REGULATION OF SKELETAL MUSCLE SATELLITE CELL PROLIFERATION BY NADPH OXIDASE

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Abstract

M.Sc. **Mahroo Mofarrahi** **Microbiology and Immunology**

Skeletal satellite cells are adult stem cells located among muscle fibers. Proliferation, migration and subsequent differentiation of these cells are critical steps in the repair of muscle injury. We document in this study the roles and mechanisms through which the NAPDH oxidase complex regulates skeletal satellite cell proliferation. The NADPH oxidase subunits Nox2, Nox4, $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ were detected in primary human and murine skeletal muscle satellite cells. In human satellite cells, NADPH oxidase-fusion proteins were localized in the cytosolic and membrane compartments of the cell, except for $p47^{phox}$, which was detected in the nucleus. In proliferating subconfluent satellite cells, both Nox2 and Nox4 contributed to O_2^- production. However, Nox4 expression was significantly attenuated in confluent cells and in differentiated myotubes. Proliferation of satellite cells was significantly reduced by antioxidants (N-acetylcysteine and apocynin), inhibition of $p22^{phox}$ expression using siRNA oligonucleotides, and reduction of Nox4 and $p47^{phox}$ activities with dominant-negative vectors resulted in attenuation of activities of the Erk1/2, PI-3 kinase/AKT and NFκB pathways and significant reduction in cyclin D1 levels. We conclude that NADPH oxidase is expressed in skeletal satellite cells and that its activity plays an important role in promoting proliferation of these cells.

Résumé

M.Sc. **Mahroo Mofarrahi** **Microbiology and Immunology**

Les cellules satellites squelettiques sont des cellules souches adultes localisées parmi les fibres musculaires. La prolifération, la migration et la différentiation subséquentes de ces cellules sont des étapes critiques se produisant lors de la réparation pendant une blessure musculaire. Dans cette étude, nous documentons les rôles et mécanismes par lesquels le complexe NADPH oxydase régule la prolifération de cellules satellites squelettiques. Les sous-unités Nox2, Nox4, p22phox, p47phox, et p67phox sont détectées dans les cellules primaires musculaires squelettiques humaines et de souris. Dans les cellules satellites humaines, les protéines de fusion NADPH oxydase sont localisées dans le cytosol et les compartiments membranaires de la cellule, excepté pour p47phox qui a été détecté dans le noyau. Dans les cellules en prolifération sous-confluentes, Nox2 et Nox4 contribuent à la production d'oxygène. Toutefois, l'expression de Nox4 est significativement atténuée dans les cellules confluentes et les myotubes différentiés. La prolifération de cellules satellites est réduite significativement par les antioxydants (N-acétyl cystéine et apocyanine), l'inhibition de l'expression de p22phox utilisant les oligonucléotides SiARN, et la réduction des activités de Nox4 et de p47phox avec des vecteurs dominants négatifs résultant dans l'atténuation des activités des voies Erk1/2 PI-3 kinase /AKT et NFKB, et par une réduction significative des niveaux de cycline D1. Nous concluons que la NADPH oxydase est exprimée dans les cellules satellites squelettiques, et que son activité joue un rôle important en favorisant la prolifération de ces cellules.

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Table of Contents:

Page

List of figures and tables

CHAPTER 1

INTRDOUCTION

SUMMARIZED INTRODUCTION:

It has been well established that relatively low levels of reactive oxygen species (ROS) play important roles as signaling molecules in a wide range of physiological and pathophysiological responses. However, excessive production of ROS leads to cell injury and apoptosis and is involved in the pathogenesis of many diseases, including diabetes, hypertension and atherosclerosis. ROS are primarily generated as byproducts of mitochondrial oxidative phosphorylation, with O_2^- as the main product. Endoplasmic reticulum cytochrome P-450 enzymes, xanthine, and aldehyde oxidases, nuclear electron transport systems, flavoproteins, and lipooxygenases are also capable of producing ROS.

In phagocytes, NADPH oxidase is a multimeric enzyme complex consisting of four essential subunits ($p22^{phox}$, $gp91^{phox}$ (Nox2), $p47^{phox}$ and $p67^{phox}$) and two additional subunits ($p40^{phox}$ and Rac2). $p22^{phox}$ and Nox2 form an integral membrane-bound complex (flavocytochrome b_{558}) that is responsible for catalytic activity, while $p47^{phox}$ and $p67^{phox}$ are normally localized in the cytosol. Recent studies have indicated that NADPH oxidase is also a major source of ROS in non-phagocytes, including vascular and airway smooth muscles, fibroblasts, endothelial and epithelial cells. In these cells, NADPH oxidase produces ROS under basal conditions but, upon stimulation, ROS are produced intracellularly at much lower levels than they are in phagocytes. Another difference between phagocytes and non-phagocytes relates to the fact that several homologues of Nox2 (including Nox1, Nox3, Nox4 and Nox5), and of $p47^{phox}$ (NOXO1) and $p67^{phox}$ (NOXOA1), are expressed in various non-phagocytic cells, indicating that multiple NADPH oxidase forms might exist in these cells. In

addition, there is increasing evidence that in non-phagocytes, Nox-derived ROS function as second messengers in the signaling of tyrosine kinase growth factor receptors, cytokine receptors and G-protein coupled receptors, such as those associated with angiotensin II.

Skeletal muscles have a relatively high regenerative capacity due to the presence of satellite (muscle precursor) cells, which are mononuclear progenitor cells located between the basal lamina and cell membranes of mature muscle fibers. Under normal conditions, satellite cells are quiescent. However, in response to muscle injury or during degenerative muscle diseases, precursor cells are activated and go through rounds of cell division to produce a pool of myogenic precursors known as myoblasts. A number of these myoblasts replenish satellite cell reserves, but the majority proliferate further, fuse, and finally differentiate into multinucleated myotubes and, eventually, myofibers.

Little information is as yet available regarding the expression and functional significance of NADPH oxidase-derived ROS in skeletal muscle precursor cells. Two recent studies have confirmed the presence of Nox2, $p47^{phox}$ and $p67^{phox}$ mRNA and proteins in immortalized rat L6 myoblasts and myotubes and that the activity of NADPH oxidase in these cells generates ROS to regulate insulin signaling and Ca^{++} flux. Others, however, have failed to detect NADPH oxidase expression in either immortalized mouse C2C12 or L6 myoblasts. The reasons behind these discrepancies are unclear, but may be related to methodological issues, including differences in the type of immortalized precursor cells under investigation.

In our attempts to evaluate the expression, intracellular localization and functional importance of various subunits of NADPH oxidase enzyme complex in skeletal precursor cells, we performed several pilot experiments in which we used cultured primary human and murine muscle precursor cells. Based on these preliminary results and the well-documented involvement of Nox proteins in the regulation of proliferation of several non-phagocytic cell types, we tested in this study the following hypotheses: 1) The NAPDH oxidase enzyme complex is present in mature skeletal muscle fibers and skeletal muscle precursor cells; 2) Both Nox2 and Nox4 contribute to total NAPDH oxidase activity in proliferating primary skeletal muscle precursor cells; 3) NADPH oxidase-derived ROS promote proliferation of muscle precursor cells by activating the Erk1/2, PI-3 kinase/AKT and the NFκB pathways.

DETAILED INTRODUCTION:

1.1 NADPH oxidase complex in phagocytes

1.1.1 Structure and subunit composition: NADPH oxidase is a multi-meric enzyme complex, which is expressed in phagocytes such as neutrophils and macrophages. The NADPH oxidase is inactive in resting phagocytes, however, when phagocytes are activated in response to exposure to pathogens, this enzyme becomes active and produces ROS with superoxide anion (O_2^-) being the main product and other forms of ROS such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\bullet$), and hypochlorous acid (HOCl) as secondary products. The NADPH oxidase is indispensable for clearing infections and patients with chronic

granulomatous disease (CGD) suffer from recurrent and life-threatening infections due to lack of NAPDH oxidase activity and reduces O_2^- production in phagocytes. Phagocyte NAPDH oxidase consists of two membrane-bound components, the $gp91^{phox}$ (also known as Nox2) and $p22^{phox}$, which together form the cytochrome b_{558}. In addition to these two subunits, NAPDH oxidase has several regulatory cytosolic subunits including the $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ and the small GTPases Rac1 or Rac-2.

The $gp91^{phox}$ (the catalytic subunit of the phagocytic NADPH oxidase enzyme) is a highly glycosylated protein which consists of six transmembrane α-helices. In addition, this protein has two heme groups that are attached to the histdine residues located in the third and fifth helices thereby placing one heme group on the outer and the other on the inner-membrane side of the cell. The C-terminal (cytoplasmic domain) of $gp91^{phox}$ contains the FAD- and NADPH-binding sites. The structure and the components of the $gp91^{phox}$ protein make it the redox center of the NADPH oxidase enzyme, where electrons are transferred from NADPH on the cytoplasmic side to O_2 in the extracellular via FAD and the two hemes (177). Although the $gp91^{phox}$ contains all the required components of a ROS producing system, it is unable to initiate the electron transfer from NADPH to O_2 in the absence of the regulatory subunits $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac proteins.

The $p22^{phox}$ is a membrane-bound non-glycosylated protein, with four transmembrane domains. This protein forms the cytochrome b_{558} complex with the $gp91^{phox}$ subunit and represents a link between $gp91^{phox}$ and cytosolic components of the NADPH oxidase enzyme. The $p22^{phox}$ protein has a proline-

rich region (PRR) by which it recruits the $p47^{phox}$ subunit to the membrane. It also contains three binding sites for $p67^{phox}$ one of which overlaps with the binding site of $p47^{phox}$. Since the $p67^{phox}$ has a lower affinity for $p22^{phox}$ compared with $p47^{phox}$, the direct interaction between $p22^{phox}$ and $p67^{phox}$ might not have any physiological significance, however it might become important in conditions where $p47^{phox}$ is either absent or dysfunctional as in the case of neutrophils in patients with CGD (52).

The $p47^{phox}$ subunit, which resides normally in the cytosol of resting phagocytes, contains two tandem SH3 domains and an autoinhibitory region (AIR), which interacts and masks these SH3 two domains thereby preventing interactions between these domains and comparable domains in other proteins. In addition to these domains, the N-terminal of $p47^{phox}$ contains a phosphoinositide-interacting PX domain, which binds to phosphoinositides such as phosphatidylinositol 3, 4-bisphosphate in activated cells. During phagocytosis, $p47^{phox}$ undergoes phosphorylation on multiple serines located in the autoinhibitory region resulting in conformational changes exposing both SH3 domains and the PX domains. As a result, the SH3 domains of $p47^{phox}$ bind to the proline-rich region of the $p22^{phox}$ while the PX domain of $p47^{phox}$ interacts with the membrane phosphoinositides eventually resulting in the recruitment of $p47^{phox}$ protein to cytochrome $_{b558}$ (177).

While the $p47^{phox}$ subunit organizes the assembly of NAPDH oxidase by providing binding sites for the other cytosolic factors, the $p67^{phox}$ acts as an activator of NAPDH oxidase by activating the electron transfer within the flavocytochrome. The $p67^{phox}$ is comprised of four tetratricopeptide repeat (TPR)

motifs in the N-terminal, SH3 domains in the center and C-terminal and a PB1 domain between the to SH3 domains. Upon cell stimulation, the SH3 domain located in the C-terminal of the $p67^{phox}$ subunit binds to the C-terminal of $p47^{phox}$ (proline-rich region) and causing a translocation of $p67^{phox}$ to cytochrome$_{b558}$ (177). Studies shave shown that in the presence of $gp91^{phox}$ inhibitors, the translocation of $p67^{phox}$ to the membrane was more inhibited compared with $p47^{phox}$. In addition, it has been demonstrated that NADPH oxidase could be activated in the absence of $p47^{phox}$ and in the presence of relatively high expression of $p67^{phox}$, a result which suggests that $p67^{phox}$ can interact directly with cytochrome $_{b558}$ (53). The complex formed in this manner is fully active and produces large amounts of O_2^-. Adding $p47^{phox}$ to this protein complex only enhanced O_2^- turnover by increasing the affinity of $p67^{phox}$ and Rac for the cytochrome $_{b558}$ by two orders of magnitude. Subsequent investigations into the role of $p67^{phox}$ in NADPH oxidase activation have shown that $p67^{phox}$ activates electron transfer in the flavocytochrome by regulating the reduction of FAD (106, 142).

Among the several isoforms of Rac proteins (Rac1, Rac1b, Rac2, and Rac3), Rac1 and Rac2 have been extensively studied in terms of their roles in activating the NADPH oxidase enzyme complex. Rac2 is exclusively expressed in hematopoietic cells and plays an important role in the activation of $gp91^{phox}$ in neutrophils. By comparison, Rac1 is ubiquitously expressed and is the main Rac GTPase involved in the activation of the NADPH oxidase in monocytes and non-phagocytes (94). Several studies have documented that Rac proteins are indispensable component of an active NADPH oxidase enzyme. Indeed, knocking

down Rac2 expression in intact phagocytes inhibited O_2^- by NADPH oxidase (62). Furthermore, O_2^- production by NAPDH oxidase is selectively impaired in Rac2-null mice (154). Finally, in human neutrophil immunodeficiency syndrome, the inability of neutrophils to produce O_2^- has been attributed to an inhibitory mutation in Rac2 gene (7).

In un-stimulated cells, cytosolic Rac is bound to an inhibitory protein known as RhoGDI (41). Upon stimulation, Rac is released from the GDI complex and is translocated to the membrane in kinetics which are similar to that of O_2^- production (150). Although activated Rac (GTP-bound) is capable of a direct interaction with the N-terminal tetratricopeptide repeat (TPR) domain of $p67^{phox}$ (105), this interaction has low affinity (61). By comparison, the direct interaction between Rac and cytochrome$_{b558}$ results in positioning of Rac in a more appropriate position to interact with $p67^{phox}$ and it may also induce certain conformational changes in Rac which increases the affinity of this protein for $p67^{phox}$ (59). It has been shown that Rac is involved in two electron transfer steps in the NADPH oxidase complex, the electron transfer from NADPH to cytochrome$_{b558}$-associated FAD, and then to cytochrome$_{b558}$ heme. For this reason, Rac-$p67^{phox}$ interaction is necessary for a complete transfer of electrons from NADPH to cytochrome$_{b558}$ (59).

The $P40^{phox}$ protein was the last cytosolic component of NADPH oxidase to be discovered (199). It contains a PX domain in the N-terminal, a single SH3 domain, and a PB1 domain in the C-terminal (149, 199, 201). The SH3 domain interacts with the SH3 domain of $p67^{phox}$ and the proline-rich domain of $p47^{phox}$ (72). In un-stimulated cells, $p40^{phox}$ is located in the cytosol and is bound to

$p67^{phox}$. These two subunits interact with each other through their PB1 domains (186). Several studies have shown that this interaction stabilizes both proteins as documented in studies in which recombinant $p67^{phox}$ becomes unstable in the absence of $p40^{phox}$ (72) and as indicated by the reduction of $p40^{phox}$ expression in patients deficient in $p67^{phox}$ (187). In stimulated cells, 40^{phox} translocates to the membrane alongside with $p67^{phox}$ and $p47^{phox}$ resulting in an increase the activity of the NADPH oxidase enzyme, mainly by facilitating the translocation of $p47^{phox}$ and $p67^{phox}$ to the membrane (107).

