CHAPTER - 7

Elucidation of the molecular mechanism of skeletal muscle protein loss under hypoxic conditions using cell line.

1. Introduction

Hypoxia is invariably associated with loss of skeletal muscle mass under various circumstances such as exposure to high altitude or in pathological conditions like chronic obstructive pulmonary, cancer disease, sepsis, burn etc. (MacDougall et al, 1991; Howald and Hoppler, 2003; Caron et al, 2007). Skeletal muscle atrophy attributable to such conditions contributes to weakness, fatigue, and loss of mobility in affected individuals.

Hypoxia induced muscle atrophy has gained much attention and a number of underlying mechanisms have been proposed. The most prominent underlying mechanism is persistent hyper-catabolic response of muscles to hypoxia (Acharyya and Guttridge, 2007; Caron et al, 2009; Pooja et al, 2012). Enhanced muscle protein degradation has been shown to result in muscle mass loss under hypoxic conditions (Mitch and Goldberg, 1996) which relies on the increased activities of various proteolytic pathways. A role for the autophagic-lysosomal degradation has been demonstrated under some circumstances (Mammucari et al, 2007). The ubiquitous calcium activated proteases calpains m and µ are assumed to play a key role in the disassembly of sarcomeric proteins that occurs in muscle atrophy as well as in the necrosis process accompanying muscular dystrophies (Attaix et al, 2005; Enns et al, 2007). The third, and the most relevant proteolytic pathway to muscle wasting, is the ATP-ubiquitin- dependent proteolytic system through which contractile proteins are degraded leading to muscle atrophy.
(Cai et al, 2004; Reid 2005). An increase in mRNA encoding for key enzymes and proteins of this pathway is a hallmark of hypoxia mediated muscle atrophy in several in-vivo and in-vitro models (Mitch and Goldberg, 1996; Li et al, 2003). We also have previously reported that increased activities of ubiquitin–proteasome pathway and calpains result in enhanced protein degradation and consequently muscle atrophy under hypoxic conditions (Pooja et al, 2012).

Recently oxidative stress has also been reported to contribute in muscle atrophy under disuse atrophy (Powers et al, 2007), cancer cachexia (Buck and Chojkier, 1996; Mastrocola et al, 2008), COPD (Wust and Degens, 2007) and aging (Muller et al, 2006; Jang et al, 2010). Oxidative stress can increase protein catabolism by directly modifying proteins to increase their susceptibility as substrates in catabolic pathways, by increasing the synthesis of key proteins involved in catabolic pathways and by affecting the regulation of catabolic pathways, for example, increased NO production cause protein nitrotyrosination and favour protein degradation through the ubiquitin-proteasome pathway (Espat et al, 1994). ROS has been shown to upregulate the gene expression of key components of the ubiquitin-proteasome pathway (Li et al., 2003). ROS has also been shown to enhance the activity of calpains (Sharma and Rohrer, 2007; Hill et al, 2008) by increasing calcium ion concentration via formation of reactive aldehydes that inhibit plasma membrane Ca\(^{2+}\) ATPase activity (Siems et al, 2003). An oxidative stress-mediated decrease in Ca\(^{2+}\) ATPase activity inhibits the removal of calcium from the cell resulting in intracellular calcium accumulation that promotes activation of calcium-dependent proteases. ROS can also cause reversible redox modifications to ion channel proteins, such as calcium channels to enhance calcium influx (Viola et al, 2007). Many reports point towards enhanced oxidative stress under hypoxic conditions (Singh et al, 2001; Magalhaes et al, 2004). Thus, hypoxia induced muscle protein loss can be correlated with increased oxidative stress.
The signals and molecular steps of enhanced muscle protein degradation under various pathological conditions are the subjects of intense research. These include the induction of myostatin expression (Mcfarlane et al, 2006; Hayot et al, 2011), Foxo (Sandri et al, 2004; Crossland et al, 2008), Murf-1 (Koyama et al, 2008; Eddins et al, 2011), atrogin-1 (Gomes et al, 2001; Julie et al, 2012) and NF-KB (Testelmans et al, 2010; Sriram et al, 2011). Interestingly, most of these emerging pathways appear to mediate their effects through the activation of the ubiquitin proteasome system. These findings reinforce that efforts should remain focused on targeting proteasome activity, but it also suggests that the identification of signaling molecules lying upstream of the proteasome should also be considered as additional therapeutic targets for the treatment muscle mass loss.

