To evaluate the therapeutic efficacy of curcumin in inhibiting muscle mass loss in rat skeletal muscle.

Results of phase III studies showed promising capabilities of curcumin in ameliorating hypoxia induced muscle protein loss. Thus, further studies were carried out to investigate its therapeutic potential in-vivo. Since in PI & PII results, maximum changes were observed in 14 days exposed group, the therapeutic potential of curcumin was evaluated in rats exposed to 14 days of hypobaric hypoxia.

1. Introduction

High altitude expedition has been associated with decrease in capability of performing physical activity (Fulco et al, 2002). This decreased physical activity is a consequence of skeletal muscle atrophy (Pooja et al, 2012). Recent studies have suggested the manifestation of high altitude induced oxidative stress in skeletal muscle atrophy (Magalhaes et al, 2004) which consequently results in early attainment of muscle fatigue. Calbet et al, (2009) have stated that even a brief reduction in oxygen supply due to hypoxia leads to acutely diminished muscle performance. Many researchers have provided evidences for high altitude hypoxia triggered oxidative stress in (Askew, 2002; Bakyoni and Radak, 2004; Jefferson et al, 2004; Dosek et al, 2007). Hypobaric hypoxia induced oxidative stress is believed to be the trigger for many physiological and biochemical consequences. In addition to imposing cellular damage, excessive reactive oxygen species has also been shown to have an adverse effect on skeletal muscle contractile function and exert a negative impact on muscle performance (Reid and Durham,
Six months of intermittent exposure to high altitude (4000m) resulted in decreased activity and protein content of mitochondrial SOD in skeletal muscle of rats (Radak et al, 1994). Other researchers have also found increased Malondialdehyde (MDA), decreased SOD and catalase in rats exposed to hypobaric hypoxia for 21 days at simulated height of 5500 m (Nakanishi et al, 1995). Similarly, Ilavazhagan et al, (2001) have reported that high altitude exposure decreased the level of reduced glutathione (GSH) and increased oxidized glutathione (GSSG) thereby providing evidence for diminished antioxidative capacity at high altitude.

Keeping in mind the muscle atrophy associated depredation such as weakness, fatigue and physical disability, several attempts have begun to identify the potential therapeutic targets and to elucidate the molecular mechanisms of oxidative stress mediated muscle wasting. Growing evidence suggests that NF-KB may play an important role in this process as NF-KB has been shown to upregulate the ubiquitin proteasome pathway under different conditions where oxidative stress is encountered (Tisdale, 2005; Jin B and Li YP, 2007). The phase III results of this study provided the molecular mechanism of oxidative stress mediated skeletal muscle atrophy. It has been found that oxidative stress inducted NF-KB results in upregulation of MURF-1 and ubiquitination of proteins alongwith a significant increase in the expression level as well as biochemical activity of calpains which subsequently enhanced muscle proteolysis and thus lead to muscle atrophy.

Curcumin (diferuloylmethane) is a well known antioxidant and inhibitor of NF-KB (Jobin et al, 1999). Recently, the role of curcumin in ameliorating skeletal muscle atrophy during a number of catabolic conditions has gained much attention. Conflicting results have been obtained regarding the influence of curcumin on skeletal muscle atrophy. In-vitro studies showed that treatment of myotubes with curcumin prevented the increase in protein degradation caused
by a cachectic factor purified from experimental tumors in mice (Wyke et al, 2004). On the contrary, treatment of rodents with curcumin did not prevent muscle atrophy caused by muscular dystrophy (Durham et al, 2006), muscle unloading (Farid et al, 2005), or experimental cancer (Busquetset al, 2001, Wyke et al, 2004). Despite increasing interest of researchers in curcumin’s ability to inhibit muscle atrophy, its therapeutic potential in ameliorating hypobaric hypoxia induced muscle atrophy still remains unexplored.