1.1.2 Regulation of NADPH oxidase activity in phagocytes: In resting phagocytes the components of the NADPH oxidase complex are disassembled and reside in the membrane and the cytosolic compartments. When the phagocytes are stimulated, for example during phagocytosis, various subunits of this enzyme are assembled to make an active, O_2^--producing enzyme (6). The relatively large amount of ROS produced in this process, known as "the respiratory burst", is involved in clearing pathogens from the host (11). To meet this purpose, a transient activation of this enzyme is sufficient (2). If the enzyme activity is uncontrolled, the enormous amount of O_2^- produced can cause serious cellular damage and participate in several pathologies including atherosclerosis (174), ischemic stroke (193), aging (14), ischemic liver necrosis (144), and Parkinson disease (202). The termination of the activity of this enzyme is also important in non-phagocytic NADPH oxidase, as the ROS produced in these systems is mainly involved in cell signaling events.

The activity of the NADPH oxidase enzyme is regulated at different levels including the assembly, initiation, maintenance, and finally deactivation

(termination) (56). In response to specific stimuli as in the case of opsonized bacteria, protein kinases particularly protein kinase C (PKC) isoforms are activated resulting in the phosphorylation of $p47^{phox}$ subunit of NADPH oxidase (141, 156). Phosphorylation of $p47^{phox}$ leads to its conformational change, making it capable of interacting with cytochrome$_{b558}$ ($gp91^{phox}$ and $p22^{phox}$)(80, 146). This interaction then facilitates the translocation of the other cytosolic components ($p67^{phox}$ and $p40^{phox}$) and Rac to cytochrome$_{b558}$ (90). Following the assembly of the enzyme complex, NADPH binds to the assembled enzyme resulting in the electron transfer and the production of superoxide anions from molecular oxygen.

The activity of the NADPH oxidase enzyme is regulated by different factors including for example the availability of its substrates. Many reports have shown that the concentration of O_2 doesn't have a major effect on the activity of phagocytic NAPDH oxidase (73). By comparison, NADPH levels are crucial for the activity of this enzyme. However, NAPDH oxidase activity is not completely terminated when cells are depleted of NADPH (101). The effect of availability of NADPH on the function of NAPDH oxidase is highlighted in the case of a hereditary defect where O_2^- production is impaired due to low affinity of NADPH for the binding site of NAPDH oxidase (116). As the activation of the NADPH oxidase enzyme depends on the activity of certain kinases, several investigators have focused their attention on the roles played by several phosphatases in deactivating NADPH oxidase. It has been shown for example that treating activated neutrophils with inhibitors of PKC (staurosporine or H-7) triggers a significant inhibition of O_2^- production (89). Since the $p47^{phox}$ subunit is the main target of the PKC isoforms, it was concluded that sustained phosphorylation of

$p47^{phox}$ is required to maintain NAPDH oxidase activity. It is logical to assume that phosphatases can deactivate NADPH oxidase on the basis that de-phosphorylation of $p47^{phox}$ destabilizes and disassembles NAPDH oxidase complex (51, 89). In addition, phosphatases can inhibit the translocation of the $p47^{phox}$ from the cytosol to the membrane, thereby preventing the formation of new enzyme complexes (51, 89). In agreement with these findings, it has been shown that phosphatase inhibitors not only prolong the activity of NADPH oxidase but in some cases they can also trigger a respiratory burst by lifting the restraint from the kinases (27). Another important regulator of NAPDH oxidase activity in phagocytes is Rac2. In vitro (1) and in vivo (154) studies have revealed that this small GTP protein is one of the indispensable elements of the NADPH oxidase complex. Finally, it has been established that the level of expression of various subunits components also regulates the NADPH oxidase enzyme. Neutrophils have an active transcription system, which can be altered extensively to make drastic changes in the expression of specific genes (100). In this respect, the expression of the genes coding for the different components of the NADPH oxidase enzyme is regulated both during neutrophil maturation (95) and in response to cytokines and inflammatory mediators in mature neutrophils (170).

1.2 NADPH oxidase complex in non-phagocytes: As a result of the development of more sensitive assays in measuring relatively low levels of ROS, many reports have documented that ROS production increases in non-phagocytic

cells in response to growth factors (178), hormones (79), cytokines (118), high glucose (99), and mechanical stress (206). So far, many members of the Nox family proteins have been identified in non-phagocytic cells including Nox1 to Nox5, and Doux1 and Duxo2. These proteins can be classified in three groups according to their resemblance to the $gp91^{phox}$ (Nox2) subunit of NAPDH oxidase (93). Nox1, Nox3 and Nox4 are almost identical to $gp91^{phox}$ in both size and structure whereas Nox5 has the basic structure of Nox2, but it also has an amino-terminal calmodulin-like domain that contains four binding sites for calcium (18). The Doux enzymes, by comparison, have an amino-terminal peroxidase-homology domain in addition to the four binding sites for Ca^{++} (65).

1.2.1 Nox1: Nox1, was cloned in 1999 (176) and is expressed mainly in vascular smooth muscle cells (111) and colon epithelium (38). It requires several proteins for its optimal activity including $p22^{phox}$, Nox organizer1 (NOXO1 or $p41^{nox}$), Nox activator1 (NOXA1 or $p51^{nox}$) and Rac proteins. NOXO1 and NOXA1 are the homologues of $p47^{phox}$ and $p67^{phox}$, respectively (181). Because NOXO1 lacks the autoinhibitory and the phosphorylation sites of $p47^{phox}$, it exists in a complex with Nox1 and $p22^{phox}$ and NOXA1 even in the absence of cell activation (15). The influence of Rac proteins on the activity of Nox1 complex appears to be mediated through an interaction with NOXA1 subunit (181). At the transcriptional level, the expression of Nox1 can be induced in response to different stimuli. For example, in rodent vascular smooth muscle, Nox1 mRNA is induced in response to angiotensin II, platelet-derived growth factor (PDGF), and phorbol esters (111, 176). In human colon epithelial cells, the expression of Nox1 increases in the

presence of IFN-γ (75). The biological roles of Nox1 are still being investigated. Early reports suggested that Nox1 has a role in host defense in epithelial cells (75). Moreover, Nox1 also play a role in the proliferation of mouse lung epithelial cells where it promotes proliferation through the induction of cyclin D1 (151). These studies suggest that the biological role of Nox1 is determined by its cellular location of this protein.

1.2.2 Nox3: This homologue of Nox2 was first identified in fetal liver, kidneys and the middle ear structures (38). Nox3 is able to produce O_2^- in the presence of $p22^{phox}$. By comparison, expression of $p47^{phox}$ or NOXO1 only mildly enhances the activity of Nox3 (188). Experiments with the dominant negative form of Rac1 showed that the Nox3 produces ROS in a Rac-independent manner (188). The fact that Nox3 is abundantly expressed in the vestibular and cochlear epithelia of the inner ear and is involved in the morphogenesis of otoconia suggest that this Nox homologue plays an important role in regulating body balance (16). Otoconia are small calcium carbonate crystals that are necessary for perception of balance and gravity. The association between Nox3 and body balance was based on the observation that mice with the head *tilt* (*het*) phenotype have defects in the otoconia formation due to the mutations in Nox3 (145).

1.2.3 Nox4: Nox4 was first named Renox as it was initially identified in the adult and fetal kidney tissue (74). It is now recognized that Nox4 is also expressed in other adult tissues including ovaries, testes, pancreas, placenta, and skeletal muscles (38). Nox4 alone is capable of producing ROS (74) and the presence of the $p22^{phox}$ subunit further facilitates the ROS production by this enzyme (5). The activity of Nox4 is independent of Rac1 and the presence of either the organizers

($p47^{phox}$ or NOXO1) or the activators ($p67^{phox}$ or NOXA1) does not affect Nox4 activity (5, 128). Since few regulatory subunits are involved in Nox4-derived ROS production, the activity of this enzyme is determined mainly by the level of its expression or by its post-translational modifications (109). One such example of such regulation is the observation that angiotensin II exposure both in vitro and in vivo elicits significant induction of Nox4 expression (200).

1.2.4 Nox5: The catalytic core of Nox5 is similar to that of $gp91^{phox}$, however, Nox5 also has four Ca^{++}-binding sites (EF-hands) in its N-terminal which render its activity to be dependent on the presence of Ca^{++} (18). Knocking down $p22^{phox}$ expression using siRNA oligonucleotides had no effect on ROS-producing activity of Nox5 suggesting that this Nox homologue does not require $p22^{phox}$ for its activity (104). Although Nox5 is abundantly expressed in human T- and B-lymphocytes and in the sperm precursors, its biological role is still unknown (17). It is interesting to know that no orthologue for Nox5 was found in either the mouse, or the rat genomes (177).

1.2.5 Duox1 and Duox2: Duox proteins (dual oxidases) were first identified in the thyroid gland and were initially designated as thyroid oxidases (63). In addition to the NADPH oxidase domain in their C-terminal, the Duox enzymes contain peroxidase-homology domains in their N-terminal and two EF-hand motifs between these two regions (63, 76). The presence of EF-hands in these enzymes is an indication of the regulatory role of Ca^{++} in these enzymes (136). Duox enzymes can also be activated in a Ca^{++}-independent manner (70). It has been shown that $p22^{phox}$ associates with Duox proteins; however, the significance of this interaction is not yet clear (195). The superoxide produced by the NADPH

oxidase domain in these enzymes is not detectable due to the rapid conversion of the superoxide to H_2O_2 by dismutation. Accordingly, H_2O_2 is the major product of the Duox enzymes (71).

The production of thyroid hormones in mammals requires H_2O_2 and the Duox enzymes have been identified as the source of this H_2O_2 (55, 63). As the two members of this group of enzymes (Duox1 and Duox2) are highly homologous, the reason for their coexistence in the thyroid glands is not yet clear. The role of the Duoxes in the thyroid hormone synthesis is further highlighted in the case of a severe congenital hypothyroidism where reduced thyroid hormone synthesis is associated with mutations in Duox2 (139). It should be emphasized that the expression of the Duox enzymes is not restricted to the thyroid glands. Duox2 mRNA has been detected in human and rat salivary glands, caecum, ascending colon and rectum (76). In addition, a relatively abundant Doux1 mRNA expression has been detected in the pseudo-stratified epithelium of trachea and bronchi (164). Moreover, cultured normal human bronchial epithelial cells produce H_2O_2 in response to the calcium ionomycin in a Duox1-dependent manner (71). The fact that Duox proteins are expressed on the lung mucosal surfaces suggests that these enzymes may be involved in host defense.

1.3 Biological Functions of NADPH Oxidase: The cellular responses to external stimuli depend on the nature of the signaling pathways being activated. Activation of different signaling pathways involves complex interplay between various processes such as receptor dimerization, protein phosphorylation, and the

synthesis of second messengers. There is a wide range of intracellular signaling intermediates, which include cAMP, intracellular Ca^{++}, H^{+}, proteins, and ROS that exist in different forms including O_2^{-}, H_2O_2, HO^{-} and $ONOO^{-}$. ROS are ideal signaling intermediates due to their small size, rapid diffusion and short half-life (68). Other characteristics, which make ROS more suited to the role of secondary messengers in cell signaling including their rapid production in response to various agonists, their rapid elimination by different catabolic systems, and finally their ability to elicit reversible and functional alterations in the targeted proteins (46). Numerous enzymes such as mitochondrial oxidoreductase, cyclooxygenases, xanthine oxidases and NADPH oxidases produce ROS. The NADPH oxidase enzyme was initially identified in human neutrophils more than 20 years ago. Since the phagocytic NADPH oxidase is involved in the clearance of the pathogens by catalyzing the "respiratory burst", it has a key role in innate immunity (12). The recent discovery that the NADPH oxidase enzyme system is also present in a wide variety of non-phagocytic cells with vastly different functions suggests that ROS produced by this enzyme are involved in multiple and essential cellular events, other than microbial clearance. Several studies have shown that NADPH oxidase produces ROS in response to a variety of stimuli such as insulin (125), transforming growth factor β1 (143, 185), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) (13, 178), angiotensin II (79) and pro-inflammatory cytokines including tumor necrosis factor (TNF) α (121), and interleukin-1 (IL-1) (133).

1.3.1 Receptor Signaling: Protein tyrosine phosphatases (PTPs) and protein tyrosine kinases (PTKs) are the main regulators of the signal transduction events

in various cell functions such as movement, apoptosis, growth, differentiation, and cytoskeletal rearrangement. Signaling pathways are activated not only by protein phosphorylation but also oxidation by ROS derived from several enzymes including NADPH oxidase. Protein tyrosine kinases and protein tyrosine phosphatases are among the proteins that are regulated by ROS. It has been estimated that there are 112 protein tyrosine phosphatases in the human genome (4). The members of the protein tyrosine phosphatase superfamily contain a CX_5R active site and a wide range of structural domains, which include SH2, extracellular ligand binding domains, and PDZ domains (196, 208). Protein tyrosine phosphatases fall into two major groups, the tyrosine-specific and the dual-specific phosphatases. The substrate of the tyrosine-specific phosphatases is phosphotyrosine-containing proteins, whereas the dual-specific phosphatases comprise of two groups, receptor-like (RPTPs) and cytosolic (cPTPs) phosphatases. The dual-specific phosphatases can act on proteins that contain phosphotyrosine, phosphoserine, and phosphothreonine (8).

Protein tyrosine kinases are another group of enzymes, which have a key role in the regulation of the signaling events in differentiation, metabolism, cell migration and growth. In the human genome, 90 of these enzymes have been identified; 58 of them belonging to the trans-membrane receptor family and the other 32 to the non-receptor family (29). The receptor protein tyrosine kinases contain an extracellular region that interacts with polypeptide ligands, a trans-membrane helix, and a tyrosine kinase domain in the cytoplasm (97). This group of kinases can be typified by receptors of epidermal growth factor (EGF) receptors, insulin, platelet-derived growth factor (PDGF), and fibroblast growth

factors. About two thirds of the protein tyrosine kinases contain cell-surface receptors, the remaining belong to the cytoplasmic protein tyrosine kinase family (cPTK). The non-receptor tyrosine kinases lack the trans-membrane domain. These kinases act downstream of the receptor tyrosine kinases and are located in the cytoplasm. Focal adhesion kinase (FAK), Janus kinases, Ab1 and Src belong to this family of kinases (39).

Several studies have shown that the activity of protein tyrosine phosphatases can be regulated in a redox-dependent manner. In response ROS, the catalytic cysteine residues of the protein tyrosine phosphatases are oxidized and intra-molecular S-S bonds are formed thereby causing reversible inactivation of these enzymes (22). The activity of the protein tyrosine kinases is regulated not only by protein tyrosine phosphatases and autoregulatory mechanisms, but also by the redox status of the cell. Unlike protein tyrosine phosphatses, the protein tyrosine kinases are activated in the presence of ROS through two mechanisms. First, direct activation of protein tyrosine kinases by oxidants is triggered through oxidization of specific cysteine residues, formation of S-S bonds, and dimerization. Second, protein tyrosine kinases could be activated indirectly by oxidants through an inhibitory effect of oxidants on protein tyrosine phosphotases (40). It should be emphasized ROS play a major roles in terminating cellular signaling events through enhanced activation of protein tyrosine kinases which themselves trigger phosphorylation of protein tyrosine phosphatases, thereby inducing a state of super-activation of the phosphatases upon reduction of the disulfide bonds (39).