Although the molecular aspects of atrophy have received increased attention in the past few decades, the underlying mechanism for hypoxia induced enhanced muscle protein loss and role of oxidative stress in mediating the negative muscle mass balance has not yet been fully understood.

Advances in understanding the mechanisms responsible for muscle atrophy under hypoxia may pave the way to new and perhaps more effective treatments of a large number of pathological conditions where hypoxia is encountered. This study was therefore designed to elucidate the molecular mechanism of hypoxia mediated muscle protein degradation. Taking into consideration the importance of hypoxia, oxidative stress and NF-KB in muscle atrophy we hypothesized that effects of hypoxia on muscle protein degradation might involve enhanced activities of proteolytic pathways by up-regulation of oxidative stress mediated NF-KB. Further, to reconfirm our hypothesis, we used curcumin to attenuate muscle protein degradation as curcumin is known to have NF-KB inhibiting properties (Jobin et al, 1999; Sagi et al, 2008).
2. Methodology

Myoblasts were differentiated into myotubes and then were exposed to different durations of hypoxia. Cell viability was assessed using MTT assay. Different protein fractions and activities of proteolytic pathways were measured using standard protocols. The expression levels of proteolytic proteins were studied using immunofluorescence. The detailed protocols are described in chapter-3. All the results are presented as mean ± SEM. The data was analyzed using ANOVA followed by Student Newman Keuls test. Significance level was set at $P<0.05$. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS Inc., version 15.0, Chicago, IL).

3. Results

3.1. Myotube differentiation

Culture medium was replaced with differentiation medium once the cells reached 70-80% confluent stage after day 1 there was only increase in the confluency of cells which started to fuse after 2 days. After 3 days of medium change, small myotubes were visible in the phase contrast microscope followed by fully formed myotubes after 4 days.

3.2. Cell viability assay

Cell viability was quantitatively assessed by MTT assay after exposing the myotubes to 6 h, 12 h, 24 h, 48 h and 72 h of hypoxia (0.5% $O_2$) in a humidified chamber. In-vitro exposure of cells to hypoxia caused time dependent loss in cell viability. MTT metabolism declined with increasing duration of hypoxia and less than 60% viability was observed after 72 h of hypoxic exposure when compared with the normoxic control (at 21% $O_2$) as shown in figure 3.2A.
LDH leakage that has been reported to be a marker of cytotoxicity and membrane damage also showed significant change following exposure to hypoxia. LDH leakage increased significantly with increased duration of hypoxia exposure when compared with the normoxic control that was considered 100% as depicted in Fig. 3.2B.

3.3. Total protein estimation

To evaluate the impact of hypoxia on muscle protein content, total protein concentration was measured in cell extract using Lowry’s method. After 24 hr, a significant reduction of 20% was observed which further got reduced by 27% following 48 h exposure when compared to normoxic control (Fig. 3.3).

Since maximum change was observed in 48 h of hypoxia exposed group with approximately 80% viable cells, further experiments were conducted in 48 h hypoxia exposed group. Myotubes were exposed to different oxygen percentages viz., 5%, 3%, 1% and 0.5% (data not shown). The morphological and biochemical changes were maximum in myotubes exposed to 0.5% oxygen for 48 h whereas higher oxygen percentage did not bring significant effects in myotubes. Therefore, final protocol involved exposure of myotubes to 0.5% oxygen for 48 h.

3.4. Myofibrillar protein, sarcoplasmic protein and protein degradation

Fractions of total protein were estimated to ascertain which fraction actually contributed to decreased protein content on the muscle cell under hypoxia. The decrease was found significant in myofibrillar protein content (Fig. 3.4A) after 48 h of hypoxia exposure while sarcoplasmic protein (Fig. 3.4A) decreased only non-significantly as compared to the normoxic group. The results showed that increased protein degradation is responsible for decreased protein
content in the muscle cells. A significant increase in the protein degradation was observed after 48 h of hypoxia exposure (Fig. 3.4B).

