 Conclusion

This study demonstrated a significant decrease in NOX subunit gene expression and O_2^- levels in response to the SOD1 mutation, in the skeletal muscle of SOD1^{G93A} mice. With evidence of OS and NOX enzyme involvement in the progressive degenerative disease, this study is suggestive of a system response in a condition of severe muscle atrophy. Furthermore, this study is indicative of a decrease in the NOX/O_2^-/NF-κB pathway, in atrophied skeletal muscle that has been suggested to induce muscle atrophy. While it still remains unclear as to the exact contribution that NOX plays in this model of severe muscle atrophy, our results are suggestive of an important skeletal muscle cellular system that is compromised in this particular disease. The consequences of this change in the cellular system is yet to be elucidated, however with the current knowledge of this multifunctional enzyme system, we have eluded to its role as an important redox-sensitive signaling enzyme for skeletal muscle growth pathways. Therefore, we propose regulated responses in severely atrophied skeletal muscle of SOD1^{G93A} mutant mice that indeed requires further investigation.

Chapter 7 Final Conclusions & Future Directions

7.1 Conclusions

These studies aimed to investigate a role for the NOX and antioxidant enzyme systems in the development of skeletal muscle aging sarcopenia and cancer-induced cachexia. These conditions demonstrated progressive degeneration of skeletal muscle, indicated by a marked decrease in muscle mass that was established, in these mouse models. While these conditions of skeletal muscle wasting are distinct in their development, the outcome of severe protein breakdown contributes significantly to poor quality of life and higher mortality in the elderly and cancer patients exhibiting these conditions. ROS are generated in highly metabolic tissue, such as skeletal muscle, and inappropriate production has been suggested to induce protein catabolism. Cumulative evidence suggests a role for ROS in the progressive degenerative nature of these skeletal muscle wasting conditions. ROS are recognised as central mediators of various cellular redox-sensitive signaling pathways and a number of internal and external stimulants have been suggested to activate ROS generating enzymes, such as the NOX enzyme system, particularly in response to disease. Although there are a number of sources of ROS and both sarcopenia and cachexia are indeed associated with metabolic changes, a strong body of evidence is suggestive of a more complex regulated release of ROS, associated with the induction of skeletal muscle atrophy, rather than lifetime accumulation or random degeneration of skeletal muscle protein.

The proposed primary function of the NOX enzymes, as a O_2^- generating system in response to regulated stimuli, establishes an interesting and potentially important role for NOX in non-phagocytes. Its highly regulated release of O_2^- and expression in various tissues, including skeletal muscle, establishes many roles for the NOX enzyme systems and involvement in a number of cellular redox-sensitive pathways. We therefore sought to determine changes in the NOX2 and NOX4 enzyme systems and O_2^- levels in skeletal muscle of sarcopenic and cancer-induced cachectic animal models. While evidence suggests that OS is a major contributor to skeletal muscle wasting, commonly developed in both of these conditions, we proposed that the NOX enzyme systems are major contributors to ROS generation and OS. However, while we found an intricate NOX subunit profile in skeletal muscle from these conditions that indeed revealed interesting and potentially important roles for the NOX enzymes, what was interesting was the significant contribution to tissue OS by SOD, due to the lack of compensation to increases in O_2^- levels, in wasting skeletal muscle.

7.1.1 A Role for the NOX Enzymes in Skeletal Muscle Wasting

The results from these studies are indicative of a role for the NOX enzyme systems in skeletal muscle wasting however, the complexity of these results suggest its involvement in various pathways within the cell. In particular, the age-associated sarcopenic and cancer-induced cachectic studies established a role for the NOX2 enzyme system with the upregulation of the important subunit components that regulate the assembly and activation of the O_2^- generating enzyme system. What was of particular interest was the upregulation of the NOX2 enzyme subunits, at the time of significant change and decline in skeletal muscle mass, at 18 and 24 months. Interestingly, the preliminary cancer-induced cachectic study 1 showed a similar

pattern of upregulation of the NOX2 enzyme subunits in cachectic skeletal muscle. These results alone are indicative of NOX2 enzyme involvement and potential for NOX2-dependent ROS production in skeletal muscle wasting. However, our results revealed a more complex mechanism by which the NOX and antioxidant enzyme systems interact to contribute to OS in these skeletal muscle wasting conditions.