1.3.2 Cell cycle control: ROS have a wide range of cellular effects ranging from apoptosis and senescence to cell growth and transformation. The sources, levels and the nature of ROS as well as the cellular context and the antioxidant status determine the fate of a stimulated cell (67). Relatively low concentrations of ROS have been shown to promote cell proliferation. For example, in vascular smooth muscle cells, PDGF promotes proliferation by inducing the production of low amounts of H_2O_2 (178). ROS-induced cell growth is mediated through activation of transcription factors as well as through regulation of protein kinases and phosphatases, which modulate various phases of the cell cycle. For example, in fibroblasts, low concentrations of H_2O_2 increases the expression of cyclin D1, which, in turn, facilitates the cell-cycle transition through the G_1/S checkpoint leading eventually to increasing cell proliferation (127). ROS could also regulate cell cycle and cell proliferation indirectly through inhibiting the inhibitors of cell cycle progression. One such example is the influence of oxidants on $P27^{Kip1}$, a negative regulator of cyclin/cyclin-dependent kinases interactions that inhibits phosphorylation of transcription factors necessary for the entry into the S phase. It has been shown that exogenous H_2O_2, in low concentrations, promotes proliferation in prostate tumor spheroids by reducing the expression of $p27^{Kip1}$ (197). Moreover, relatively low levels of H_2O_2 endogenously produced as in the case of over-expression of Nox1 has also been shown to promote cell proliferation (9). In accordance with these results, antioxidants have been shown to inhibit proliferation and cell-cycle progression. For instance, fibroblasts treated with N-acetyl-L-cysteine (NAC), a strong antioxidant, are arrested in the G1 phase of the cell-cycle due to down-regulation of cyclin D1 expression, hypophosphorylation

of the retinoblastoma protein (Rb), and an increase in the expression of $p27^{Kip1}$ (135).

Unlike the above-mentioned examples of the positive regulation of cell cycle by relatively low levels of ROS, moderate levels of these species result in growth arrest and at high concentrations can even trigger necrosis and/or apoptosis (58). These effects of moderate and high levels of ROS could be mediated through the same targets that are activated by relatively low levels of oxidant. Indeed, sustained production of reactive oxygen species in mouse fibroblast induce cell-cycle arrest by eliciting the up-regulation of the cell cycle inhibitor $p21^{Cip1}$ and by suppressing the expression of cyclins that promote cell cycle progression such as cyclin D1 (21).

1.4 Skeletal muscle satellite cells:

1.4.1 Definition and localization: In several pathologies such as cancer, heart failure, and sepsis, imbalance between the catabolic and anabolic processes in skeletal muscles leads to muscle loss, diminished muscle strength, immobility, impaired respiratory function, and increased morbidity (123). The main components of skeletal muscle pathogenesis in these conditions are progressive loss of muscle tissue and inability to regenerate and replace the damaged myofibers (171). The process of tissue repair involves four phases: degeneration, inflammation, regeneration, and fibrosis (140). In the course of healing, a wide range of inflammatory cells is recruited to the damaged muscle to clear cellular debris by phagocytosis (171). The activated inflammatory cells accelerate the inflammatory response by secreting various cytokines such as IL-1β, IL-6, IL-8,

and TNF-α (96). In addition to the immune-cell-derived cytokines, skeletal muscles are capable of producing several cytokines including IL-4, IL-6, IL-8 and IL-10 (190). Circulating and muscle-derived cytokines could contribute to increased proteolysis and inhibition of protein synthesis leading eventually to muscle wasting. This has been well documented in the case of TNF-α which has been identified as an important mediator of muscle wasting (120).

Adult mammalian skeletal muscle fibers are terminally differentiated tissues with relatively low mitotic activity as indicated by a nuclei turnover rates that do not exceed 1 to 2% per week (159). Despite the scarcity of mitotic events in muscle fibers due to the presence of postmitotic myonuclei which are unable to proliferate, the regenerative capacity of the skeletal muscle tissue in case of severe damage is relatively high (second only to bone marrow)(93). This relatively high regenerative capacity is attributed to the presence of muscle-specific progenitor cells termed the satellite cells which are located between the basal lamina and the cell membrane of mature myofibers (130). The reduced organelle content, small nuclei, and high nuclear to cytoplasmic ratio in these cells suggest that they are quiescent, both metabolically and mitotically (130). However, in response to direct injuries to muscle or degenerative muscle diseases, satellite cells exit the quiescent stage and start proliferating (81, 87). After proliferation, most satellite cells fuse to each other and then differentiate to form new myofibers, while the remaining undifferentiated cells restore the pool of these stem cells under the basal lamina (162). The process of satellite cell activation includes the up-regulation of muscle specific genes and muscle transcription factors, which in turn are regulated by various mechanisms such as cell-matrix and cell-cell interactions

as well as secreted factors. The initiators of satellite activation are found in the extracts from crushed muscles (36) and molecules secreted by invading macrophages (115) and released by the connective tissues (175).

1.4.2 Proliferation of satellite cells: There are also a number of growth factors and cytokines that have been shown to maintain the balance between the growth and differentiation of these cells. For example hepatocyte growth factor (HGF) (103), insulin- like growth factor (IGF), fibroblast growth factor (FGF), transforming growth factor-β (TGF-β) (3), TNF-α and IL-6 all play important roles in regulating both proliferation and differentiation of skeletal muscle satellite cells (87). Once satellite cells are activated, they re-enter the cell cycle and go through rounds of cell division to produce a pool myogenic precursor cells (myoblasts). A number of these cells will replenish the satellite cell reserve but the majority of them will proliferate further before they fuse and differentiate into multinucleated myotubes and eventually myofibers.

1.4.3 Effects of hepatocyte growth factor (HGF): HGF, first isolated from the sera of partially hepatectomized rats, has a key role in organ regeneration including skeletal muscles (207). In regenerating muscles, the degree of upregulation of HGF expression is proportional to the degree of the injury (180). Immunomeutralization experiments have identified HGF as the primary activator of quiescent satellite cells (183). In addition to its role in triggering the entry of the quiescent satellite cells into the cell cycle, HGF has been shown to promote satellite cell migration to the site of the injury (179). HGF acts through its

receptors, *c-met*, which are expressed in both quiescent and activated satellite cells (50).

1.4.4 Effects of fibroblast growth factors: In addition to HGF, a number of fibroblast growth factors (FGFs) play important roles in satellite cell proliferation (64). For example FGF-6 is exclusively expressed in muscle and its expression is upregulated during muscle regeneration (57). By comparison, FGF-2 is a potent myoblast activator *in vitro* and is present in the basal lamina which surrounding mature and developing myotubes (48). Injection of FGF-2 in *mdx* mice enhances satellite cell proliferation and muscle regeneration (114). Contrary to its effect on muscle regeneration in *mdx* mice, injecting FGF-2 had no effect on the repair process of injured muscles in mice (138). These results suggest that the effect of this growth factor on muscle regeneration depends on the nature of the injury. In addition to the regulation of satellite cell proliferation, FGFs contribute to muscle regeneration through their angiogenic properties by promoting revascularization in injured muscles (113). FGFs exert their effects through specific receptors (FGF receptors, FGFRs). Among the FGFRs, FGFR-1 and -4 are the most abundant in satellite cell. FGFRs and *c-met* are transmembrane receptor tyrosine kinases which in response to their agonists and the presence of heparan sulphate proteoglycans (HSPGs), undergo auto-phosphorylation and subsequently activate intracellular signaling pathways resulting eventually in satellite cell activation (152).

1.4.5 Effects of insulin-like growth factors (IGFs): IGFs are another group of growth factors involved in the regulation of muscle growth and repair through both paracrine and autocrine mechanisms. IGF-I and -II are capable of promoting

proliferation and differentiation in cultured mouse myoblasts (42). In addition, increased levels of IGF-I in muscle cells induce muscle hypertrophy not only by activating the satellite cells, but also by augmenting the protein synthesis in the existing myofibers (19, 23, 112). Since motor neurons are also responsive to IGFs, the role of these growth factors is also implicated in the reinnervation process in the injured muscle (191). Both *in vivo* and *in vitro* studies have shown that cytokines play important roles in regulating satellite cell proliferation. For example, injection of leukemia inhibitory factor (LIF) in skeletal muscles of mice with muscle dystrophy (mdx mice) improves muscle regeneration by inducing satellite cell proliferation and also results in increased regenerated myofibers in both number and size (10, 20, 172). In addition, in vitro studies have shown that this cytokine promotes myoblast proliferation by activating the JAK2-STAT3 signaling pathway (172).

1.4.6 Effects of tumor necrosis factor alpha (TNF-α): An important cytokine that modulate satellite cell proliferation and differentiation is TNF-α. The importance of this cytokine in the regulation of skeletal muscle regeneration stems from many observations showing elevated TNF-α expression in injured and regenerated muscle fibers (43, 54, 157, 184). TNF-α acts through two trans-membrane receptors, TNF receptor type 1 (TNF-R1 or p55R) and TNF receptor type 2 (TNF-R2 or p75R). Both receptors interact with membrane-bound TNF-α, soluble TNF-α and the related protein lymphotoxin-α (LT-α or TNF-β) (194). Many reports have shown that TNF-R1 and R2 could mediate opposing effects in the same cells. For example in neuronal tissues, while TNF-R1 exacerbates the

process of tissue destruction, TNF-R2 elicits a protective effect by activating the PKB/Akt signaling pathway (69). It should be emphasized that cellular outcomes of activation of these receptors by TNF-α depend on the cell type, the TNF-R arrangement, the concentration and the duration of their exposure to their ligand (192).

TNF-R1 harbors a cytoplasmic death domain (DD), which makes it a typical death receptor and the most potent inducer of cytotoxic signals (182). In response to TNF-α, TNF-R1 recruits various signaling molecules to form complexes I and II. Complex I is composed of TNF-R associated death domain (TRADD), TNF-R associated factor 2 (TRAF2), and receptor interacting protein 1 (RIP1) (137). Formation of this complex leads to the activation of the transcription factor NF-κB and different mitogen-activated protein kinase (MAPK) cascades, which lead to the activation of the extracellular, regulated kinases (Erk), c-Jun activating kinases (JNK) and the p38 MAPK signaling pathways (28, 88). The Erk signaling pathway is involved in cell proliferation, differentiation and survival (117), whereas the JNK signaling pathway is involved in inflammatory responses (205). Finally, the p38 MAPK signaling pathway has important roles in inflammation, cell growth, differentiation, and apoptosis (108). The cytosolic complex II is comprised of internalized complex I, Fas associated death domain (FADD) and caspase-8 (137). Formation of complex II initiates the mitochondria-dependent apoptosis pathway by activating caspase-8 which in turn cleaves Bid to tBid followed by the induction of cytochrome c release by

permeabilizing the mitochondria leading eventually to the activation of other caspases and apoptosis (28).

In intact skeletal muscles, genetic deletion of TRAF2 (TRAF2$^{-/-}$ mice) elicits a significant decline in muscle mass (124). This observation along with the observation that knocking out TNF-α is associated with severe muscle atrophy in mdx mice confirm the importance of TNF-α in maintaining muscle mass (173). In cultured satellite cells, TNF-α treatment stimulates DNA synthesis by accelerating the transition of the cells from the G_1 to the S phase of the cell cycle (119). This effect is likely to be mediated through NF-κB activation which upregulates cyclin D1 expression (85). In addition, the mitogenic effect of TNF-α could also be mediated through activator protein-1 (AP-1) transcription factor as a result of induction of AP-1 subunit, c-Fos (119).

1.4.7 satellite cell activation and quiescence: For many years, identification of satellite cells was based on their unique location under the basal lamina of each myofiber and electron microscopy was the only tool available for this purpose. The recent discovery of various markers such as, CD34, Pax7, Pax3, M-cadherin, c-met, syndecan-3 and syndecan-4, on these cells has facilitated not only their identification by light microcopy, but also their purification (24, 48, 87, 153, 165). The quiescence of satellite cells is under a tight transcriptional control and is associated with the expression of certain proteins and their isoforms. One of these proteins is the growth/differentiation factor 8 (GDF8 or myostatin), a member of the transforming growth factor-β superfamily. Genetic deletion of GDF8 (GDF8$^{-/-}$) in mice is associated with increased activity and proliferation of satellite cells

suggesting that GDF8 promotes quiescence of these cells (131). By comparison, syndecan-3, and -4, CXCR4 and c-met receptors promote satellite cell activation and proliferation (48, 169, 183). Syndecan-3 and -4 are members of the heparin sulfate protein glycans that required for the activation of various receptors tyrosine kinases. Syndecan-4 deficiency in satellite cells results in a delay in the early stages of their proliferation (49).

The major signaling pathway involved in the activation and proliferation of satellite cell is the Notch signaling pathway (45). In the case of muscle injury, the expression of Delta-1 (the Notch ligand) is increased not only in the site of the injury, but also in the membranes of the myofibers distant form the site of injury (44). The latter could be an explanation for the 'activation at a distance' phenomenon, one of the characteristics of satellite cells (163). Activated satellite cells show an increase in the expression of the myogenic regulatory factor (MRF), such as Myf-5, which is required for the proliferation and self-renewal of the satellite cells, and MyoD which is required for differentiation of the myogenic precursor cells (myoblast) (47). In $MyoD^{-/-}$ mice, the process of muscle regeneration is severely impaired and the myoblast fail to fuse and differentiate (132). The importance of MyoD in myoblast differentiation is further confirmed by finding that cultured $MyoD^{-/-}$ satellite cells continue to proliferate and fail to differentiate even after the removal of serum. Upon activation, satellite cells go through several rounds of proliferation and the majority of them proceed along a myogenic lineage. The fact that the number of reserved quiescent satellite cells in an adult muscle remains the same suggests that these cells are capable of self-renewal (161). The exact mechanism of the satellite cell replenishment is to be

discovered. Since the continuous activation of the Notch signaling pathway maintains the cells in their progenitor stage, it is thought that Numb, the Notch inhibitor, might be involved in this process by its asymmetric distribution in a dividing myogenic progenitor cell (35, 168). In this situation, the daughter cell that harbors Numb advances along the myogenic lineage, while the other cell becomes quiescent and remains in the basal lamina as a new satellite cell.