Skeletal muscle atrophy has profound effects on overall health and viability. The diminution in skeletal muscle mass leads to multiple consequences which includes a decrease in physical strength, increased fatigability and in severe case, inability to perform any physical task. Identifying the mechanisms leading to muscle mass loss is therefore an elementary step to develop therapeutic strategies. The aim of present study is therefore to study the role of curcumin in ameliorating oxidative stress mediated muscle atrophy under hypobaric hypoxia.

2. Methodology

Effects of curcumin on physical performance, total protein and myofibrillar protein were estimated. Calpain activity, proteasome activity were measured in muscle homogenates. Protein expressions were studied using western blot analysis. Oxidative stress markers, and antioxidants were also measured alongwith CPK activity and calcium levels. 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. Effect of curcumin on physical performance and muscle protein degradation

As shown in Fig. 3.1A, exposure to 14 days of hypobaric hypoxia resulted in a significant decrease in physical performance. Oral administration of curcumin to rats daily during the hypoxia exposure period resulted in dose dependent increase in their physical performance as evaluated by fatigue time during running on treadmill. The protection from hypoxia induced fatigue was conferred almost equally by both 100 mg/kg body weight and 200 mg/kg body weight; however 50 mg/kg body weight dose was less effective in restoring the physical performance. Similarly, 14 days of hypobaric hypoxia exposure also resulted in a significant increase in muscle protein degradation. Attenuation of muscle protein degradation conferred by 100 mg/kg of curcumin was equivalent to that of higher dose while a dose of 50 mg/kg was found to be less effective (Fig. 3.1B).

3.2. Effect of curcumin on total protein and myofibrillar protein

Exposure to hypobaric hypoxia for 14 days resulted in a significant decrease in the total protein content and myofibrillar protein content of gastrocnemius muscle as shown in Fig. 3.2A. Curcumin administration prevented the loss of total protein following exposure to hypobaric hypoxia for 14 days (Fig. 3.2A). About one half of total muscle protein is myofibrillar protein. Thus, as predicted, similar results were obtained for myofibrillar protein content (Fig. 3.2B). Significant decrease in the myofibrillar loss was observed in rats administered with 100 mg/kg body weight and 200 mg/kg body weight curcumin. However, the lowest dose (50 mg/kg body weight) did not show any significant change in the myofibrillar protein content when compared to the hypoxia group without drug.
The protection from hypoxia induced changes was conferred almost equally by both 100 mg/kg body weight and 200 mg/kg body weight; however 50 mg/kg body weight dose was less effective in restoring the alterations. Therefore further experiments were carried out in 100 mg/kg body weight drug dose group.

3.3 Effect of curcumin on biochemical activity of ubiquitin-proteasome pathway and calpain pathway

Exposure to chronic hypobaric hypoxia for 14 days resulted in increased biochemical activities of the proteolytic core and calpains which were significantly lowered following curcumin administration as shown in Fig. 3.3A and 3.3B respectively.

3.4. Effect of curcumin on expressions of ub-proteasome pathway proteins and calpain

The western blot results also showed that 14 days of hypobaric hypoxia resulted in a significant increase in the expressions of MURF-1 (Ub E3 ligase) (Fig. 3.4A), ubiquitinated proteins (Fig. 3.4B) and calpain (Fig. 3.4C). However, curcumin inhibited the upregulation of these proteins significantly.

3.5. Effect of curcumin on expressions of NF-KB and HIF-1α

Exposure of rats to 14 days of hypobaric hypoxia increased the expression of the transcription factor NF-KB. Curcumin administration however, decreased the hypobaric hypoxia induced expression levels of NF-KB which plays the key role in upregulation of proteolytic pathways. It also resulted in a significant decrease in the expressions of HIF-1 α (Fig. 3.5) which is an indicator of the extent of hypoxia induced oxidative stress.
3.6. Effect of curcumin on oxidative stress markers and antioxidants

Different oxidative stress markers were studied biochemically in rat skeletal muscle homogenates. Curcumin administration decreased the extent of oxidative stress under hypobaric hypoxia as shown in Fig. 3.6 (A-D). Rats administered with 100 mg/kg body weight during 14 days of exposure to hypobaric hypoxia exhibited an increased antioxidant status when compared to the hypoxia exposed rats with curcumin administration (Fig. 3.6, E-F).