3.5. Expression of ubiquitin proteasome pathway and calpains in muscle cells by immunofluorescence

As depicted in Fig. 3.5, phase contrast microscopy showed normoxic myotubes as elongated cylindrical tubes, however, exposure to hypoxia results in alterations in the myotube with their size decreased after hypoxia. Expressions of MURF-1 and ubiquitinated proteins were studied to confirm the upregulation of ubiquitinated pathway as MURF-1 is an ubiquitin E3 ligase which tags the target protein with ubiquitin moities and ubiquitinated proteins are then degraded via proteasome core. Hypoxia increased the expression of ubiquitin and MURF -1 in the cells with concomittantly increased expression of calpains as observed by immunofluorescence (Fig. 3.5).

3.6. Oxidative stress markers and antioxidant status

3.6A. Reactive oxygen species (ROS) generation

To investigate the involvement of free radicals mediated oxidative stress during hypoxia, generation of ROS was measured in muscle cells. Results showed significant increase in ROS generation after 48 h of hypoxia (Fig. 3.6A).
3.6B. Lipid peroxidation

The lipid peroxidation was measured by quantitating the malondialdehyde formed by 2-thiobarbituric acid reaction. Results displayed a significant increase in lipid peroxidation on exposure to hypoxia (3.6B)

3.6C. Nitric oxide (NO) estimation

Nitric oxide was estimated using a colorimetric method by Hageman (1980). A significant increase of about 25% was observed in the hypoxia exposed group as compared to the normoxic control (Fig. 3.6C).

3.6D. Antioxidant status

The antioxidant status was measured by estimating reduced glutathione and oxidized glutathione in cell lysates. The results indicated a significant decrease in the GSH level with concomitant increase in the GSSG levels following 48 h of hypoxia exposure (Fig. 3.6D).

3.7. Myosin heavy chain (MHC) expression using western blot

Myosin heavy chain is the major structural protein in skeletal muscles. Exposure to 48 h of hypoxia caused a significant decrease in the expression of myosin heavy chain in the myotubes (Fig. 3.7).

3.8. HIF-1α and NF-KB expression along with curcumin as NF-KB blocker

Oxidative stress is known to play a role in activation of NF-κB which subsequently leads to activation of ubiquitin-proteasome pathway. To assess the magnitude of oxidative stress, expression of HIF-1α was analysed using western blot (Fig. 3.8A). There was a significant
increase in HIF-1α expression after 48 h of hypoxia exposure. Similarly, NF-κB was also found to be increased significantly after the exposure (Fig. 3.8B). Curcumin was used as an antioxidant and NF-κB inhibitor. Curcumin at the effective dose of 2 µM inhibited the increase in NF-κB expression as well as HIF-1α expression. β-actin was used as loading control (Fig. 3.8C).

3.9. Expressions of MURF-1 of Ubiquitin-proteasome pathway and calpains

A significant increase in the expression of MURF-1, which is an ubiquitin E3 ligase, was observed in lysates of cells exposed to normoxic hypoxia for 48 h (Fig. 3.9A). MURF-1 ligates ubiquitin molecules to the target proteins which further get degraded by the proteasome core. Similar alterations in the expression of calpains were also observed after 48 h of hypoxia exposure (Fig. 3.9B). However, treatment with curcumin inhibited the hypoxia induced increase in MURF-1 as well as calpain expressions thus resulting in inhibition of the ubiquitin-proteasome pathway and calpain pathways.

3.10. Proteolytic activities of ubiquitin proteasome pathway and calpains

The biochemical activities of the proteolytic pathways were also studied. The activity of chymotrypsin like enzyme of the proteasome core increased significantly after 48 h hypoxia which decreased following curcumin administration (Fig. 3.10A). Similar trend was observed in the calpain activity (Fig. 3.10B).
Fig. 3.1. Differentiation pattern of L6 myotubes.

Fig. 3.2. (A) MTT expressed in percentage of control (B) LDH leakage expressed as percentage of control. All data represent mean ± SEM of three independent experiments done in triplicate. ‘a’ indicates significance at p<0.05 versus normoxia for each time point.
**Fig. 3.3.** Total protein in cell lysate expressed as percentage of control. All data represent mean ± SEM of three independent experiments done in triplicate. ‘a’ indicates significance at p<0.05 versus normoxia for each time point.