1.5 NAPDH oxidase in skeletal muscles and satellite cells: Little information is yet available regarding the expression and functional significance of NADPH oxidase-derived ROS in both skeletal muscle fibers and skeletal satellite cells. Javeshghani *et al* (102) were the first to identify the presence of NADPH oxidase subunits in skeletal muscles. These authors reported the presence of $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ proteins in rat skeletal muscles and described intracellular localization inside muscle fibers in a fashion similar to that of dystrophin suggesting that these NAPDH oxidase subunits were expressed in close proximity to the sarcolemma. Javeshghani *et al* (102) also found that skeletal muscle NAPDH oxidase subunit expression was not altered by exposure to bacterial lipopolysaccharides (LPS), however, the contribution of NADPH oxidase-derived to overall skeletal muscle ROS production increased significantly in rats injected with bacterial lipopolysaccharides suggesting that NAPDH oxidase may contribute to the development of oxidative stress inside skeletal muscles in sepsis. More recently, Hidalgo *et al.* (91) described the localization of $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$ and $p67^{phox}$ proteins in the transverse tubules of mouse

and rabbit skeletal muscles. These authors also found that NAPDH oxidase-derived ROS contribute to enhanced muscle contractility by modifying the opening of ryanodine receptors and increasing Ca++ flux in response to depolarization of muscle sarcolemma. Although these studies have improved our knowledge about the presence of NAPDH oxidase in skeletal muscle fibers, more questions remains unanswered regarding this presence. For instance, it is unknown whether other Nox proteins apart from $gp91^{phox}$ (Nox2) are also present in skeletal muscle fibers. Also not known are the abundance and the association of these various subunits with each other.

As in the case of skeletal muscles, little is known about the structure and functional importance of NAPDH oxidase subunits in skeletal satellite cells. Wei *et al.* (198) reported that angiotensin II (Ang II) markedly enhanced NADPH oxidase activity and consequent ROS generation in L6 myotubes (differentiated rat myoblasts). This effect was mediated through translocation of NADPH oxidase cytosolic subunits $p47^{phox}$ and $p67^{phox}$ to the plasma membrane. In addition, Ang II abolished insulin-induced tyrosine phosphorylation of insulin receptor substrate 1, activation of Akt, and glucose transporter-4 translocation to the plasma membrane (198). All these effects were reversed by apocynin (inhibitor of NAPDH oxidase). Wei *et al.* concluded that NADPH oxidase activation and ROS generation play a critical role in in Ang II-induced inhibition of insulin signaling in skeletal muscle cells. In a recent study, Espinosa *et al.* (66) have demonstrated that electrical field stimulation or exposure to K^+ elicited a significant increase in DPI-sensitive ROS generation in primary rat skeletal myoblasts and reported the presence of $p47^{phox}$ and $gp91^{phox}$ proteins in these

cells. Unlike the above-described studies which confirmed the presence of subunits of NAPDH oxidase in skeletal satellite cells, Hutchinson *et al.* (98) failed to detect NADPH oxidase expression in either immortalized mouse C2C12 or L6 myoblasts. The reasons behind these discrepancies with respect to the presence of NAPDH oxidase subunits are unclear. We speculate that the use of different methodologies, different antibodies to detect NAPDH oxidase proteins and the use of different immortalized murine and rat satellite cells might have contributed to these differences.

1.6 Objectives: The general aim of this thesis was to identify the molecular structure, activity and function of NADPH oxidase in skeletal muscle fibers and satellite cells. Specific goals included: 1) characterization of subunit expression and localization of the NADPH oxidase enzyme complex in both skeletal muscle fibers and skeletal muscle satellite cells; 2) assessing the influence of satellite cell density and differentiation into myotubes on NAPDH oxidase subunit expression and ROS-generating capacity of this enzyme; 3) investigation of whether NAPDH oxidase-derived ROS play any role in the proliferation of skeletal satellite cells; and 4) identifying signaling pathways through which NAPDH oxidase-derived ROS regulate satellite cell proliferation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animal preparations: Six-week old male C57/BL6 mice were sacrificed with an overdose of pentobarbital sodium. Diaphragm, extensor digitorum longus (EDL), tibialis anterior and soleus muscles were immediately excised, frozen in liquid nitrogen and stored at -80°C until further analysis.

2.2 Cell culture:

2.2.1 Primary human myoblasts: Primary human satellite cells (human myoblasts) immortalized by expression of the E6E7 early region from human papillomavirus type 16, were generously provided by Dr. E. Shoubridge (McGill University, Montréal, QC),and cultured in SkBM culture medium (SkBM® Bullet Kit, Cambrex, East Rutherford, NJ) supplemented with 15% inactivated fetal bovine serum (FBS)(122). To induce differentiation into myotubes, myoblasts (90% confluent) were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Inc., Carlsbad, CA) supplemented with 2% inactivated horse serum (HS) for 6 days. Differentiation was evaluated, using immunoblotting, by the morphological appearance of cells and by measuring the expression of myogenin and muscle-specific proteins, including myosin heavy chain and creatine kinase.

2.2.2 Primary murine myoblasts: Primary murine satellite cells (mouse myoblasts) derived from diaphragm and tibialis anterior muscles were established as described by Rosenblatt *et al.* (155). Briefly, diaphragm and tibialis anterior muscle strips were extracted from 6-week-old C57/BL6 mice, digested with collagenase (0.2% at 37°C for 60 min), then triturated to break muscle tissues into single fibers. Individual fibers were washed in (DMEM) and phosphate-buffered

saline, transferred into matrigel-coated (1mg/ml in DMEM) 6-well plates and maintained in DMEM supplemented with 1% penicillin/streptomycin and 0.2% amphotericin B, 10% HS and 0.5% chick embryo extract (MP Biomedicals, Aurora, OH) for 4 days, which allowed myoblasts to attach to the substratum. Myoblasts were then grown in growth medium (DMEM supplemented with 20% fetal bovine serum, 10% HS, 1% chick embryo extract) for 6 days. Identical growth medium was used to maintain immortalized murine C2C12 cells. Total RNA was extracted from primary human and murine as well as C2C12 myoblasts.

2.3 Reverse transcriptase-PCR (RT-PCR) and real-time PCR: Total RNA from mouse diaphragm, EDL, tibialis anterior and soleus muscles and mouse and human myoblasts and myotubes was extracted using a GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich Co., Oakville, ON). Total RNA (2μg) was reverse transcribed using 200 Superscript II Reverse Transcriptase (Invitrogen) in a reaction mixture containing 0.5mM dATP, dCTP, dGTP, and dTTP, 40 units of RNase inhibitor, 50pmol random hexamers, 3mM $MgCl_2$, 75mM KCl, 50mM Tris-HCl (pH 8.3), and DTT 20mM in a total volume of 20μl. The resultant cDNA was PCR amplified using TaqDNA polymerase (Invitrogen) and oligonucleotide primers specific for murine and human $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, Nox1-Nox5 and cyclophilin B transcripts (control) (Table 1). For all genes, except Nox4, the amplification cycles consisted of initial denaturation at 94°C for 3min followed by 35 cycles of 30s at 94°C, 30s at 57°C, and 30s at 72°C. For Nox4, the annealing temperature was 50°C. RT- PCR products were analyzed on a 2% agarose gel.

Real-time PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) and primers designed to amplify human and mouse transcripts of Nox2, Nox4, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and GAPDH (Table 1). One μl of the reverse transcriptase reaction was added to 25μl of SYBR Green PCR Master Mix (Qiagen Inc., Valencia, CA) and 3.5μl of each 10μM primer. The thermal profile was as follows: 95°C for 10min and 40 cycles of 95 °C for 15s, 57 °C for 30s, and 72°C for 33s. A melt analysis for each PCR experiment was used to assess primer-dimer formation or contamination. Results were analyzed using the comparative threshold cycle (C_T), the value where the amplification curve crosses the threshold line. C_T values were then used to calculate absolute copy numbers based on standard curves generated by plasmids containing full-length coding sequences of NADPH oxidase subunits. All real-time PCR experiments were performed in triplicate.

2.4 NADPH oxidase activity: NADPH oxidase activity in human cells was measured using lucigenin-enhanced chemiluminescence in 96-well plates using an LMax II luminometer (Molecular Devices Corp., Sunnyvale, CA). Briefly, 70% confluent myoblasts or myotubes were washed with PBS and scraped off the plate into cold HBSS buffer. Cells ($50x\ 10^4$) were loaded in each well of a 96-well plate. NADH or NADPH (100μM) and lucigenin (10μM) were added to each well and luminometer output (relative light unit) was measured for a 20min period. NADPH oxidase activity (total signal minus background signal) was measured as the area under the curve. In a few experiments, N-acetylcysteine (NAC, 10mM), which is a general antioxidant, apocynin (1mM), which is an inhibitor of the association of $p47^{phox}$ with $p22^{phox}$ and Nox2, and diphenyleneiodonium (DPI, 10μM) which is an

Table 1: List of oligonucleotides primers used for RT-PCR and Real-Time PCR amplification of NADPH oxidase subunits and Nox proteins in human and murine skeletal myoblasts and myotubes. * Primers used for Real-time PCR.

Target	Product size bp	Sequences	Species
$p22^{phox}$	164	Forward-GTACTTTGGTGCCTACTCCA Reverse-CGGCCCGAACATAGTAATTC	H*
$p22^{phox}$	176	Forward-TCTATCGCTGCAGGTGTGCT Reverse-AGGCACCGACAACAGGAAGT	M*
Nox1	281	Forward-CTTCCTCACCGGATGGGACA Reverse-TGACAGCATTTGCGCAGGCT	M&H
Nox2	321	Forward –TGTCCAAGCTGGAGTGGCAC Reverse-GCACAGCCAGTAGAAGTAGAT	H
Nox2	156	Forward-CCAGTGAAGATGTGTTCAGCT Reverse-GCACAGCCAGTAGAAGTAGAT	H*
Nox3	708	Forward-GAGTGGCACCCCTTCACCCT Reverse- CTAGAAGCTCTCCTTGTTGT	H
Nox4	1741	Forward-GCCGCCGCCATGGCTGTGTCCTGG Reverse-GGCATAACACAGCTGATTGATTCCGCTGAG	H
Nox4	211	Forward-AGTCAAACAGATGGGATA Reverse-TGTCCCATATGAGTTGTT	M&H
Nox4	285	Forward TTGTCTTCTACATGCTGCTG Reverse AGGCACAAAGGTCCAGAAAT	M&H*
Nox5	240	Forward-AAGCATACTTGCCCCAGCTG Reverse-CAGGCCAATGGCCTTCATGT	M&H
$p47^{phox}$	207	Forward-TTGAGAAGCGCTTCGTACCC Reverse-CGTCAAACCACTTGGGAGCT	H
$p47^{phox}$	93	Forward-CCCAGCCAGCACTATGTGTA Reverse-GGAACTCGTAGATCTCGGTG	M&H*
$p67^{phox}$	245	Forward-CAGTTCAAGCTGTTTGCCTG Reverse-TTCTTGGCCAGCTG	M&H*
CyclophilinB	265	Forward-ATGGCACAGGAGGAAAGAGC Reverse-ATGATCACATCCTTCAGGGG	M&H
GADPH	166	Forward-AAGAAGGTGGTGAAGCAGGCG Reverse-ACCAGGAAATGAGCTTGACAA	M&H*

inhibitor of electron transporters, were added 1h prior to the addition of NADPH and lucigenin.

2.5 Subcellular fractionation: Confluent human myoblasts were washed twice with ice-cold PBS and lysed on ice in HEPES buffer (HEPES 50mM, NaCl 150mM, NaF 100mM, EDTA 5mM, triton X-100 0.5%) containing protease inhibitors (aprotinin 5mg/ml, leupeptin 2mg/ml and PMSF 100mM) and sonicated at 100W for 15s on ice. The homogenate was centrifuged at 250g for 5min followed by 20min centrifugation at 20,000g to separate the mitochondria. The supernatant was then centrifuged for 60min at 100,000g to obtain detergent-resistant proteins in the pellet and detergent-soluble proteins in the supernatant. All fractions were stored at -80°C until further analysis.

2.6 Transfection with plasmids and fluorescence microscopy: To identify subcellular targeting of NADPH oxidase subunit proteins in human skeletal myoblasts, expression vectors containing enhanced cyan (ECFP), and enhanced yellow (EYFP) fluorescence proteins were used following methods for the construction of ECFP-Nox2, EYFP-$p22^{phox}$, and EYFP-Nox4 plasmids that have been previously described (148). ECFP-$p47^{phox}$ and EYFP-$p67^{phox}$ plasmids were generated by cloning human $p47^{phox}$ and $p67^{phox}$ cDNA into pECFP-C1 and pEYFP-C1 vectors, respectively (BD Biosciences Inc., Mississauga, ON). Transient transfections were performed with human skeletal myoblasts cells seeded on $22mm^2$ cover slips in 24-well tissue culture plates using Lipofectamine LTX (Invitrogen) at a 1:2 ratio of DNA/Lipofectamine in Opti-MEM® I Medium (Invitrogen), in accordance with the manufacturer's recommendations.

Fluorescence was visualized 48h later using a Zeiss LSM-510 META laser scanning microscope with a 40× oil immersion lens in a multitrack mode with dual excitation (458nm for ECFP and 514nm for EYFP) and emission (BP 470–500nm for ECFP, and BP 530–600nm for EYFP) filter sets.

2.7 Adenoviral infection of myoblasts: Human skeletal myoblasts ($15x10^4$) were seeded in 24-well plates and infected for 5h with adenoviruses (multiplicity of infection was 500 to 1500) in basal medium. These conditions resulted in uniform expression of transgenes in ~90% of the cells, as assessed by green fluorescence protein (GFP) fluorescence. The viruses were then removed, and cells were allowed to recover in complete medium for 24h. To inhibit $p47^{phox}$, adenoviruses expressing a mutant form of $p47^{phox}$, which is defective in the first Src homology 3 (SH3) domain (p47W(193)R), were used (83). To evaluate the functional importance of Nox4, myoblasts were infected with dominant-negative Nox4 adenoviruses lacking FAD-NAD(P)H binding domains (Ad-ΔFAD/NADPH-Nox4), which were generously provided by B.J. Goldstein, Thomas Jefferson University, Philadelphia, PA (125). To evaluate involvement of the NFκB pathway in myoblast proliferation, myoblasts were infected with adenoviruses expressing dominant-negative mutant forms of IκB kinase (IKK) α (Ad-dnIKKα) and IKKβ (Ad-dnIKKβ), as previously described (158). Adenoviruses expressing GFP were used as controls in all experiments involving adenoviruses.

2.8 Transfection with small inhibitory RNA (siRNA) oligonucleotides: Double-stranded siRNA oligos targeting the 5′-AAT TAC TAT GTT CGG GCC GTC-3′ region of the human $p22^{phox}$ coding sequence were synthesized and

purified by Qiagen. A fluorescein-labeled non-silencing control siRNA oligo (Alexa Fluor488, Qiagen) was used as a control. Myoblasts were transfected with siRNA oligos using HiPerFect Transfection Reagent (Qiagen) in skeletal muscle growth serum (SkGM) and were examined 24h later for mRNA expression and 48h later for protein expression.

2.9 Myoblast proliferation: Myoblast proliferation was assessed using two different assays: cell count and bromodeoxyuridine (BrdU) incorporation. For the cell count, myoblasts ($15x10^5$ cells) were plated into 12-well plates and maintained for 24h in complete culture medium containing 15%FBS in the absence and presence of NAC (10mM), apocynin (1mM), PI-3 kinase inhibitors (LY294002 at 300nM and wortmannin at 100nM), a mammalian target of the rapamycin (mTOR) pathway inhibitor (rapamycin at 50ng/ml), an Erk1/2 inhibitor (PD98059 at 30μM), and an NF-κB inhibitor (BAY11-7082 at 1μM). Cells were then trypsinized (0.5% trypsin-EDTA) and stained with trypan blue. Viable cells were counted by hematocytometer. For BrdU incorporation, a Cell Proliferation ELISA, BrdU colorimetric kit was used (Roche Applied Science, Laval, Quebec). Myoblasts were plated into 96-well plates at a density of $5x10^3$ cells/well in 100μl of full culture medium for 24h in the absence and presence of antioxidants and various inhibitors. Cells were then pulsed with 10μM of BrdU for 4h, fixed and then labeled, according to the manufacturer's instructions. Absorbance (370nm) was measured 10min after the addition of substrate.

