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Skeletal muscle constitutes a large portion of the body, responsible for 40% of body weight. Myofibrillar proteins constitute 50-55% of total muscle protein. Muscle protein is constantly being degraded and synthesized. The mechanism of muscle protein degradation is still unclear. It is believed that calpains play important roles in myofibrillar protein degradation. Hence, control of calpains activities may be important to control of muscle growth.

The goals of this study were to investigate the role of calpains and lysosomal proteinases in myofibrillar and non-myofibrillar protein degradation and to elucidate the possible mechanism by which IGF-1 reduces muscle protein degradation. Three approaches have been used in this study which included antisense oligonucleotide, antisense RNA and proteinase inhibitors. Due to the limitation of using antisense oligonucleotide and antisense RNA, we found that it was not appropriated to use these two approaches to regulate calpain activities. A cell-penetrating proteinase inhibitor, calpain inhibitor II (CI-II), and a lysosomal proteinase inhibitor, chloroquine (CQ),

were chosen to investigate the functions of proteinases in muscle cells. Total protein degradation was reduced 40%, 15% and 21% by CI-II, CQ and insulin-like growth factor 1 (IGF-1) respectively. CI-II reduced myofibrillar protein degradation; however, CQ and IGF-1 did not alter degradation of this pool. CI-II increased myofibrillar protein synthesis but had no effect in non-myofibrillar protein synthesis. Concomitant with these effects, CI-II, but not CQ or IGF-1, caused accumulation of myofibrillar proteins. In addition, CI-II stabilized some myofibrillar proteins and CQ and IGF-1 did not affect the stability of individual myofibrillar proteins. These results demonstrate that the decrease in total protein degradation caused by CI-II was primarily mediated by the reduction in myofibrillar protein breakdown, and the reduction in total protein degradation caused by CQ and IGF-1 was mediated by a reduction in non-myofibrillar protein degradation. We conclude that calpains play crucial role in myofibrillar protein degradation and that lysosomal proteinases are important to non-myofibrillar protein degradation. IGF-1 regulates degradation of non-myofibrillar protein pool via a lysosomal mechanism. Finally, this study documents validity of the concept that protein accretion will occur if myofibrillar protein degradation is reduced and protein synthesis is enhanced.

The Role of Calpains in Muscle Protein Degradation

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CHAPTER I

INTRODUCTION

Skeletal muscle makes up a larger proportion of body tissue than any other tissue and is the single largest protein pool in the body. Muscle protein is in a constant state of flux. Protein is constantly being degraded and synthesized. Usually, the rate of protein degradation is 5 to 10 percent per day. To maintain or increase muscle mass, muscle would have to synthesize an amount of protein over 5 to 10 percent of total protein content on a daily basis (Allen, 1986). In muscle, proteins generally are grouped into three categories based on their solubility: (1) the sarcoplasmic proteins, which constitute 30-35%, by dry weight, of the proteins in muscle and comprise almost all of the cytoplasmic proteins in the cell; (2) the myofibrillar proteins, which constitute the myofibril in striated muscle cells; these constitute 50-55% of total muscle protein and are the major class of muscle proteins both in a physiological sense and in the use of muscle as meat; and (3) the stromal proteins, which constitute 15-20% of total muscle proteins and which are largely extracellular because most of the stromal proteins consist of collagen and elastin (Goll et al., 1989). Because myofibrillar proteins are responsible for most of the desirable properties of muscle when used as food, study of muscle protein turnover in domestic animals should focus on the myofibrillar class of muscle proteins.

The turnover of myofibrillar protein in skeletal muscle occurring during growth can be regulated by changing the rates of both protein synthesis and degradation. Protein synthesis in muscle is almost certainly an important site of regulation, being particularly sensitive to dietary and hormonal changes. However, it appears that a substantial degree of change in muscle protein levels can be achieved through the control of the degradation process. Overall, protein degradation may be manipulated by changing conditions of the nutritional or hormonal environment (Hershko and Ciechanover, 1982). Promoting an increase in growth rate, for example, by administration of trenbolone acetate or clenbuterol in rats, causes rates of muscle growth to increase by decreasing the rate of muscle protein degradation with little or no change in the rate of muscle protein synthesis (Vernon and Buttery, 1978; Reeds et al., 1986; Forsberg et al., 1989). However, very little is known about the mechanism of intracellular protein degradation and how this process is regulated in muscle cells. It is unfortunate that the rate of muscle protein degradation has not received more attention in growth studies because decreasing the rate of muscle protein degradation would have two direct beneficial effects: (1) it would increase the rate of muscle protein accretion; and (2) it would reduce the amount of feed required per unit of gain because less feed would be needed to replace degraded muscle protein. Hence, altering the rate of muscle protein degradation can have significant effects on the rate of muscle growth. Goll

(1991) estimated that reducing the rate of muscle protein degradation in a market-size pig by 10% would increase the rate of live weight gain by 50%.

Degradation of myofibrillar proteins is a highly regulated process. It is believed that the regulation of this process may take place at the level of the protein substrate itself (through modulation of protein structure by intrinsic and extrinsic factors) and at the level of the degradative machinery, which would include branding enzymes, sequestration organelles and proteolytic systems (Benyon and Bond, 1986). Regulation of proteolytic systems, which include many factors such as compartmentalization, hormonal and nutritional environment, and proteinase inhibitors may be the most important level for regulation of proteolysis as shown in studies of myofibrillar protein degradation (Benyon and Bond, 1986).

PROTEOLYTIC SYSTEMS IN SKELETAL MUSCLE

Several proteolytic systems have been identified in skeletal muscle. These include three major proteolytic systems: (1) lysosomal proteases, which include cathepsins B, D, H and L, (2) proteasome and (3) calcium-dependent proteases, which include μ- and m-calpains.