**Fig. 3.4.** (A) Myofibrillar protein (MP), sarcoplasmic protein (SP) and (B) protein degradation (PD). All data represent mean ± SEM of three independent experiments done in triplicate. ‘a’ indicates significance at p<0.05 versus normoxia.
Fig. 3.5. (A1) Image of control (normoxic) myotubes as observed by phase contrast microscope (A2) expression of ubiquitin in normoxic control (A3) Image of hypoxia exposed myotubes as observed by phase contrast microscope (A4) expression of ubiquitin in hypoxic cells (B1) Image of control (normoxic) myotubes as observed by phase contrast microscope (B2) expression of MURF-1 in normoxic control (B3) Image of hypoxia exposed myotubes as observed by phase contrast microscope (B4) expression of MURF-1 in hypoxic cells (C1) Image of control (normoxic) myotubes as observed by phase contrast microscope (C2) expression of calpains in normoxic control (C3) Image of hypoxia exposed myotubes as observed by phase contrast microscope (C4) expression of calpains in hypoxic cells.
Fig. 3.6. (A) ROS generation expressed as percentage of control (B) MDA formation expressed as percentage of control (C) Nitric oxide (NO) expressed as percentage of control (D) GSH and GSSG expressed as percentage of control. All data represent mean ± SEM of three independent experiments done in triplicate. ‘a’ indicates significance at p<0.05 versus normoxia.
Fig. 3.7. (A) Expression of myosin heavy chain (MHC) (B) expression of actin which was loading control. Corresponding densitometric graphs show the change in expressions of respective proteins when compared to control. All data represent mean ± SEM of three independent experiments done in triplicate. ‘a’ indicates significance at p<0.05 versus normoxia.
Fig. 3.8. (A) Expression of HIF-1α (B) expression of NF-κB (C) expression of actin which was loading control. Corresponding densitometric graphs show the change in expressions of respective proteins when compared to control. All data represent mean ± SEM of three independent experiments done in triplicate. ‘a’ indicates significance at p<0.05 versus normoxia and ‘#’ represents significant difference between hypoxia exposed without drug (H) and hypoxia exposed with drug (HT) groups.
Fig. 3.9. (A) Expression of MURF-1 (B) expression of calpains (C) expression of β-actin. Corresponding densitometry graph show the change in expressions of respective proteins when compared to control. All data represent mean ± SEM of three independent experiments done in triplicate. ‘a’ indicates significance at p<0.05 versus normoxia and ‘#’ represents significant difference between hypoxia exposed without drug (H) and hypoxia exposed with drug (HT) groups.
Fig. 3.10. (A) Chymotrypsin like enzyme activity expressed as percentage of control (B) Calpains activity expressed as percentage of control. All data represent mean ± SEM of three independent experiments done in triplicate. ‘a’ indicates significance at p<0.05 versus normoxia and ‘#’ represents significant difference between hypoxia exposed without drug (H) and hypoxia exposed with drug (HT) groups.
4. Discussion

The present study aimed to investigate the involvement of oxidative stress induced NF-kB in the pathogenesis of muscle depletion under hypoxia.

Different doses of curcumin (0.5 -3 µM) were used to inhibit the NF-KB mediated increase in protein degradation in the L6 myotubes under hypoxia. NF-KB expression levels, cell viability assay, total protein and protein degradation were measured to determine the dose of curcumin which could effectively inhibit NF-KB and protein degradation without causing considerable loss of cell viability. The dose of 2 µM was found to significantly reduce the hypoxic effects in the myotubes and therefore this dose was used in further cell culture experiments.

Though considerable advances have been made in understanding the molecular mechanism of muscle atrophy under various conditions, most of the studies remained focused on induction of certain pathological conditions such as cancer cahexia (Gomes et al, 2001), sarcopenia (Hall et al, 2011), chronic obstructive pulmonary disease (Plant et al, 2010), sepsis (Hasselgren et al, 2005) and disuse atrophy (Hunter et al, 2002). Since, hypoxia is common phenomenon in most of these conditions; elucidation of the molecular mechanism of hypoxia induced muscle protein loss would pave way for effective treatment of many pathological conditions.