2.10 Serum-induced proliferation in myoblasts: To evaluate the mechanisms through which oxidants influence serum-induced proliferation of human skeletal

myoblasts, the activation status of the p38 and Erk1/2 members of mitogen activated protein kinases (MAPKs), protein kinase B (AKT), mTOR, and NFκB pathways were measured. Subconfluent human skeletal myoblasts plated into 6-well plates were serum starved for 4h in serum-free medium (SkBM) and then exposed to 15% FBS for 5, 15 and 60 min. Cells were then lysed and underwent immunoblotting for total and phosphorylated p38, Erk1/2, AKT, mTOR, and the p65 subunit of NFκB. Some serum starved myoblasts were stimulated for 15min with 15% FBS in the presence 0.02% dimethyl sulphoxide (DMSO, vehicle), NAC (10 mM) or apocynin (1 mM). Cells were then collected and phosphorylation of the above-mentioned pathways was evaluated using immunoblotting.

2.11 Immunoblotting: Crude cell lysates or cell fractions (30-80μg total protein) were boiled for 5min and then loaded onto tris-glycine SDS-polyacrylamide gels. Proteins were electrophoretically transferred onto polyvinylidene difluoride membranes, blocked for 1h with 5% non-fat dry milk, and incubated overnight at 4 °C with primary antibodies. Nox2, $p47^{phox}$ and $p67^{phox}$ proteins were detected with polyclonal antibodies (102), whereas $p22^{phox}$ protein was identified using a monoclonal antibody (mAb48), generously provided by Dr. D. Roos (University of Amsterdam, Netherlands). Nox4 protein was detected with an affinity-purified polyclonal antibody (78). Activation of Erk1/2 was assessed with polyclonal antibodies specific to active (dually phosphorylated at $Thre^{183}$ and Tyr^{185}) and total Erk1/2. p38 and Stress activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) phosphorylation was monitored with polyclonal phospho-p38

(Thre^{180}/Tyr^{182}), phospho-SAPK/JNK (Thre^{183}/Tyr^{185}) and total p38 and total SAPK/JNK antibodies. Phosphorylation of the p65 subunit of NFκB (RelA) was assessed using a phospho-Ser^{536} antibody. Phosphorylation of mTOR at Ser^{2448} and AKT at Ser^{473} was detected with polyclonal antibodies. All antibodies were purchased from Cell Signaling Technology (Danvers, MA). Myogenin (A4.74) and myosin heavy chain (MF20) protein levels were assessed using monoclonal antibodies (Developmental Studies Hybridoma Bank, University of Iowa). Proteins were detected with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies and ECL reagents (Chemicon Inc., Temecula, CA). Blots were scanned with an imaging densitometer, and optical densities of the protein bands were quantified with ImagePro software (Media Cybernetics, Carlsbad, CA). Predetermined molecular weight standards were used as markers. Protein concentration was measured by the Bradford method with bovine serum albumin as a standard.

2.12 Data analysis: Results are shown as means± SEM. For immunoblotting experiments, at least three independent measurements were performed within each group. Six independent measurements of cell proliferation and NADPH oxidase activity were performed. Comparisons between different groups were performed with a One-Way Analysis of Variance where probability (P) values less than 0.05 were considered significant.

CHAPTER 3

RESULTS

3.1 Expression of NADPH oxidase subunits: Transcripts of Nox2, Nox4, $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ subunits were detected in the diaphragm, EDL, tibialis anterior and soleus muscles of normal mice using RT-PCR. Table 2 lists the expression levels of these subunits expressed as copies per ng total RNA and reveals two main observations: First, the abundance of NADPH oxidase subunits in the diaphragm was relatively higher than that measured in other muscles; second, in a given muscle, the $p22^{phox}$ subunit was the most abundant, as compared to other subunits. Transcripts of Nox2, Nox4, $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ subunits were also detected in primary human satellite cells and murine satellite cells (myoblasts) derived from the diaphragm and tibialis anterior muscles (Figure 1). In these cells, the relative abundance of the $p22^{phox}$ subunit was substantially higher than in other subunits. Nox4 was the second most abundant subunit (Table 3). In addition, between murine and human myoblasts, relative expressions of NADPH oxidase subunits were similar, although murine C2C12 myoblasts showed substantially lower $p22^{phox}$ mRNA expression than primary myoblasts (Table 3). No detectable mRNA transcripts of Nox1, Nox3 and Nox5 were observed in either murine or human myoblasts (data not shown). Immunoblotting analysis of primary murine and human myoblasts using specific antibodies confirmed the presence of 91, 22, 47 and 67 kDa protein bands equivalent to Nox2, $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ subunits, respectively (Figure 2A). In addition, anti-Nox4 antibody detected a strong band at 64 kDa, which disappeared when the antibody was pre-incubated with the immunizing peptide. A non-specific band was also detected at 55 kDa (Figure 2B).

Table 2: Expression of mRNA of NADPH oxidase subunits in mouse skeletal muscles expressed in copies/ng total RNA.

	Nox2	$p22^{phox}$	$p47^{phox}$	$p67^{phox}$	Nox4
Diaphragm	52±15	262±2	28±4	7±0.5	22±1
EDL	1±0.5	13±6	1.1±0.4	0.1±0.05	2.0±0.3
TA	11±2	182±10	11±4	2.5±0.7	3.0±0.1
Soleus	5.0±0.3	255±18	8±2	4.0±0.8	5±1

Values are means (3 independent samples) ± SEM. EDL: Extensor Digitorium Longus; TA: Tibialis Anterior.

Table 3: Expression of mRNA of various NADPH oxidase subunits in skeletal myoblasts expressed in copies/ng total RNA.

	Nox2	$p22^{phox}$	$p47^{phox}$	$p67^{phox}$	Nox4
H. Myoblasts	0.1	1444	0.05	0.3	16
M. diaphragm myoblasts	0.8	1260	0.1	0.3	42
M. Tibialis myoblasts	0.2	426	0.1	0.3	13
C2C12	0.7	0.1	0.03	6.1	42

Values are means of 3 independent samples.

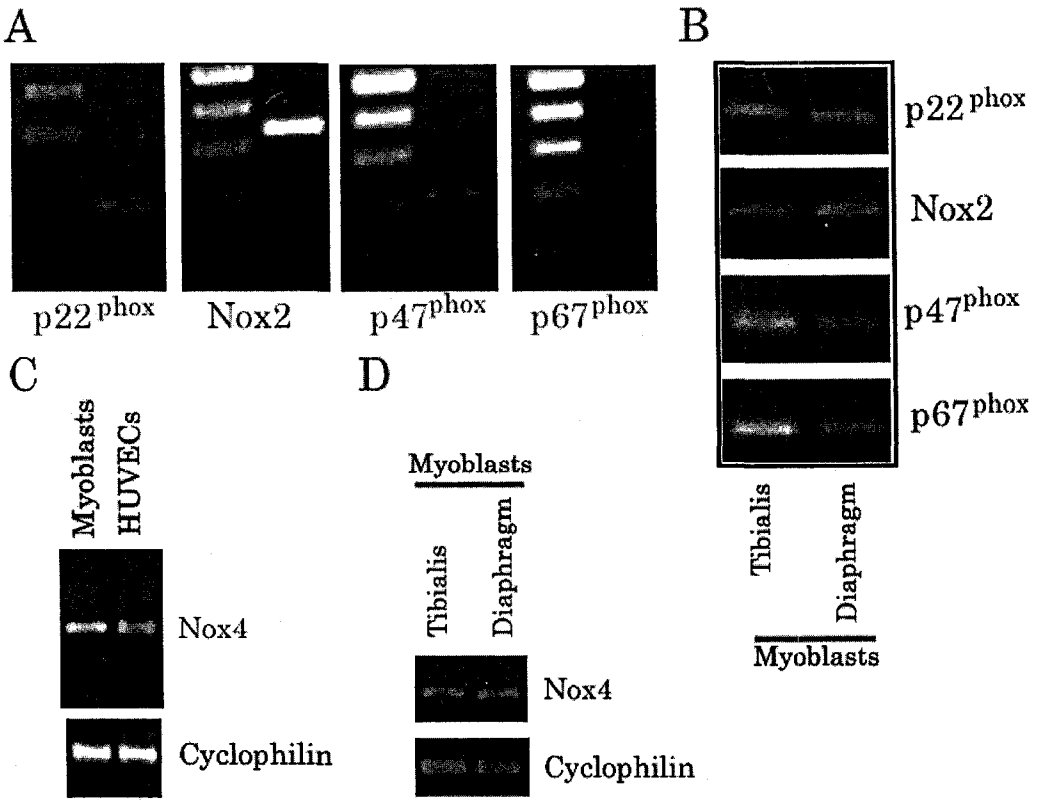

Figure 1: RT-PCR amplification of $p22^{phox}$, Nox2, $p47^{phox}$ and $p67^{phox}$ transcripts in human skeletal myoblasts (A) and primary murine myoblasts derived from tibialis anterior and diaphragm muscles (B). In each lane, PCR products are shown to the right and the DNA ladder is shown to the left. C & D: Amplification of Nox4 mRNA using RT-PCR in human myoblasts (C) and in murine myoblasts derived from diaphragm and tibialis anterior muscles (D). Human umbilical vein endothelial cells (HUVECs) were used as positive controls.

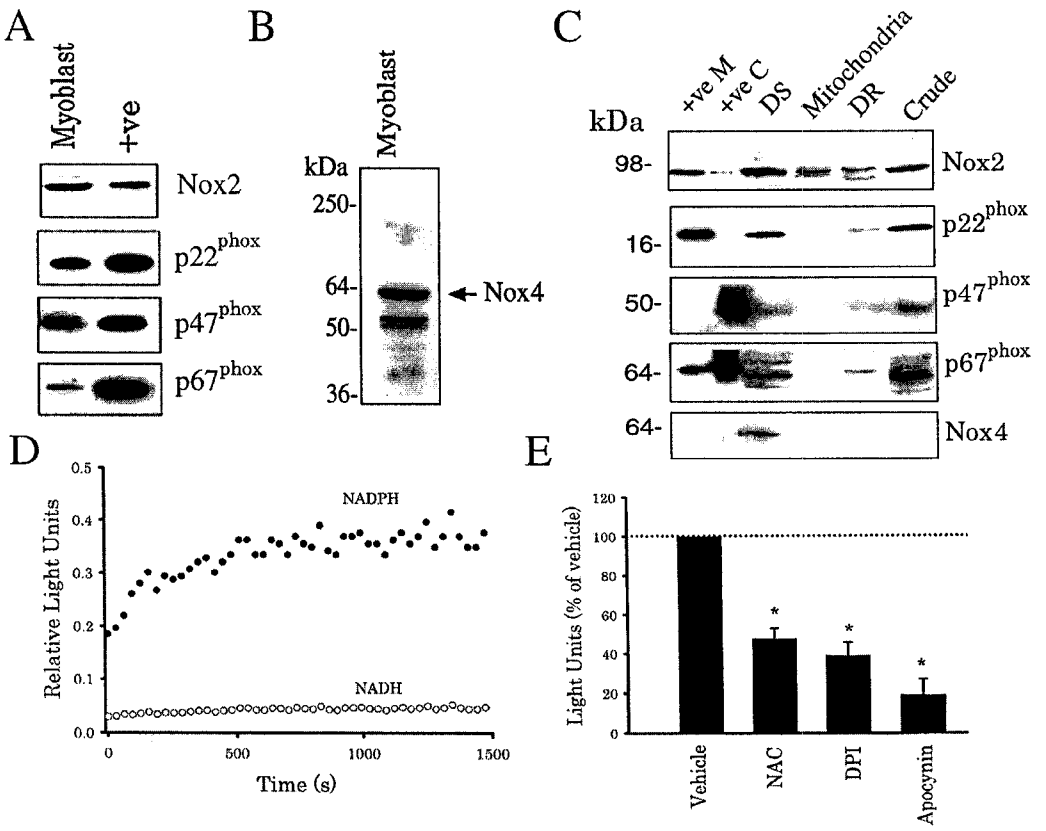

Figure 2: A & B: Detection of Nox2, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$ and Nox4 in total cell lysates of human myoblasts using immunoblotting and selective antibodies. +ve refers to human neutrophil lysates. C: Expression of NADPH oxidase subunits in crude lysates, detergent-soluble (DS), mitochondrial and detergent-resistant (DR) fractions of human myoblasts. +veM and +veC refers to membrane and cytosolic fractions of human neutrophils. D: Production of O_2^- in human myoblasts measured with lucigenin enhanced chemiluminescence. E: Influence of NAC, DPI and apocynin on O_2^- production in human myoblasts (expressed as percentage of that measured in the presence of vehicle, DMSO). *P<0.05 compared with vehicle.

3.2 Localization of NADPH oxidase: Fractionation of human myoblast lysates into subcellular fractions revealed the presence of Nox2, Nox4, $p22^{phox}$, $p67^{phox}$,$p47^{phox}$ proteins in detergent-soluble fraction. Only weak expressions of Nox2, $p67^{phox}$ and $p47^{phox}$ were detected in the detergent-resistant fraction (Figure 2C). Nox2 protein was also detected in the mitochondrial fraction (Figure 2C).

3.3 NADPH oxidase activity: Measurements of O_2^- anion levels in intact human skeletal myoblasts using lucigenin chemiluminescence revealed activity that was dependent on the presence of NADPH rather than NADH (Figure 2D). Pre-incubation with NAC, DPI or apocynin for 1 h significantly attenuated NADPH oxidase activity in these cells (Figure 2E).

3.4 Localization of NAPDH oxidase fusion proteins: To investigate the targeting of NADPH oxidase subunits to various intracellular compartments, human myoblasts were transfected with plasmid constructs that express ECFP and EYFP fused to NADPH oxidase subunit proteins. Nox2, Nox4 and $p22^{phox}$ proteins primarily exhibited an endoplasmic (ER)-like expression pattern (Figure 3). In addition, nuclear membranes stained positive for $p22^{phox}$ (Figure 3). The $p67^{phox}$ protein was localized to the cytosol in a pattern similar to that of the cytoskeleton, with weak membrane-associated staining evident in a few cells (Figure 3). In comparison, diffuse cytosolic staining in addition to strong nuclear staining was detected for the $p47^{phox}$ fusion protein (Figure 3).