7.1.2 Oxidative Stress (OS) in Skeletal Muscle Wasting

An interesting pattern of NOX enzyme subunit gene expression, O_2^- levels and SOD activity was revealed in sarcopenic skeletal muscle. While there was an increase in NOX2 activity potential at 18 and 24 months, skeletal muscle O_2^- levels were only significantly increased at 24 months, compared to the younger groups. The increase in SOD1 activity at 18 months therefore appeared to control the increase in O_2^-, while the decrease in SOD activity at 24 months seems to have contributed to the increase in skeletal muscle O_2^- levels. Collectively, these results point towards uncontrolled ROS production and contribution to OS in skeletal muscle wasting. While we did not measure the O_2^- levels in the preliminary cancer-induced cachectic study 1, no change was observed in total SOD activity in the cachectic skeletal muscle from this study, despite the upregulation of the NOX2 enzyme subunits. These results are suggestive of a lack of compensation by SOD for the potential increase in NOX generated O_2^-.

Further investigations in the cancer-induced cachectic model however, revealed differential changes in the NOX2 enzyme system. The results from this further study (cancer-induced cachectic study 2) found a marked decrease in NOX2 subunit expression in cachectic skeletal muscle. However, these findings most probably point towards the differences in the mouse model itself. We established the MAC16 model

of cancer-induced cachexia from cell lines that were also used in our preliminary study. Differences in the criteria for tissue collection may explain the differences that we observed in the oxidative and antioxidative systems in MAC16-induced cachectic skeletal muscle. Tissue was collected in the cancer-induced cachectic study 1, 25 days post MAC16 cell implantation and muscle from animals with varied weight loss from 9-25% were included in the study. Criteria for tissue collection in the cancer-induced cachectic study 2 was according to the degree of weight loss, ranging from 15-20%, approximately 20 days post MAC16 cell implantation. Therefore the varying degree of weight loss in the cancer-induced cachectic study 1 may have contributed to the differences in the NOX and SOD enzyme system changes, in the two cachectic models. While this proposes interesting speculation of NOX2 enzyme involvement during early stages of disease and with regards to the onset of muscle wasting, we demonstrated an interesting increase in skeletal muscle O_2^- in the cachectic skeletal muscle, despite the decrease in NOX subunit expression. Although this finding does not appear to be NOX-dependent, the increase in O_2^- in cachectic skeletal muscle can be attributed to the decrease in SOD activity that we observed in this study.

7.1.3 The Contribution of SOD in Skeletal Muscle Wasting

Regulation of cellular ROS and OS by antioxidant enzymes is an important process for cellular redox signaling pathways and maintenance of homeostasis. These studies revealed an important contribution by the SOD enzyme system, in these conditions of skeletal muscle wasting. While an increase in O_2^- indeed requires a compensatory increase in O_2^- dismutation by SOD, the cachectic and sarcopenic muscle from these models suggest a lack of compensation by SOD to increases in O_2^- levels. Therefore, the results from these studies demonstrated the important role that the SOD enzyme

system plays in skeletal muscle wasting and suggested a need to investigate the SOD enzyme system further. This was made possible through a model of skeletal muscle wasting, caused by genetic overexpression of the primary cytosolic O_2^- dismutator, SOD1. The proposed toxic 'gain-of-function' by the genetic mutation in SOD1 is an interesting model of alterations in oxidative homeostasis, and this alteration is known to significantly alter redox-related cellular function. The phenotypic skeletal muscle wasting and dysfunction, seen in these models overexpressing SOD1, indeed establishes an important role for SOD1 in maintaining skeletal muscle function, and further supports a role for ROS in degenerative disease.