The results of this study showed that exposure of muscle cells to hypoxia results in decreased total protein which is attributed to enhanced muscle protein degradation. As observed in the immunofluorescence studies, the pathways responsible for increased proteolysis under
hypoxic conditions are ubiquitin-proteasome pathway and calpains both of which have also been shown to result in muscle atrophy in rats exposed to chronic hypoxia (Pooja et al, 2012). The hypoxia exposed myotubes exhibited increased expression of MURF-1 (ubiquitin E3 ligase) which has been proposed to trigger muscle protein degradation via ubiquitination (Koyama et al, 2008). In response to conditions that promote muscle atrophy, elevated skeletal muscle MURF-1 facilitates the ubiquitination and degradation muscle components (Cohen et al, 2009). Recent studies have shown that there is an interactive involvement of calpains and ub-proteasome pathways in muscle proteolysis (Jackman and Kandarian, 2004; Debigare et al, 2010). Our study shows that exposure to hypoxia also results in upregulation of calpains in the muscle cells, thereby further providing evidence for the inter-related functioning of the ub-proteasome pathway and calpains.

Furthermore, there was a significant increase in oxidative stress as observed by increase in ROS generation, lipid peroxidation and nitric oxide production. ROS can also promote protein catabolism by oxidatively modifying proteins, which enhances their susceptibility to catabolism via ub-proteasome and calpain proteolytic pathways (Jung et al, 2007; Hill et al, 2008). Alongwith increase in the oxidative stress markers, a decreased level of antioxidants was also observed in the hypoxia exposed cells thereby accentuating the effect of hypoxia induced oxidative stress. The extent of hypoxia was measured by HIF-1α expression which was found to be significantly increased in the exposed cells.

Many studies have shown that about one half of the total muscle protein is myofibrillar protein, which is lost at a faster rate than other proteins during atrophy (Munoz et al, 1993). Recent results (Acharyya et al, 2004) show a massive decrease in expression of the core my-
ofibrillar protein responsible for muscular contraction, myosin heavy chain, in response to cachectic factors such as tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), whereas other core proteins, such as actin, troponin, and tropomyosin, are unaffected, indicating a high degree of selectivity. Our results showed that hypoxia exposure also results in loss of myosin heavy chain thereby decreasing the total myofibrillar protein content of skeletal muscle under such conditions.

Increased oxidative stress has recently been correlated with increased NF-κB under many pathological conditions. In this study, we speculated the possible role of oxidative stress and NF-κB in causing muscle proteolysis under hypoxic conditions. The key result of present study is enhanced NF-κB expression in hypoxic myotubes which was suppressed in presence of curcumin, a well known NF-κB inhibitor. To find out whether there is any relation between NF-κB and the proteolytic pathways, we further studied the expressions of murf-1, ubiquitinated proteins and calpains in absence and in presence of curcumin. In accordance to our hypothesis, we found a significant increase in the expressions of these proteins in absence of curcumin under hypoxia. However, curcumin pretreated cells showed a decline in the expressions and a subsequent decrease in the biochemical activities of both the pathways. Though many studies show that calpains activates NF-κB (Scholzke et al, 2003; Lee et al, 2005; Hamelet et al, 2009), vice-versa is rarely reported (Poylin et al, 2008).

Thus, in conclusion, our results reveal that exposure of muscle myotubes to hypoxia results in increased oxidative stress which causes activation of NF-κB in the muscle cells. Increased NF-κB leads to up-regulation of ubiquitin –proteasome pathway and calpains subsequently leading to their enhanced biochemical activities which ultimately results in
augmented muscle proteolysis under hypoxic conditions. By inhibiting NF-κB with curcumin, we found the hypoxic responses to be reversed thus confirming the role of oxidative stress inducted NF-κB in upregulation of proteolytic pathways and increased muscle protein degradation. Since, NF-κB has found to be playing a significant role in muscle proteolysis, the present study has opened a new area for developing therapeutic strategies for prevention of muscle protein loss. Moreover, since this study has shown that by inhibiting NF-κB, calpain activation has also been inhibited, it opens a wide new field for studying the dependence of calpain pathway on NF-κB. The molecular mechanism of hypoxia mediated muscle proteolysis has been explained in the diagram (Fig. 3.11).

![Diagram](image)

**Fig. 4.11.** Molecular mechanism of hypoxia mediated muscle atrophy.