3.5 NADPH oxidase expression and myoblast differentiation: Differentiation into myotubes was induced by incubation of myoblasts in differentiation medium (DMEM plus 2% HS) and was associated with the appearance of myogenin transcription factor and myosin heavy chain protein within 1 and 4 days,

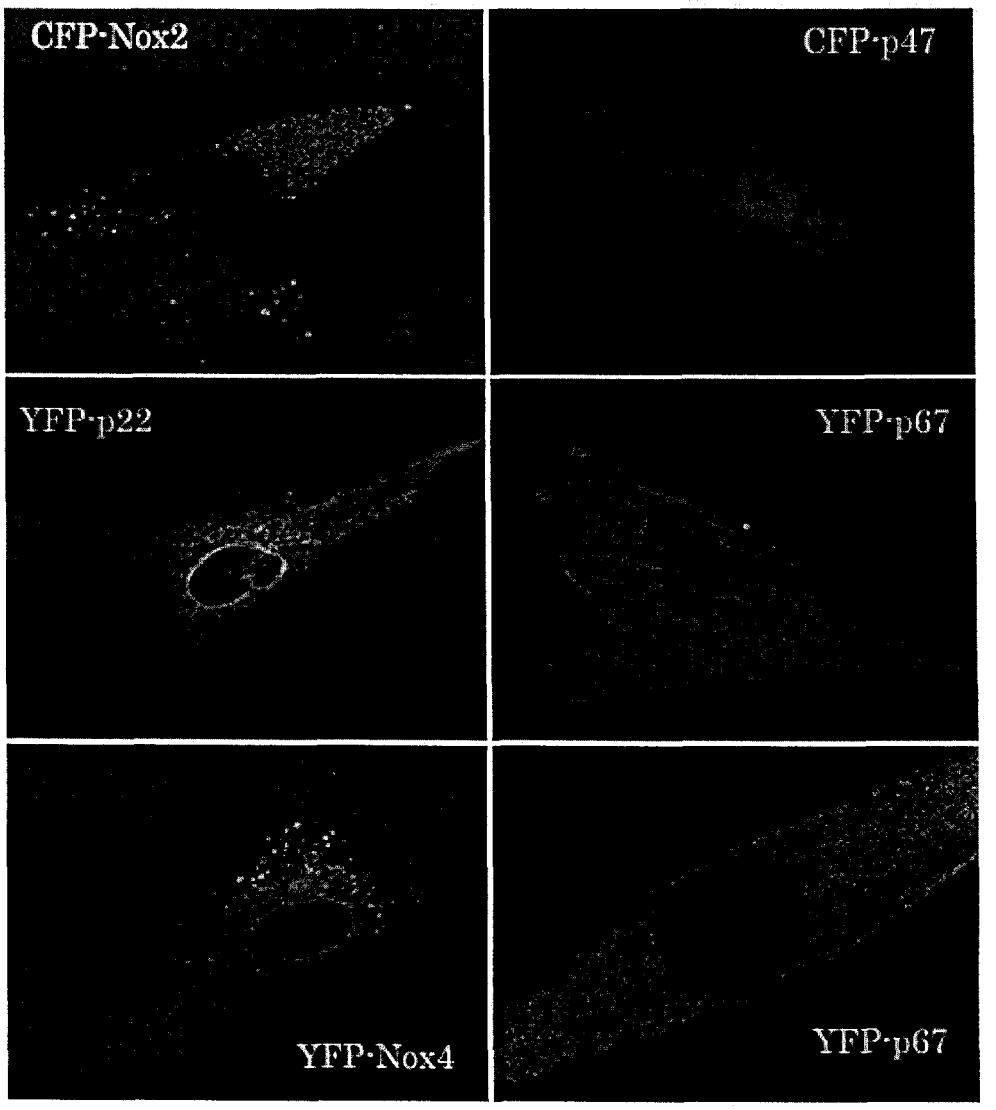

Figure 3: Intracellular localization of CFP-Nox2, CFP-$p47^{phox}$, YFP-Nox4, YFP-$p22^{phox}$, and YFP-$p67^{phox}$ proteins in human myoblasts. Cells were visualized (400x) with a confocal microscope. Note the endoplasmic reticulum-like distribution of Nox2, Nox4 and $p22^{phox}$ and diffuse cytosolic distribution of $p47^{phox}$ and $p67^{phox}$. Also note the nuclear localization of $p47^{phox}$ (upper right) and membrane-associated distribution of $p67^{phox}$ (lower right).

respectively (Figure 4A). Differentiation into myotubes elicited a rapid decline in Nox4 and $p67^{phox}$ levels, a slower reduction in $p47^{phox}$ expression, and a rapid increase in $p22^{phox}$ and Nox2 expression (Figures 4A and B). In addition, differentiation into myotubes was associated with a significant decline in O_2^- production, as measured by lucigenin-enhanced chemiluminescence (Figure 4C).

3.6 Cell density and NADPH oxidase expression: Increasing cell density from 50 to 100% confluency had no effect on mRNA and protein levels of Nox2, $p22^{phox}$, and $p47^{phox}$ but significantly attenuated Nox4 and $p67^{phox}$ mRNA and Nox4 protein levels (Figure 4 D and E).

3.7 Regulation of cell proliferation by ROS: Figure 5 shows that NAC and apocynin significantly attenuated cell number and BrdU incorporation in skeletal myoblasts grown for 24h in the presence of 15% FBS ($P<0.05$ compared with vehicle). Transfection with $p22^{phox}$ siRNA oligonucleotides reduced $p22^{phox}$ protein expression by 75%, attenuated myoblast O_2^- production and reduces cell number and BrdU incorporation by 26 and 24%, respectively (Figure 6). Similarly, expression of dominant-negative $p47^{phox}$ (p47W(192)R) protein attenuated O_2^- production, cell number and BrdU incorporation, as compared with cells expressing GFP (Figure 7). Finally, expression of dominant-negative Nox4 (ΔFAD/NADPH Nox4) protein also attenuated O_2^- production and significantly reduced BrdU incorporation by 16% in human myoblasts (Figure 8).

To evaluate the pathways involved in serum-induced proliferation of myoblasts and the influence of NADH oxidase-derived ROS on these pathways,

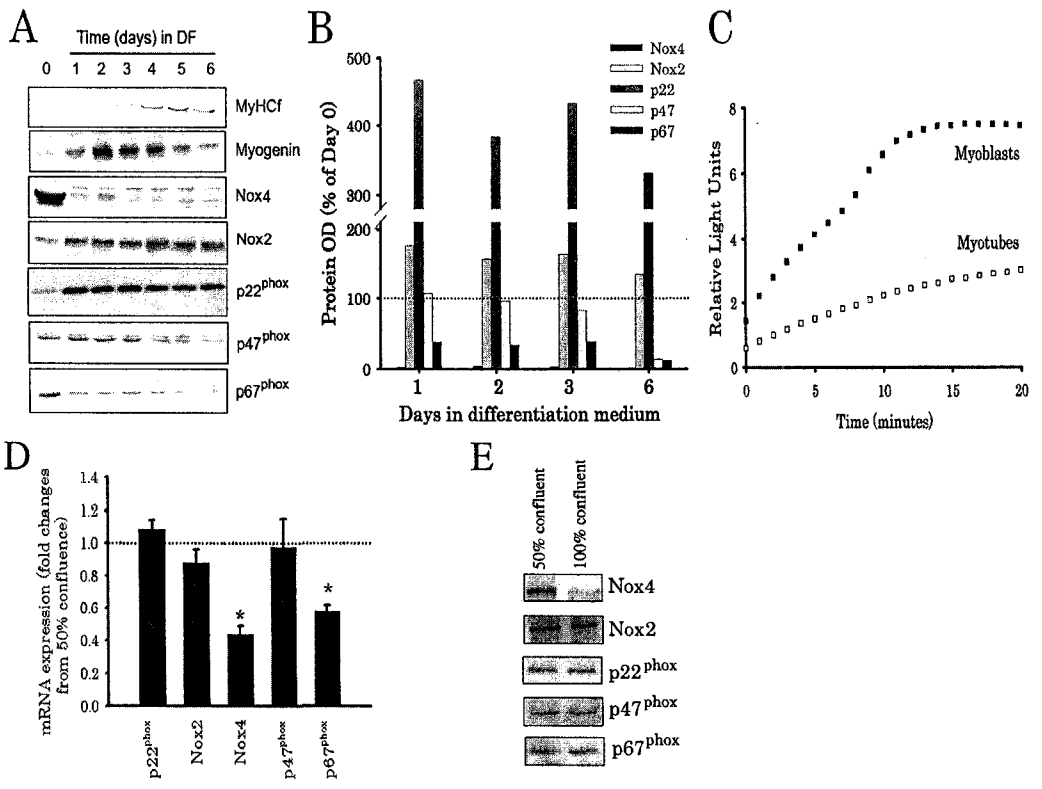

Figure 4: A & B: Myoblasts grown in differentiation medium (DF) for 1 to 6 days and expression of NADPH oxidase subunits, myogenin and myosin heavy chain (MyHCf) proteins measured with immunoblotting. Day 0 designates undifferentiated myoblasts. Representative immunoblots are shown in panel A. Mean values of protein optical density are shown in panel B. C: Production of O_2^- anions in proliferating myoblasts and differentiated myotubes (day 6) using lucigenin enhanced chemiluminescence. D & E: Regulation of NADPH oxidase expression by changes in cell density. Myoblasts were grown at 50 and 100% confluency. Expressions of mRNA (D) and protein (E) of NADPH oxidase subunits were measured with real-time PCR and immunoblotting, respectively. *P<0.05 compared with values measured at 50% confluency.

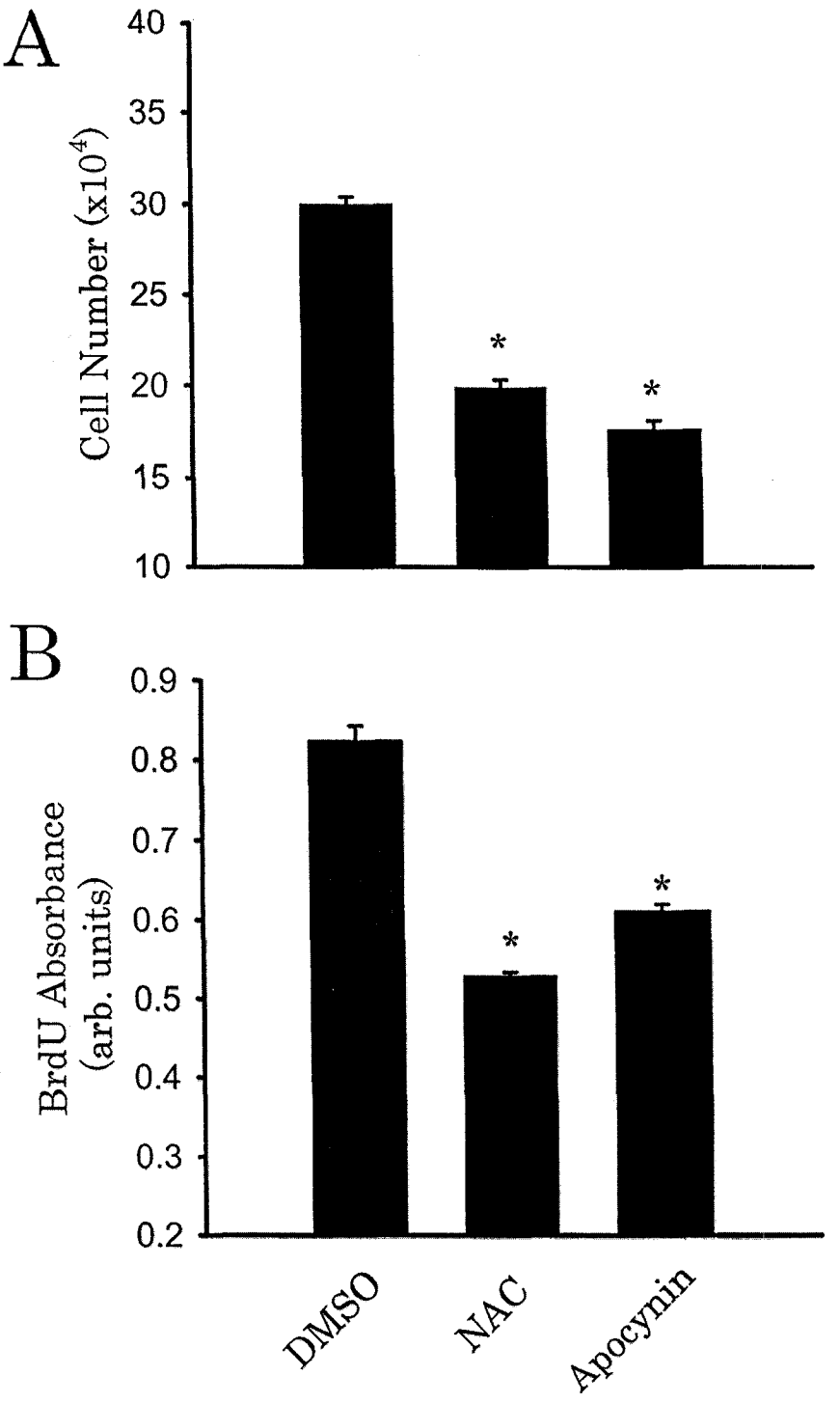

Figure 5: Regulation of proliferation in human skeletal myoblasts grown for 24h in full medium in the presence of DMSO (vehicle), NAC or apocynin. Cell count and BrdU incorporation mean values shown in panels A and B. *P<0.05 compared with vehicle.

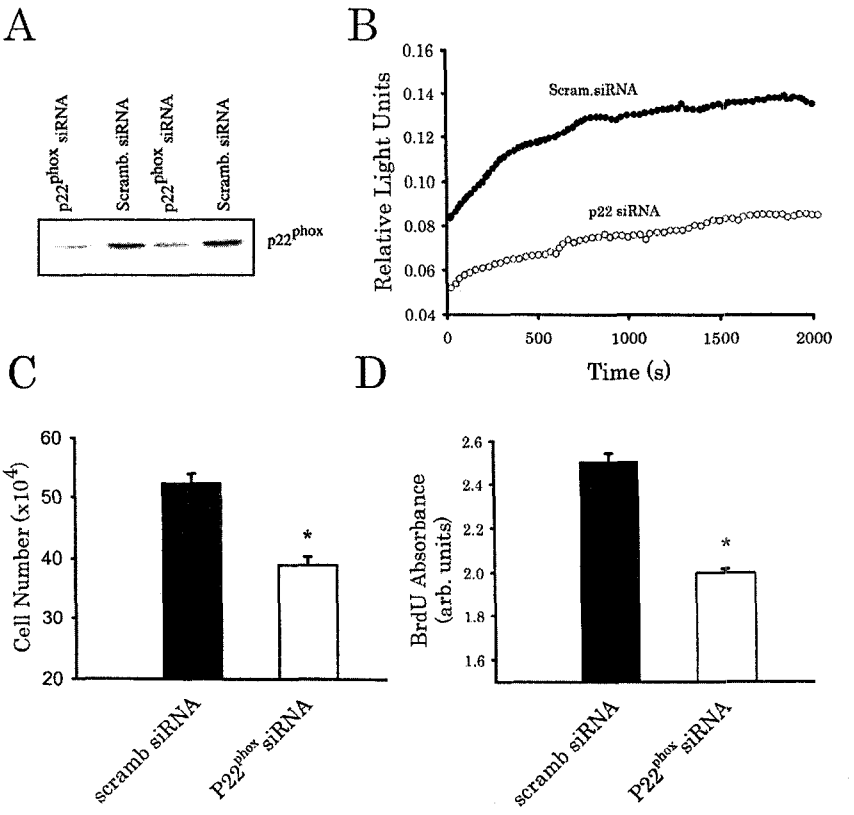

Figure 6: Expression of $p22^{phox}$ (A), O_2^- production (B), cell count (C) and BrdU incorporation (D) in human myoblasts transfected with siRNA oligonucleotides selective to $p22^{phox}$ or scrambled siRNA oligonucleotides. *P<0.05 compared with cells transfected with scrambled siRNA oligonucleotides.