Interestingly, while research has demonstrated an increase in the NOX enzyme system in response to SOD1 overexpression, a marked decrease was observed in the important NOX2 enzyme subunits and O_2^- levels, in the skeletal muscle of the SOD1^{G93A} mutant mice. However, the antioxidant enzymes catalase and GPx were significantly upregulated in the skeletal muscle from this model, indicating an increase in H_2O_2. While we are aware that H_2O_2 can be generated from sources other than the dismutation of O_2^- by SOD, it could be speculated that the increase in SOD1 activity, as a result of genetic overexpression, contributed significantly to the decrease in skeletal muscle O_2^-, observed in the SOD1^{G93A} model, consequently resulting in an increase in H_2O_2.

7.1.4 A Role for ROS & SOD in Skeletal Muscle Wasting

While the phenotypic changes appear to be similar in the three models, used in these investigations of significant skeletal muscle wasting, the underlying mechanism appears to involve changes in the oxidative and antioxidative systems that we measured, in the development of skeletal muscle wasting. With the knowledge that ROS are involved in important cellular signaling pathways in skeletal muscle, particularly in atrophy and hypertrophy related pathways, both the ROS generating NOX and antioxidant SOD enzyme systems are important regulators of skeletal muscle growth. Our results from the three investigations of skeletal muscle wasting are indicative of cellular signaling by ROS such as O_2^- and H_2O_2, via O_2^- dismutation by SOD. However, our results have revealed a complex system of changes that not only suggest the induction of atrophy, through redox-sensitive signaling pathways, but also suggest potential compensatory responses to maintain skeletal muscle integrity. This was particularly evident in age-associated sarcopenia, through differential changes to the oxidative associated systems in aging skeletal muscle, despite the increase in NOX activity potential.

It is evident that the NOX enzyme subunits are regulated at the gene level and require stimulation to regulate the active oxidase. The differential changes that we observed in the NOX and antioxidant enzyme systems, in the three models of skeletal muscle wasting, are suggestive of specific disease related redox-sensitive stimulation. Indeed the three conditions of skeletal muscle wasting investigated that include aging, cancer and SOD1 overexpression, would induce many complex changes in the patient and contribute to the differential changes that we observed in the NOX enzyme subunits.

It is important to recognise however, that ROS are important intracellular signaling molecules and that an increase in their production may actually be necessary for the cell to function, in response to changes in their environment, particularly in response to disease. Therefore, the changes that we observed in the oxidative and antioxidative enzyme systems, in the three conditions of skeletal muscle wasting investigated, may be a result of signaling compensation to prior muscle damage for regeneration.

The significant changes that we observed in the three conditions of skeletal muscle wasting, suggest the involvement of the NOX and antioxidant enzyme systems for the production of ROS, and their involvement in redox-sensitive skeletal muscle atrophy and hypertrophy pathways. It is evident that the systemic changes in the aging and pathological conditions that we investigated have the potential to influence these systems in skeletal muscle, through redox-sensitive signaling. While the balance between atrophy and hypertrophy is crucial to skeletal muscle, the cellular responses that we observed in skeletal muscle from the three conditions indicate a potential induction of atrophy but also a compensatory change indicative of hypertrophy and regeneration. Therefore, these investigations support a regulated system of skeletal muscle atrophy and hypertrophy that involves central mediation by NOX-dependent ROS, through regulated external and internal stimulation in response to disease, but also reveals the importance of SOD regulation, in conditions of skeletal muscle wasting. To our knowledge these studies are the first to investigate the NOX and associated SOD enzyme systems in the skeletal muscle of these three wasting conditions specifically, which has provided a basis for future investigation. The results from our studies have indicated a role for the NOX and SOD enzyme systems in these three conditions of skeletal muscle wasting and indeed, future investigations

into the exact contributions that these systems play would contribute to the current knowledge and understanding of these systems and potential targets for patient therapy.