3.7. Effect of curcumin on calcium levels and CPK activity

Since calpain activity is regulated by calcium concentration, the calcium level was measured in rat skeletal muscle homogenates. Curcumin significantly reduced the calcium level in skeletal muscle homogenates (Fig. 3.7A) under hypobaric hypoxic conditions. Hypobaric hypoxia leads to increased muscle permeability which results in leakage of the CPK from muscle to the bloodstream. Thus, the decreased creatinine phosphokinase activity in skeletal muscle homogenates, as shown in these results, is an indicator of the muscle permeability under chronic hypobaric hypoxia. The CPK activity was restored in the skeletal muscle following curcumin administration (Fig. 3.7B).

3.8. Effect of curcumin on expression of myosin heavy chain (MHC) and muscle histology

As shown in Fig. 3.8A, exposure to 14 days of hypobaric hypoxia decreased the expression of myosin heavy chain (MHC) which is the major myofibrillar protein. Curcumin administration however, restored the expression of MHC. Histological examination of sections from gastrocnemius muscles were used to evaluate the effects of curcumin in attenuation of muscle atrophy under chronic hypobaric hypoxia. Muscles were removed from different groups
of rats and analyzed for the potential presence of histopathological signs. The 40X high power
photomicrograph of muscle biopsy from control group showed transverse cut muscle fibers with
uniform size and shape revealing the thin, delicate endomysial connective tissue, fibers of
uniform size, and the peripherally placed nuclei. Muscle sections from control group which was
given curcumin for 14 days (CT) also showed similar arrangement of muscle fibres. However, in
the hypoxia exposed group (H) fibers were smaller in size and had relatively more space between
them (indicated by arrow) as compared to control fibers. They also showed centralization of
nuclei (indicated by arrowhead). Photomicrograph of muscle tissue from rat exposed to 14 days
of hypobaric hypoxia, and administered with curcumin during the exposure period (HT) showed
increase in number of muscle fibres with presence of the endomysial layer. Thus, it is clearly
evident in these images that chronic hypobaric hypoxia results in loss of muscle mass of rat hind
limb gastrocnemius muscle and curcumin administration restored the muscle mass under these
conditions.
Fig. 3.1. (A) Physical performance on treadmill running experiment (b) protein degradation rate as expressed in nmol/mg protein/2 hr. The results are expressed as mean ± SEM. ‘a’ indicates comparison with control (C), ‘#’ indicates comparison with hypoxia (H) at p<0.05. CT50: Control + drug (50 mg/kg BW); CT100: Control + drug (100 mg/kg BW); CT200: Control + drug (200 mg/kg BW); HT50: 14 days hypoxia + drug (50 mg/kg BW); HT100: 14 days hypoxia + drug (100 mg/kg BW); HT200: 14 days hypoxia + drug (200 mg/kg BW).
Fig. 3.2. (A) Total protein expressed as mg/g muscle and (B) myofibrillar protein (MP) expressed as mg/g muscle. The results are expressed as mean ± SEM. ‘a’ indicates comparison with control (C), ‘#’ indicates comparison with hypoxia (H) at p<0.05. CT50: Control + drug (50 mg/kg BW); CT100: Control + drug (100 mg/kg BW); CT200: Control + drug (200 mg/kg BW); HT50: 14 days hypoxia + drug (50 mg/kg BW); HT100: 14 days hypoxia + drug (100 mg/kg BW); HT200: 14 days hypoxia + drug (200 mg/kg BW).