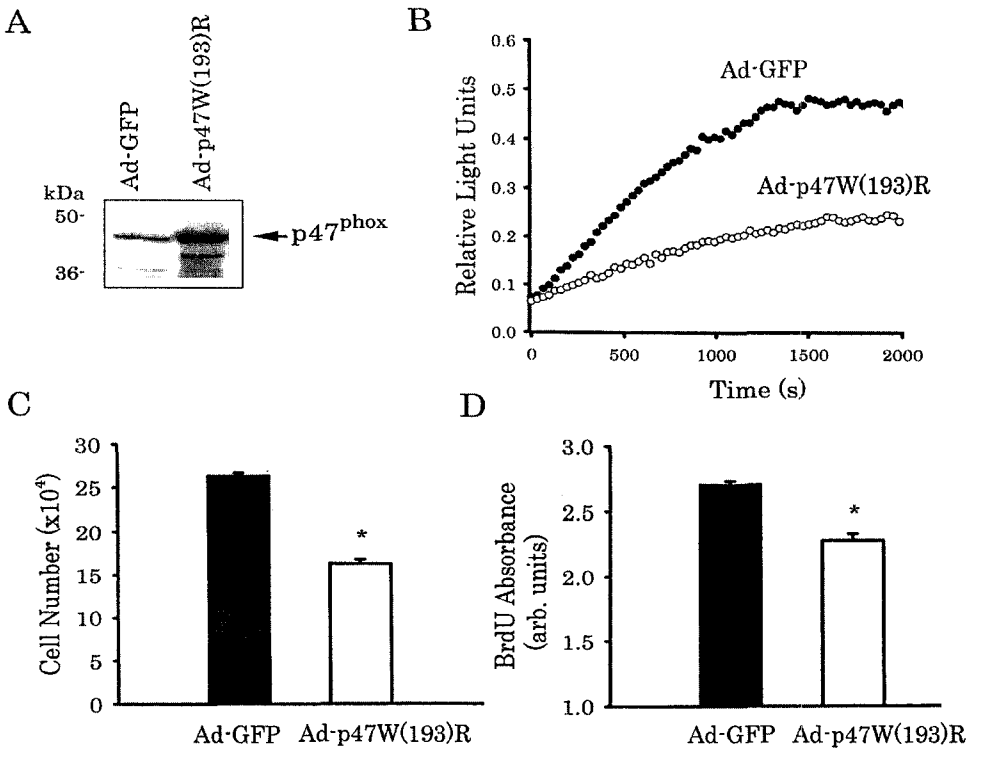

Figure 7: Expression of $p47^{phox}$ (A), O_2^- production (B), cell count (C) and BrdU incorporation (D) in human myoblasts infected with adenoviruses expressing GFP or a dominant-negative form of $p47^{phox}$ (p47W193R). *P<0.05 compared with cells infected with GFP adenoviruses.

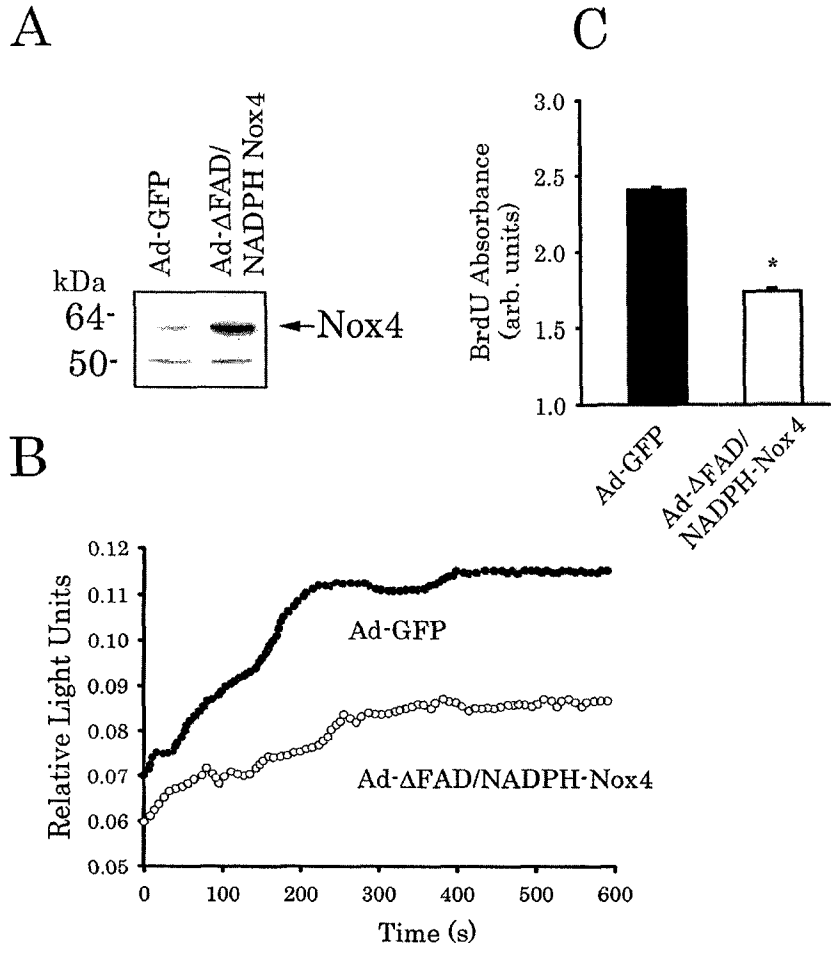

Figure 8: Expression of Nox4 (A), O_2^- production (B), and BrdU incorporation (C) in human myoblasts infected with adenoviruses expressing GFP or a dominant-negative form of Nox4 (ΔFAD/NADPH Nox4). *P<0.05 compared with cells infected with GFP adenoviruses.

serum-starved myoblasts were first exposed to 15%FBS, which triggered a rapid (within 5min) increase in phosphorylation of p38, Erk1/2, AKT, and mTOR proteins and a delayed (within 60min) augmentation of phosphorylation of the p65 subunit of NFκB (Figure 9A). Serum-induced phosphorylation of Erk1/2, AKT, mTOR, and p65 NFκB proteins was significantly attenuated while that of p38 protein rose substantially when NAC and apocynin were present (Figures 9B and C). These results indicate that serum-induced activation of the Erk1/2, PI-3 kinase/AKT, mTOR and the p65 NFκB pathways is redox-sensitive. To evaluate the roles of these pathways in myoblast proliferation, cell counting of myoblasts cultured in full culture medium in the presence of DMSO (vehicle) or selective inhibitors of Erk1/2 (PD98059), PI-3 kinase (wortmannin and LY924002), mTOR (rapamycin), and NFκB (Bay11-7082) pathways was performed. All inhibitors significantly attenuated cell numbers, as compared with cells treated with vehicle (Figure 9D). In addition, expression of dominant-negative IKKα and IKKβ proteins significantly reduced cell counts, as compared with cells expressing GFP (Figure 9D). Previous studies have confirmed that oxidants regulate cell proliferation in response to serum or mitogen exposure by inducing the expression of cyclin D1, a protein which induces the transition of the cell cycle from the quiescent G0 phase to G1 phase. Figure 10 indicates that this was the case in human skeletal myoblasts, where $p22^{phox}$ expression was knocked down using siRNA oligonucleotides or where inhibition of $p47^{phox}$ and Nox4 activities using dominant-negative forms resulted in significant attenuation of cyclin D1 expression.

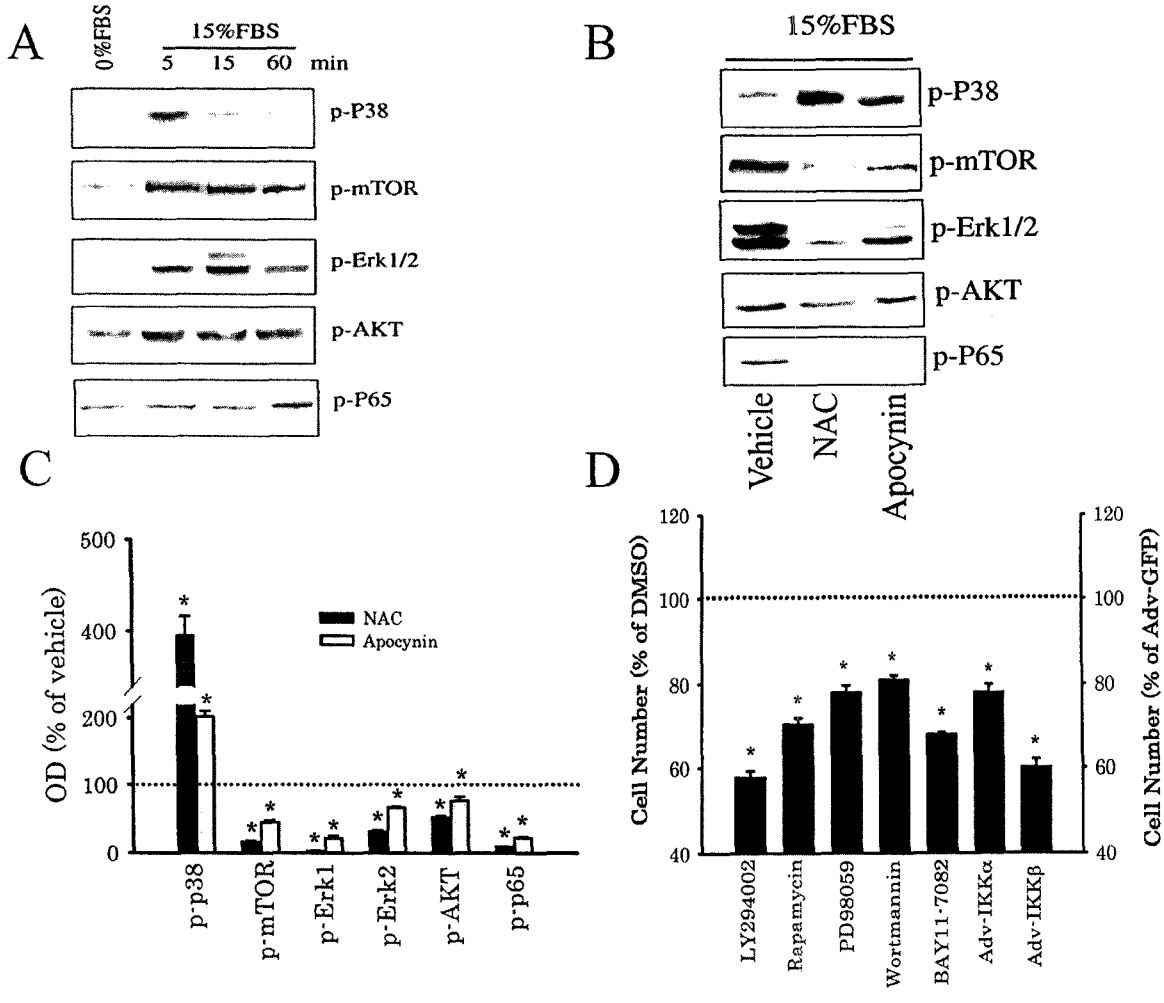

Figure 9: A: Phosphorylation of p38, mTOR, Erk1/2, AKT and the p65 subunit of NFκB after 5, 15 and 60min of addition of 15%FBS in serum-starved human myoblasts. B & C: Influence of vehicle (DMSO), NAC and apocynin on the intensity (representative example in panel B and mean values in panel C) of p38, mTOR, Erk1/2, AKT and p65 NFκB phosphorylation measured after 15min of serum exposure in serum-starved myoblasts. D: Influence of inhibitors of PI-3 kinase (LY294002, wortmannin), mTOR (rapamycin and LY294002), Erk1/2 (PD98059) and NFκB (Bay11-7082 and dominant-negative forms of IKKα and IKKβ) pathways on myoblast cell number counted after a 24h-period. Results are normalized as percentage of values measured in the presence of vehicle (DMSO) or in cells infected with GFP viruses.

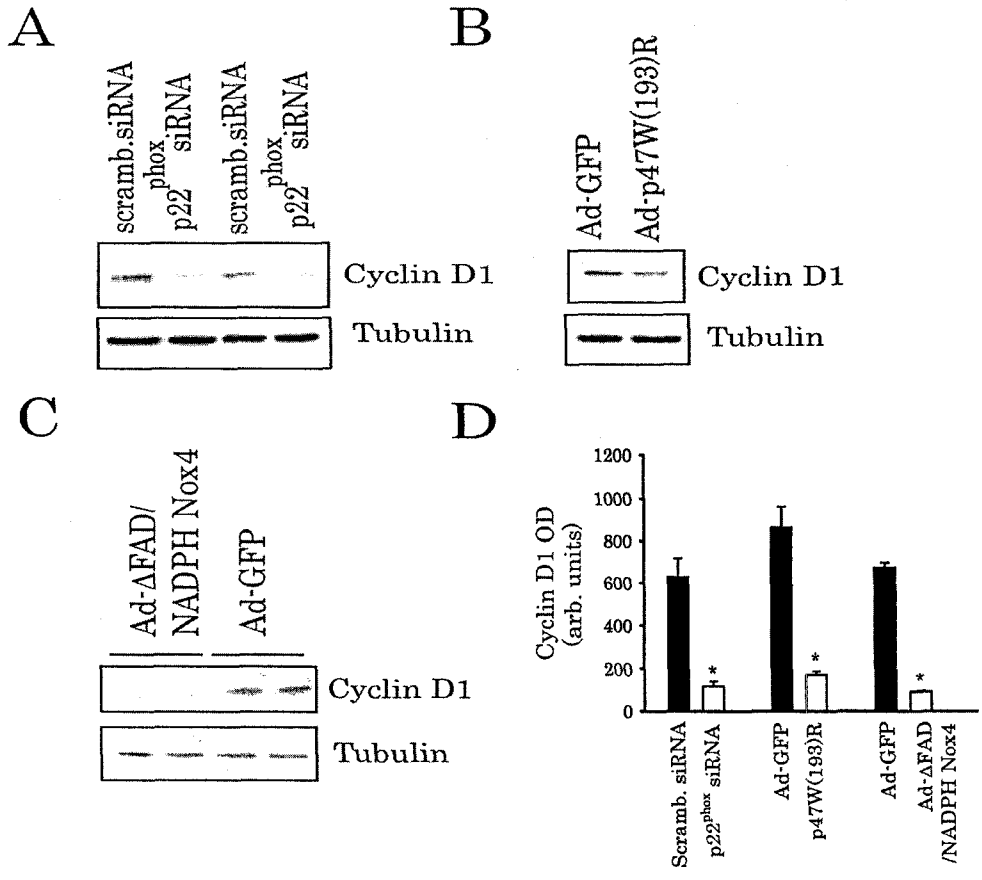

Figure 10: Expression of cyclin D1 protein in myoblasts transfected with scrambled siRNA, $p22^{phox}$ siRNA oligonucleotides (panel A), myoblasts infected with adenoviruses expressing GFP or dominant-negative forms of $p47^{phox}$ (panel B) and Nox4 (panel C). Panel D shows the mean values of cyclin D1 optical densities expressed in arbitrary units. *$P<0.05$ compared with scrambled siRNA or Ad-GFP. Note that knockdown of $p22^{phox}$ or inhibition of $p47^{phox}$ and Nox4 elicits a significant decline in cyclin D1 levels.