7.2 Future Directions

It is evident from our investigations that the NOX enzyme plays various roles in the skeletal muscle system and is indeed altered in response to changes associated with aging, cancer and SOD1 overexpression. However, its contribution in these conditions is yet to be fully established, and further investigation is required to understand its role in this cellular system, in order to better understand its role in each of the three wasting conditions. Further to the involvement of the NOX enzyme system in skeletal muscle wasting, what appears to be of particular interest for further investigation is the involvement of the SOD enzymes in the skeletal muscle system and in conditions of skeletal muscle wasting. However, before investigating the NOX and antioxidant systems further, it is of great importance to characterize the locality of the NOX enzyme in the skeletal muscle system, which may also provide a more reliable measure of NOX enzyme activity in skeletal muscle. These measures can then be applied to the three conditions of skeletal muscle wasting that we investigated to provide a greater understanding of the NOX enzyme systems in these conditions. In addition to these investigations of the NOXenzyme systems, further investigation of the catalase and GPx antioxidant enzymes and subsequent ROS production would provide a more broad understanding of the oxidative associated changes, in the three conditions of skeletal muscle wasting.

7.2.1 Further Investigations of the NOX Enzymes

Further investigations, to the existing studies, for the measurement of NOX activity and subunit proteins would provide valuable information for this enzyme system in skeletal muscle. However, what has made it particularly difficult to investigate the activity of the oxidase is that to our knowledge and experience, there appears to be no reliable measure for NOX activity in skeletal muscle, primarily due to the lack of knowledge for the localisation of the enzyme. Research has suggested that the locality of the NOX enzyme in skeletal muscle is within or on both external and intracellular membranes, adding difficulty to the measurement of NOX activity and O_2^- production. While a large body of evidence exists for NOX enzyme involvement in various functions in a variety of tissues, without a specific measure of functional activity, the NOX enzyme system cannot be fully investigated. In addition, the low abundance of NOX subunit proteins in skeletal muscle tissue, has posed problems for its measurement by popular immunohistochemistry and western blotting technique. The measurement of NOX subunit proteins, by traditional western blotting procedures, were attempted in our studies, with the use of NOX complex specific antibodies (SantaCruz, USA) in skeletal muscle homogenates, without success. Therefore, it is of interest to establish antibodies with greater specificity to detect these low abundant proteins in skeletal muscle tissue, in order to further investigate this oxidative system.

7.2.2 Localisation of the NOX Enzymes in Skeletal Muscle

To further our investigations of NOX enzymes in skeletal muscle wasting, it would be necessary to fully characterize the locality of the NOX2 and NOX4 enzymes and O_2^- production in skeletal muscle, for their contribution to the cellular system. This can be achieved through western blotting techniques that were unsuccessfully carried out in our studies, which again indicated the need for more specific antibodies designed for the detection of NOX2 and NOX4 in skeletal muscle. While it has been suggested that NOX2 is localised at the plasma membrane, similar to phagocytes, it is suggested that the direction of O_2^- release is in the intracellular compartment. Further to this, NOX4 has been suggested to localise at intracellular membranes and may therefore release O_2^- inside intracellular organelles. While the intracellular release of O_2^- has been established for the NOX enzyme systems, giving evidence for its potential role in redox-sensitive signaling, the localisation of NOX4 and generation of O_2^- in skeletal muscle is unknown. It is therefore of great interest to localise the enzyme in the cell and determine its release of O_2^-. This could be achieved through skeletal muscle extra and intracellular membrane fractionation and western blotting techniques for NOX2 and NOX4 detection. The localisation of the NOX enzyme systems in skeletal muscle would be of great value to further understand these O_2^- generating systems. It may provide further understanding and potential stimulation by extra and/or intracellular stimulants, as well as the cellular compartmental release of O_2^-. Overall, these investigations may provide further insight into the potential roles that NOX play in the cellular system and their contribution to aging sarcopenia, cancer-induced cachexia and in response to SOD1 overexpression.

7.2.3 Further Investigations of ROS and Antioxidant Enzymes

It would appear from the results of our investigations that a measure of the activity of the antioxidant enzymes, catalase and GPx would help to understand the complex oxidative changes and contribution to skeletal muscle wasting in the three conditions. The changes that we observed in the gene expression of these antioxidant enzymes in skeletal muscle wasting is suggestive of potential changes in antioxidant activity and conversion of H_2O_2 to less reactive oxidants. We found a significant lack of compensation by SOD, to the increase in O_2^-, despite an increase in gene expression in sarcopenic and cachectic skeletal muscle. Therefore, enzyme assay measures of catalase and GPx would provide a greater understanding of the antioxidant enzyme system in conditions of skeletal muscle wasting, as well as the potential ROS involved in skeletal muscle atrophy, in these three conditions. In addition to further antioxidant enzyme measures in skeletal muscle wasting, specific measures of H_2O_2, which can be measured by either Scopoletin or Amplex Red fluorescence measures (Serrander et al. 2007), would provide a broader picture of the cellular oxidative environment and potential role in these skeletal muscle wasting conditions.