**Fig. 3.3.** Effect of curcumin on (A) chymotrypsin like activity expressed as AFU/min/µg protein and (B) calpain activity expressed as pmol/µg protein. The results are expressed as mean ± SEM. ‘a’ indicates comparison with control (C), ‘#’ indicates comparison with hypoxia (H) at p<0.05.
Fig. 3.4. (A) Curcumin decreased expression of MURF-1 (B) expression of ubiquitinated protein (C) expression of calpains (D) expression of β-actin. Corresponding densitometry graph show the change in expressions of respective proteins when compared to control. The results are expressed as mean ± SEM. ‘a’ indicates comparison with control (C), ‘#’ indicates comparison with hypoxia (H) at p<0.05.
**Fig. 3.5.** Effect of curcumin on (A) Expression of NF-KB, (B) expression of HIF-1α (C) expression of β-actin. Corresponding densitometry graph show the change in expressions of respective proteins when compared to control. The results are expressed as mean ± SEM. ‘a’ indicates comparison with control (C), ‘#’ indicates comparison with hypoxia (H) at p<0.05.
Fig. 3.6. Effect of curcumin on (A) Free radical generation expressed in AFU/mg protein, (B) Lipid peroxidation expressed in nmol/g muscle, (C) Protein carbonyl expressed in nmol/mg protein, (D) Nitric oxide expressed in µmol/ml, The results are expressed as mean ± SEM. ‘a’ indicates comparison with control (C), ‘#’ indicates comparison with hypoxia (H) at p<0.05.
Fig. 3.6. Effect of curcumin on (E) Reduced glutathione (GSH) expressed in µmol/g muscle tissue, (F) Oxidized glutathione (GSSG) expressed in µmol/g muscle tissue, (G) Glutathione reductase (GR) expressed in µmol/min/mg protein, (H) Glutathione peroxidase expressed in µmol/min/mg protein. The results are expressed as mean ± SEM. ‘a’ indicates comparison with control (C), ‘#’ indicates comparison with hypoxia (H) at p<0.05.
Fig. 3.7. Effect of curcumin on (A) Calcium expressed in ng/ml (B) CPK activity expressed in mIU/mg muscle. The results are expressed as mean ± SEM. ‘a’ indicates comparison with control (C), ‘#’ indicates comparison with hypoxia (H) at p<0.05.
Fig. 3.8. Effect of curcumin on (A) Western blot results of myosin heavy chain (MHC) expression with corresponding densitometry graph (B) Hematoxylin and Eosin staining of muscle cells in rat gastrocnemius muscle. The 40X high power photomicrograph of muscle biopsy from control group showed transverse cut muscle fibers with uniform size and shape revealing the thin, delicate endomysial connective tissue, fibers of uniform size, and the peripherally placed nuclei. Muscle sections from control group which was given curcumin for 14 days (CT) also showed similar arrangement of muscle fibers. However, in the hypoxia exposed group (H) fibers were smaller in size and had relatively more space between them (indicated by arrow) as compared to control fibers. They also showed centralization of nuclei (indicated by arrowhead). Photomicrograph of muscle tissue from rat exposed to 14 days of hypobaric hypoxia, and administered with curcumin during the exposure period (HT) showed increase in number of muscle fibers with presence of the endomysial layer. Scale bar 20 mm.
4. Discussion