CHAPTER 4

DISCUSSION

4.1 Summary of findings: The main findings of this study are: 1) The NADPH oxidase subunits Nox2, Nox4, $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ are expressed in murine skeletal muscles and in primary murine and human skeletal muscle satellite cells (myoblasts); 2) NADPH oxidase-fusion proteins are localized mainly in the cytosol of satellite cells; 3) both Nox2 and Nox4 contribute to O_2^- production in proliferating human skeletal myoblasts; whereas the contribution of Nox4 is reduced significantly in confluent cells and during differentiation of myoblasts to myotubes; and 4) NADPH oxidase-derived oxidants promote myoblast proliferation through activation of the Erk1/2, PI-3 kinase/AKT, mTOR and NFκB pathways, eventually resulting in the induction of cyclin D1 expression and progression of the cell cycle.

4.2 Expression of NADPH oxidase in skeletal muscles: Little information is available regarding the activity and functional significance of NADPH oxidase in skeletal muscles. Our group was the first to report the presence of Nox2, $p47^{phox}$, $p67^{phox}$ and $p22^{phox}$ proteins in rat skeletal muscle samples (102). More recently, Hidalgo *et al.* (91) identified Nox2, $p47^{phox}$, $p67^{phox}$ and $p22^{phox}$ proteins in the transverse tubules of mouse and rabbit skeletal muscles and proposed that oxidants derived from NADPH oxidase activate ryanodine receptors through redox modifications. Our results (Table 2) confirm that murine skeletal muscles contain not only Nox2, but also Nox4 protein in addition to $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$. Furthermore, we report here that the relative expression of all NADPH oxidase subunits in the diaphragm is higher than in limb muscles (Table 2). The reasons behind this particular distribution of NADPH oxidase expression are

unclear. We speculate, however, that elevated NADPH oxidase expression in the diaphragm might be an adaptive response designed to provide an abundance of ROS to facilitate optimal Ca^{++} release from ryanodine receptors during repetitive activation of the diaphragm across breathing cycles. Another important finding regarding NADPH oxidase expression in murine skeletal muscles is that the relative abundance of the $p22^{phox}$ subunit in various murine skeletal muscles is substantially higher than that of other subunits (Table 2). We attribute this finding to the fact that $p22^{phox}$ protein binds to more than one Nox isoform and its presence is required for stabilization and optimal activity of these proteins, including Nox2, Nox1 and Nox4 (109). The presence of two Nox proteins (Nox2 and Nox4) inside muscle fibers would, therefore, justify the relatively high abundance of $p22^{phox}$ subunits in these fibers.

As in skeletal muscle fibers, little is known regarding the structure and biological roles of NADPH oxidase in skeletal muscle satellite cells. Two recent studies have confirmed the presence of Nox2, $p47^{phox}$ and $p67^{phox}$ mRNA and proteins in immortalized rat L6 myoblasts and myotubes and also have reported the involvement of NADPH oxidase-derived ROS in insulin signaling and Ca^{++} flux (66, 198). Others, however, have failed to detect mRNA expression of Nox4, Nox2, $p22^{phox}$ and $p47^{phox}$ in C2C12 and L6 immortalized murine myoblasts but have detected $p47^{phox}$ and Nox4 mRNA in primary skeletal myoblasts (98). The present study indicates that primary human and murine myoblasts derived from the diaphragm and tibialis anterior muscles express both Nox2 and Nox4 in addition to $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ at mRNA and protein levels. These results suggest that failures to detect NADPH oxidase expression in immortalized

myoblasts from C2C12 and L6 origins might be due to the relatively low expression of oxidase subunits, particularly $p22^{phox}$. These cells may not, therefore, be ideally suited to the study of the biological roles of the NADPH oxidase (Table 3).

To identify intracellular localization of the NADPH oxidase, we used two approaches, namely, subcellular fractionation and expression of NADPH oxidase fluorescent-fusion proteins. Both revealed that all subunits are expressed mainly within the cytosol, with prominent ER-like distributions for Nox2, Nox4 and $p22^{phox}$ and more diffuse staining for $p47^{phox}$ and $p67^{phox}$ (Figures 2 and 3). We also identified membrane-associated Nox2, $p47^{phox}$ and $p67^{phox}$ (Figure 3). The endoplasmic reticulum-like distribution of Nox2, Nox4 and $p22^{phox}$ in proliferating myoblasts is similar to that reported in endothelial cells (148). Moreover, the membrane-related expression of NADPH oxidase subunits in the present study is similar to that described at the leading edge of migrating endothelial cells, suggesting that, as in vascular cells, NADPH oxidase-derived ROS may be involved in regulating myoblast motility (203). Another interesting observation in the present study is that $p47^{phox}$ fluorescence fusion protein was frequently detected in the nuclei of human skeletal myoblasts (Figure 3). Although the mechanisms and functional significance of this nuclear localization remains to be determined, we speculate that $p47^{phox}$ is targeted to the nucleus due to specific interactions of the tandem SH3 domains of $p47^{phox}$ with the proline-rich mid region of the p65 subunit of NFκB (RelA) (82). This association results in potentiation of NFκB activation in response to IL-1β exposure (82).

4.3 Contribution of NADPH oxidase subunits to O_2^- production: The present study reveals for the first time that both Nox2 and Nox4 contribute to O_2^- production in proliferating human skeletal myoblasts. This conclusion is based on the observations that knocking down $p22^{phox}$ expression using siRNA and inhibiting $p47^{phox}$ and Nox4 activities using dominant-negative proteins attenuates O_2^- production in proliferating human myoblasts (Figures 6-8). Although we did not directly measure the exact contribution of Nox2 and Nox4 to O_2^- production in these cells, the observation that Nox4 mRNA expression is more than 100-fold greater than that of Nox2 in proliferating myoblasts suggests that Nox4 is a greater contributor to NADPH oxidase-derived O_2^- production than is Nox2 in these cells. However, Nox4 contribution is likely to be greatly reduced in quiescent myoblasts (100% confluent), where Nox4 expression declines significantly (Figure 4). It should be emphasized that attenuation of Nox4 expression with increasing cell density in skeletal myoblasts is similar to that of Nox1 expression in epithelial cells (147), and suggests that, in proliferating myoblasts, Nox4-derived oxidants are needed for optimal cell proliferation.

The expression, and possibly the contribution, of Nox2 and Nox4 to overall NADPH oxidase activity in skeletal myoblasts were also altered during myoblast differentiation into myotubes, as indicated by a rapid decline in Nox4 expression and simultaneous increases in Nox2 and $p22^{phox}$ levels, resulting in reduction of overall O_2^- production (Figure 4). We speculate that this decline in NADPH oxidase activity in differentiated myotubes is a response designed to promote myogenesis. Indeed, many reports have confirmed that *in-vivo* and *in-vitro* myogenesis is negatively influenced by oxidants as a result of selective

activation of the NFκB pathway (31, 110). Another pathway that is activated by oxidants and negatively affects myogenesis is the Erk1/2 pathway, which inhibits transcriptional activation of MyoD and myogenin transcription factors through upregulation of cyclin D1 (26). Our observations that NADPH oxidase-derived ROS activate both the Erk1/2 and NFκB pathways and inhibition of NAPDH oxidase expression results in downregulation of cyclin D1 suggest that the activity of NADPH oxidase must be reduced for optimal expression and activation of transcription factors required for differentiation into myotubes.

4.4 Regulation of myoblast proliferation by NADPH oxidase: It has long been established that relatively low concentrations of oxidants stimulate proliferation of mammalian cells (34). Moreover, many mitogens and growth factors acting through tyrosine kinase and G-protein coupled receptors stimulate cell proliferation through the release of ROS, particularly from non-phagocytic NADPH oxidase (25). The present study extends these observations to skeletal muscle satellite cells and confirms that NADPH oxidase-derived ROS promote serum-induced proliferation of human skeletal satellite cells and that this effect is mediated through selective signaling pathways, including the MAPK, Erk1/2, PI-3 kinase/AKT and NFκB pathways.

Many reports have confirmed that activity of the Erk1/2 pathway is redox-sensitive when cells are exposed to growth factors such as PDGF, VEGF, angiotensin II and to exogenous H_2O_2 (84, 86, 178). Likewise, our results show a strong redox-sensitive activation of the Erk1/2 pathway within 5min of serum exposure in human skeletal myoblasts. The Erk1/2 pathway initiates within

minutes of mitogenic stimulation of the immediate-early gene response by activating c-Fos protein, which then dimerizes with members of the Jun family (c-Jun, JunB and JunD) to form the AP-1 transcription factor complex. Cells then enter into the S-phase of their cycle (167).

Our results also indicate that the PI-3 kinase/AKT pathway is activated in human skeletal myoblasts by serum in a redox-dependent manner and that this pathway promotes serum-induced myoblast proliferation. The PI-3 kinase/AKT pathway regulates cell survival, motility, and migration and its activity is enhanced by exogenous H_2O_2 and by NADPH oxidase-derived ROS in a variety of cells, including cardiac muscles, endothelial and vascular smooth muscle cells (37, 189). This redox-dependent activation is attributed to reversible oxidation and inactivation of protein tyrosine phosphatase (PTP) family proteins such as PTEN, which dephosphorylates and inactivates PI-3 kinase (134). The PI-3 kinase/AKT pathway regulates cell proliferation, in part, through the mTOR protein which is a serine/threonine kinase that exists as two complexes, the rapamycin-sensitive mTORC1 complex, which contains mTOR, raptor and mLST8 proteins, and the rapamycin-insensitive mTORC2 complex, which contains mTOR, rictor and mLST8 proteins (204). The mTOR network is inhibited by two proteins, namely hamatrin (TSC1) and tuberin (TSC2). The PI-3 kinase/AKT pathway inactivates the TSC2 complex, resulting in phosphorylation of mTOR at Ser^{2448}, followed by increased activity of this protein (126, 166). Our results confirm that serum-induced phosphorylation of mTOR at Ser^{2448} is dependent on NADPH oxidase-derived ROS and that mTOR activity promotes myoblast proliferation (Figure 9).

In addition to the Erk1/2, PI-3 kinase/AKT and mTOR pathways, we report here that NFκB transcription factor is a target for NADPH oxidase-derived ROS in skeletal myoblasts. Inactive NFκB consists of p50, p65 and IκBα subunits. Upon stimulation by agonists, IκBα is phosphorylated at Ser^{32} and Ser^{36} by the IK kinases (IKKα, IKKβ and IKKγ) and is subsequently ubiquitinated and degraded by the proteosome. This eventually leads to phosphorylation of p65 and mobilization of p50 and p65 to the nucleus, where they bind their respective nucleotide sequences and transcriptionally regulate expression or repression of target genes.

Many reports have confirmed that the initial upstream response to growth factor stimulation of IKKs and subsequent degradation of IκBα are redox-dependent (129, 160). We observed an increase in p65 phosphorylation after 60min of serum exposure in human skeletal myoblasts, a response that is strongly attenuated by pre-treatment with NAC and apocynin (Figure 9). We also observed that selective inhibition of NFκB reduces myoblast proliferation, indicating that NADPH oxidase-derived ROS promote proliferation of these cells, in part through the NFκB pathway. A similar observation has been made in vascular cells (30). It should be emphasized that the targeted pathways by NADPH oxidase-derived oxidants (Erk1/2, PI-3 kinase/AKT, mTOR and NFκB pathways) all converge on cyclin D1 in regulating cell cycle phases. This labile protein is required for progression through the G1 phase in the cell cycle, and several reports have confirmed that its expression is regulated by mitogen-stimulated oxidants (32, 33). In our study, the expression of cyclin D1 was significantly reduced in human

satellite cells transfected with siRNA oligos for $p22^{phox}$ and in cells infected with viruses expressing dominant-negative forms of $p47^{phox}$ and Nox4, confirming the dependence of cyclin D1 expression in these cells on NADPH oxidase-derived ROS (Figure 10).

Transcription and stability of cyclin D1 are regulated by multiple signaling pathways, including the Erk1/2 pathway, which is sufficient on its own and in the absence of growth factors to increase cyclin D1 expression through the AP-1 transcription factor (33). In the same context, activation of the PI-3 kinase/AKT pathway stabilizes cyclin D1 by inhibiting its phosphorylation by glycogen synthase kinase 3β and, therefore, preventing its degradation through the proteasomal pathway (60). The PI-3 kinase/AKT signaling pathway has also been shown to induce the accumulation of cyclin D1 by activating its transcription (77). Finally, Guttridge *et al.* (85) have reported that NFκB promotes proliferation of C2C12 myoblasts and embryonic fibroblasts through the induction of cyclin D1 expression. This regulation is mediated through binding to two different NFκB binding sites in the cyclin D1 promoter (92).

4.5 Summary: our study reveals that skeletal muscle fibers and skeletal muscle satellite cells express two Nox isoforms (Nox2 and Nox4) along with $p22^{phox}$, $p67^{phox}$ and $p47^{phox}$, and that both Nox isoforms contribute to the production of O_2^- in proliferating skeletal muscle satellite cells. Furthermore, ROS derived from NADPH oxidase activate several pathways, including the Erk1/2, PI-3 kinase/AKT, mTOR and NFκB pathways, resulting in significant activation of the cell cycle machinery and increased cell proliferation.

CHAPTER 5

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Appendix I

List of Abbreviations:

ROS: Reactive oxygen species
Erk1/2: Extracellular regulated kinase 1 and 2
PI-3 kinase: Phosphatidy inositol 3 kinase
AKT: Protein kinase B
NFκB: Nuclear Factor κ B
EDL: Extensor digitorum longus
DMEM: Dulbecco's modified Eagle's medium
FBS: Fetal bovine serum
HS: Horse Serum
GAPDH: Glyceraldehyde phosphate dehydrogenase
DTT: Dithiothreitol
C_T: Threshold cycle
DPI: Diphenyleneiodonium
NAC: N-Acetylcysteine
EDTA: Ethylene diamine tetra acetate
ECFP: Enhanced cyan fluorescence protein
EYFP: Enhanced yellow fluorescence protein
GFP: Green fluorescence protein
IKK: IκB kinase
SiRNA: Small inhibitory RNA
ELISA: Enzyme-linked immunosorbent assay
BrdU: Bromodeoxyuridine
SAPK/JNK: Stress activated protein kinase/c-Jun NH2-terminal kinase
mTOR: Mammalian target of rapamycin
PMSF: Phenylmethylsulfonyl fluoride
ECL: Enhanced chemiluminescence

Appendix II

Permit for the use of biohazard materials.

APPENDIX III

McGill University Animal Use Protocol