7.2.4 Further Investigations in Three Models of Skeletal Muscle Wasting

A common factor in these models of skeletal muscle wasting is the progressive degenerative nature of skeletal muscle protein breakdown. While we investigated skeletal muscle at intervals over four age groups and observed a significant decline in muscle mass from 12-18 months, accompanied by significant changes to the NOX and antioxidant enzyme systems, what is of particular interest from this study is the potential changes in skeletal muscle from 12 to 18 months of age. Therefore, while we investigated skeletal muscle at 6 month intervals over a period of 18 months, it is of interest to include additional age groups by expanding the study to investigate skeletal muscle changes at 3 month intervals over an 18 month period. As we did not observe changes in skeletal muscle wasting over the period of 18–24 months of age, it would not only be of interest to investigate the NOX and antioxidant enzyme systems during this time that may give evidence for a potential shift from atrophy to hypertrophy, but to also investigate the pattern of skeletal muscle mass following 24 months. This proposed extension of the study may not be possible as our survival data suggests a limited lifespan beyond 24 months, however it would indeed be of great value to better understand the progression of skeletal muscle wasting during aging. In addition to investigating the progression of muscle wasting during aging, we propose a similar investigation into the progression of muscle wasting in the cancer-induced cachectic model. While we observed significant weight loss of up to 20%, approximately 20 days post MAC16 cell implantation, it would be of interest to observe the oxidative changes that we investigated, in this model, over the course of disease progression. This investigation would give further insight into the changes in

these cellular systems and might further explain the differences in NOX subunit expression that we observed in the two cancer-induced cachectic studies. Likewise, further investigation of these oxidative systems in the progressive stages of the SOD1^{G93A} mutant model of ALS, would give further evidence for the progression of skeletal muscle changes throughout the course of the disease.

7.2.5 Further Investigations in Aging Sarcopenia

7.2.5.1 The NOX Enzymes in Aging Sarcopenia

We demonstrated a marked increase in the NOX2 enzyme activator, p67^{phox} in skeletal muscle at 24 months and proposed a role for the NOX2 enzyme in regeneration at this time. In order to investigate the role for the NOX2 enzyme in skeletal muscle at 24 months further, the use of a p67^{phox} knockout model may provide greater insight into the role of NOX2 in age-associated skeletal muscle degeneration/regeneration. Additionally, genetic alterations in the rate limiting catalytic subunit NOX2 may provide us with a better understanding of the role of the NOX2 enzyme system in skeletal muscle during aging. In particular, we demonstrated a marked increase in NOX2 gene expression in sarcopenic skeletal muscle and therefore a study using a NOX2 knockout model of aging may show delays in the progression of skeletal muscle wasting during aging. Furthermore, with the proposed role for NOX2 enzyme in both atrophy and hypertrophy related pathways, further investigations in these knockout models may provide a better understanding of the role of NOX2 in cell signaling pathways during aging. As previously mentioned, Wu et al (2006) demonstrated a significant delay in the onset of skeletal muscle atrophy/paralysis in transgenic SOD1^{G93A}/gp91^{phox}-, which may have similar results

for NOX2 knockout mice during aging. Further to this however, studies by Looi et al (2008) and Bendall et al (2002) demonstrated a significant decrease in cardiac hypertrophy in NOX2-/- mice. To our knowledge the role of the NOX2 enzyme has not been investigated in aging skeletal muscle, however these studies demonstrating a role for NOX2 in atrophy and hypertrophy, propose its potential role in these important skeletal muscle pathways during aging that can be further investigated through the use of the NOX2 knockout model.