A loss of skeletal muscle mass is a common observation in people suffering from various pathological conditions such as cancer, COPD, burn, sepsis as well as in people exposed to high altitude hypoxia (MacDougall et al, 1991; Howald and Hoppler, 2003; Caron et al, 2007). This decreased muscle mass not only leads to muscle weakness (Schols et al, 1991; Engelen et al, 2000; Gosker et al, 2003), but may also be associated with physical disability and increased mortality under such conditions (Wust and Degens, 2007). Therefore attenuation of the loss of muscle mass is of primary concern. The aim of the present study was therefore to explore the therapeutic efficacy of curcumin in ameliorating muscle mass loss under hypobaric hypoxia.

In recent years, curcumin has gained much attention in relation to its antioxidant and anti-NF-KB properties which are the key factors responsible for muscle atrophy resulting from pathological conditions. The results of part three of this thesis have proved that hypoxia induced oxidative stress upregulated NF-Kb which in turn leads to activation of the ubiquitin-proteasome pathway and calpains resulting in increased degradation of myofibrillar proteins. This part of the study therefore, focused on ameliorating muscle atrophy and subsequent recovery using curcumin as an intervention. Different doses of curcumin (50 mg/kg body weight, 100 mg/kg body weight and 200 mg/kg body weight) were administered to rats for 14 days under normoxic or hypoxic conditions and various parameters were evaluated. Curcumin administration prevented the decrease in physical performance of rats during treadmill running exercise. Curcumin restored the total protein content of skeletal muscle by decreasing the proteolysis/degradation of myofibrillar proteins. We have earlier showed that the ub-proteolytic pathway and calpains play the major role in muscle mass loss under hypobaric hypoxia (Pooja et
al, 2012). The results of this study showed that curcumin inhibited the biochemical activities of these two pathways which were in concurrence with the decreased expression of MURF-1, ubiquitinated protein and µ-calpains. MURF-1 is an ubiquitin E3 ligase which tags the target proteins with ubiquitin moieties. Theses ubiquitinated proteins are than degraded by the proteolytic activity of the proteasome core.

The results of the present study also showed that curcumin reduced the expression of NF-KB which is the key mediator in upregulation of the proteolytic pathways. Thus, reduction in NF-Kb resulted in decreased activities of the proteolytic pathways. These results are in accordance with the recent study which demonstrated that curcumin treatment prevented the enhanced proteasome chymotrypsin-like activity during immobilization-induced muscle atrophy (Vazeille et al, 2012).

In addition curcumin also decreased hypobaric hypoxia mediated oxidative stress as observed by decreased free radical generation, lipid peroxidation and protein oxidation. It also restored the antioxidant levels in skeletal muscle homogenates. Oxidative stress has been proposed to be a key intermediary in promoting protein catabolism (Arthur et al, 2008). Enhanced oxidative stress has also been associated with increased proteolytic activities (Clarke et al, 2007; Moylan and Reid, 2007). Free radicals can also promote protein catabolism by oxidatively modifying proteins, which enhances their susceptibility to ubiquitination and catabolism by the 26S-proteasome pathway (Jung et al, 2007). The decrease in oxidative stress following curcumin treatment thus plays a major role in preventing muscle protein loss under hypobaric hypoxic condition. Calcium concentration is the prime determinant of calpain activity. Hypobaric hypoxia exposure is known enhance calcium levels which in turn activate the calpain pathway. As depicted in our results, curcumin administration inhibited the increase in calcium
under hypoxic conditions which may be one of the factors responsible for decreased calpain activity following curcumin treatment. It also restored the CPK activity in muscle homogenates of rats exposed to hypobaric hypoxia. Hypobaric hypoxia leads to increased muscle permeability which results in leakage of the CPK from muscle to the bloodstream. Thus, the restoration of creatinine phosphokinase activity in skeletal muscle homogenates, as shown in our results, provides evidence for curcumin mediated protection of muscle integrity under chronic hypobaric hypoxia.

As observed in our results, exposure to chronic hypobaric hypoxia leads to degradation of myosin heavy chain which is the core myofibrillar protein. This is in accordance with some studies which have also pointed towards decreased myosin heavy chain content under different muscle wasting conditions (Clarke et al, 2007; Cohen et al, 2009). Curcumin however, preserved the myosin heavy chain content of skeletal muscle which is evident in the expression of MHC in western blot results. This increased MHC content is attributed to increased number of muscle fibres following curcumin administration under hypobaric hypoxia as shown in the histological sections of rat gastrocnemius muscle.

In conclusion, curcumin inhibited hypobaric hypoxia mediated muscle mass loss by decreasing muscle proteolysis which is attributed to the decreased activities of the ubiquitin-proteasome pathway and calpains. The declined proteolytic activities of these pathways were mediated through decrease in hypobaric hypoxia induced oxidative stress and decreased expression of NF-KB, both of which play significant role in regulation of various proteolytic pathways. Since, curcumin is easily accessible, inexpensive, and non-toxic even at high doses, it may therefore offer an important treatment modality in muscle wasting under various pathological and physiological conditions.