7.2.5.2 Superoxide Dismutase (SOD) Enzymes in Aging Sarcopenia

We have proposed an important contribution by the SOD antioxidants in the development of skeletal muscle wasting during aging. The role of the SOD antioxidants during aging in our study was interesting, as O_2^- was shown to increase significantly at 24 months, while SOD1 and SOD2 enzyme activity was decreased. While these results were interesting in themselves, we found no further decline in skeletal muscle mass at 24 months of age compared to 18 months, when oxidative systems appeared to be balanced. Research has already shown a decrease in lifespan and age associated conditions in SOD knockout models and a significant increase in lifespan and age associated conditions in transgenic models of heterozygous SOD overexpression. However, it is of interest to further our investigations of the role of the SOD enzymes in regulating ROS for its involvement in redox-sensitive atrophy and hypertrophy related pathways in aging skeletal muscle.

7.2.6 Further Investigations in Cancer-Induced Cachexia

7.2.6.1 The NOX Enzymes in Cancer-Induced Cachexia

To further investigate the NOX2 enzyme systems in the cancer-induced cachectic condition, it would appear from our results that in addition to investigating this system in skeletal muscle during early stages of the disease, the use of a transgenic NOX2 knockout, may give further insight into the role of this NOX enzyme system in cancer-induced cachectic skeletal muscle. Our results revealed a complex pattern of NOX2 enzyme subunit expression in the two models of cancer-induced cachexia, and while the exploration of the differences between these two models is indeed of interest, the use of a NOX2 knockout model induced with the MAC16 cachectic cell line would give further insight into this complex system. The interesting subunits profiles of the NOX2 enzyme system that we found in the two models of cancer-induced cachexia, reveal alternative roles for the NOX enzyme system, as both a system involved in skeletal muscle damage and protection. Therefore, the use of a genetically altered NOX2 model of cancer-induced cachexia, may establish a role for the NOX2 in this model. In particular, the use of the NOX2 knockout mouse as an animal model of cancer-induced cachexia, through cancer cell implantation, may reveal a delayed catabolic response, induced by NOX-dependent ROS, via the induction of the Ub-proteasome pathway. Therefore, within this model it would also be of interest to explore the downstream redox-sensitive induction of skeletal muscle specific Ub ligases such as MuRF-1 and Atrogin-1, as well as the hypertrophy related growth factor IGF-1 and associated kinase Akt (Appendix 2).

7.2.6.2 Superoxide Dismutase (SOD) Enzymes in Cancer-Induced Cachexia

Further to the investigations of the NOX enzyme system, we have proposed an important contribution by the SOD antioxidants in the development of cancer-induced cachexia. The lack of SOD compensation in the two models of cancer-induced cachexia, despite the increase in O_2^- and potential for NOX generated O_2^- in the preliminary study, is indicative of an important contribution to skeletal muscle oxidative status in cachectic skeletal muscle. Therefore, the use of transgenic models of heterozygous SOD1 and SOD2 overexpression would be of particular interest for use in the cancer-induced cachectic model. These models would not only give further insight into the importance of these SOD enzymes in the skeletal muscle system during cancer-induced cachectic development, but could also provide a target for antioxidant therapy.

7.2.7 Further Investigations in the SOD1^{G93A} Model

7.2.7.1 The NOX Enzymes in the SOD1^{G93A} Model

To further our investigations of the role of the NOX enzyme system in the SOD1^{G93A} transgenic model, the addition of genetic alterations in the NOX enzymes could further our understanding of these oxidative and antioxidative systems in this model of skeletal muscle wasting. Our results revealed an interesting decrease in NOX subunit gene expression profile in the skeletal muscle of SOD1^{G93A} model at stage III of the disease. We have proposed that the decrease in NOX may be a compensatory response to prior oxidative insult and therefore it would be of particular interest to explore this further. Interestingly, a model of SOD1 overexpression coupled with

genetically altered NOX subunit expression, could reveal an interesting pattern of increased NOX-dependent O_2^- generation that was significantly lower in our SOD1^{G93A} model. Therefore, in addition to investigating the stages prior to stage III insult, the development of a transgenic model of SOD1 overexpression with NOX2 knockout, would help to investigate this further and may even delay the development of severe muscle wasting in this model. This investigation may not only provide a better understanding of the role of NOX and O_2^- in the skeletal muscle system, but also potential targets for disease therapy.

7.2.8 Further Investigations in Skeletal Muscle Wasting

Evidence from these studies in skeletal muscle wasting conditions, has questioned the role that the NOX enzymes play in skeletal muscle, in response to disease. To build upon and explore further the results of these investigations, it would be of interest to investigate these three models of skeletal muscle wasting with genetic alterations in the NOX enzyme subunits. Further to this, the potential pathways discussed in these studies propose a role for NOX and antioxidant enzymes and associated ROS generation, in both skeletal muscle atrophy and hypertrophy, and we have proposed that these pathways deserve particular attention. In particular, an interesting study by Dobrowlny et al (2005) investigated a transgenic SOD1^{G93A} model overexpressing hypertrophy related IGF-1 that showed significant delays in disease onset and progression of skeletal muscle degeneration. It would therefore be interesting to investigate the muscle specific IGF-1 (mIgf-1) transgenic model during aging and implantation of cancer-induced cachexia, to determine the changes that may be associated in the important hypertrophy pathway in these models. It would also be of interest to investigate the downstream effect of stimulated NOX-dependent redox

signaling. Therefore, an investigation of a transgenic atrophy related knockout models, such as MuRF-1-/- or Atrogin-1-/- in the three models of skeletal muscle wasting we investigated, would provide valuable information of the atrophy related pathways involved in these conditions.

7.2.9 NOX & SOD Enzymes as Targets for Therapy

It is evident from our investigations of the three models of severe skeletal muscle wasting in response to aging and disease that the oxidative and antioxidative enzyme systems that we measured, are indeed complex and requires further investigation to better understand these contributing factors in the skeletal muscle system. The investigations that we have proposed here, would not only give further insight into the complex system of changes involved in these conditions of skeletal muscle wasting, but would also help in the understanding of the role that these systems play in skeletal muscle tissue. An investigation of NOX generated ROS and downstream skeletal muscle atrophy and hypertrophy related pathways, such as IGF-1 and atrophy related Ub ligases, such as MuRF-1 and Atrogin-1 in the three models, would give further insight into the potential pathway(s) leading to skeletal muscle wasting. Furthermore, these investigations in NOX and SOD transgenic models, may give further insight into the role of ROS in skeletal muscle growth pathways, in these skeletal muscle wasting conditions. Our results propose potential NOX enzyme subunit and antioxidant enzyme targets for therapy, however it is evident that further investigation is required. The common contribution of a lack of antioxidant compensation that we observed in skeletal muscle during aging and cancer-induced cachexia, suggesting a therapeutic target for antioxidant supplementation, has been established. However, with further research and understanding of the complex oxidative and antioxidative

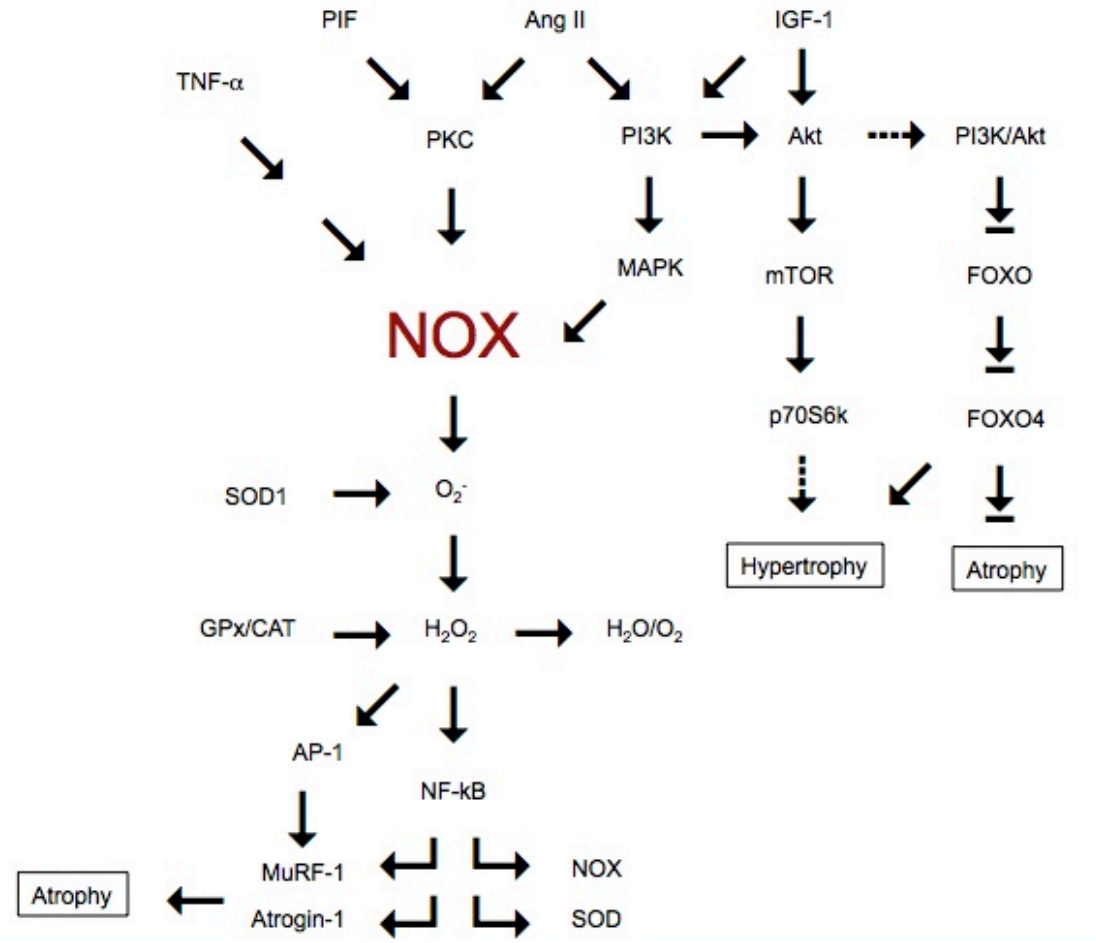
systems, in these conditions of skeletal muscle wasting, may provide potential pharmaceutical targets for successful treatment of sarcopenia and cachectic related conditions.

Appendix 1

SOD Standards			
Tube	SOD Stock (µl)	Sample Buffer (µl)	SOD Activity (U/ml)
A	0	250	0
B	5	245	0.025
C	10	240	0.050
D	20	230	0.100
E	30	220	0.150
F	40	210	0.200
G	50	200	0.250
H	60	185	0.300

Table of SOD standards for the SOD activity measurement in skeletal muscle tissue, prepared as per the manufacturers protocol (706002 Superoxide Dismutase Assay Kit, Cayman, USA). SOD standards were prepared by diluting 20µl of the SOD standard with 1.98ml of sample buffer to obtain a SOD stock standard and the SOD stock was diluted with sample buffer, according to the table above, to yeild different concentrations, representative of SOD activity levels (U/ml). The SOD standards were used to calculate SOD activity in tissue samples as follows; Standard A's absorbance, containing no SOD stock, was divided by itself and by all the other standard and sample absorbances to yield the Linearized Rate (LR). The LR was plotted for a typical standard curve and final SOD Activity (U/ml) was obtained for each sample.

Appendix 2



A model of the atrophy and hypertrophy pathways in skeletal muscle. Extra and intra-cellular stimuli such as TNF-α, PIF and Ang II are proposed to induce skeletal muscle atrophy via NOX/ROS stimulation and redox-sensitive atrophy related transcription factor, NF-κB and AP-1 activation. IGF-1 is an important component in skeletal muscle hypertrophy, stimulating the Akt/mTOR/p70S6k pathway and blocking the FOXO/FOXO4 atrophy pathway. This model demonstrates the central role for NOX-dependent ROS generation in these important skeletal muscle growth pathways.

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