Lysosomal proteinases

The most commonly-studied cathepsins include cathepsins B, H and L, which are cysteine proteinases, and cathepsin D which is an aspartyl proteinase. Traditionally, it was believed that lysosomal proteases were responsible for muscle protein degradation (Schwartz and Bird, 1977; Matsukura, 1981). However, evidence indicates that lysosomal proteases are not involved in the initial degradation of myofibrillar proteins. Libelius et al. (1979) and Gerard and Schneider (1979) have shown that products of myofibrillar degradation, such as myofilament fragments or myofibrillar protein fragments, could be detected in the lysosome, but intact myofibrils did not exist in the lysosome. This implied that lysosomal proteases can only degrade individual myofibrillar protein fragments and are not involved in the degradation of intact myofibrils. Furthermore, lysosomal proteases are contained in lysosomes and are active at acidic pH. In other words, lysosomal proteases cannot degrade myofibrils at sarcoplasmic pH. In addition, treatment of cells with lysosomal protease inhibitors failed to suppress calcium-induced protein degradation (Furuno and Goldberg, 1986).

Proteasome

An ATP-dependent proteolytic system was initially found in reticulocytes (Etlinger and Goldberg, 1977). This proteolytic system, called proteasome or multicatalytic proteinase complex, consists of at least 13 nonidentical components (Fujiwara et al., 1989) with molecular masses of 21 to 31 KDa and pI of 3 to 10 (Tanaka et al., 1988). It is most easily identified by its characteristic cylindrical shape in electron micrographs (Tanaka et al., 1988). Proteasome has been found in eukaryotes ranging from yeast to humans (Tanaka et al., 1992). Proteasome is present in large amounts in all cells that have been examined for its presence and it may be a major proteinase involved in intracellular protein turnover. It is difficult to detect proteasome's activity in cells without actually isolating the complex, because it has almost no proteolytic activity in its native state. Incubation with SDS, certain unsaturated fatty acids or certain polycations stimulates activity of the latent protease 10-fold or more. Obviously, proteolytic activity of the proteasome in vivo must be under strict control. However, the nature of this control is still unknown. Proteasome has multifunctional catalytic sites which include chymotrypsin-like peptidase, trypsin-like peptidase and peptidylglutamyl-peptidase, responsible for endoproteolytic cleavage of peptide bonds on the carboxyl side of basic, neutral and hydrophobic or acidic amino acid residues of proteins (Gottesman and Maurizi, 1992; Orlowski 1990). Proteasome has a dual role in intracellular protein turnover; 1) degrading ubiquitinated proteins when it is associated with

the ubiquitin complex (Ugai et al., 1993); and 2) degrading unubiquitinated protein when it is not associated with the ubiquitinating system (Murakami et al., 1992). Although proteasome has high activity in skeletal muscle (Driscoll and Goldberg, 1989), it has no effect on degradation of intact myofibrils (Koohmaraie, 1992). This implies that proteasome is not involved in the initial steps of myofibril disassembly. Natural substrates for proteasome have not been identified. It has been reported that proteasome activity is elevated in leukemic cells, other cancer cells and SV-40 transformed hepatocytes. This suggests that proteasome is involved in tumorigenesis and transformation (Tanaka et al., 1992). However, the precise role of proteasome in vivo and its regulation remain unknown.

Calpains

It was originally proposed in 1976 (Dayton et al., 1976a; 1976b) that calpain acted to initiate metabolic turnover of the myofibrillar proteins by disassembling these proteins from myofibrils. Currently, a mechanism for explaining myofibrillar protein degradation suggests that calpains initiate turnover of the myofibrillar proteins by removal of the N2 line. The N2 line consists of nebulin and titin, which anchor the thin and thick filaments to the myofibril. N2 line digestion is followed by dissolution of the Z-disk. α-actinin is a major Z-disk protein which anchors thick and thin filaments to the Z-disk. Calpains are proposed to degrade an unknown protein which anchors α-actinin to the Z-disk allowing α-actinin, α-actin and myosin to be released intact. This

is proposed to provide other proteolytic systems (eg. lysosomes or proteasome) with substrate (Allen, 1986; Goll et al., 1991).

Evidence supporting this belief includes the following: (1) Ca^{++}-dependent proteolytic activity was isolated from myofibrils and was shown to degrade myofibrillar proteins at the pH and ionic strengths of the intracellular environment (Busch et al., 1972). The Ca^{++}-dependent degradation was initiated at the Z-disk, a site where myofibrillar disassembly is initiated. (2) Immunohistochemical studies show that the calpains are located inside muscle and some calpains are associated with the Z-disk (Dayton et al., 1981; Goll et al., 1983; Yoshimura, et al., 1986; Kleese et al., 1987). (3) The release of myofilaments was Ca^{++}-dependent and was reduced by inhibitors of calpains (van der Westhuyzen et al., 1981; Zeman et al., 1985). (4) Treatment of muscle cells with high extracellular Ca^{++} or Ca^{++}-ionophore A23187 increased myofibrillar protein degradation (Publicover et al., 1978; Lewis et al., 1982; Zeman et al., 1985). (5) Finally, it has been shown using SDS-PAGE gels that the proteolytic changes caused by calpain treatment of myofibrils resemble the changes seen in myofibrils prepared from patients with Duchenne muscular dystrophy (Sugita et al., 1980; Obinata et al., 1981).

If the belief that myofibrillar protein degradation is initiated by calpains is accurate, the process of initial degradation should be the rate-limiting step. Though the regulatory details of these proteases have not been elucidated, a specific inhibitor of these proteases, calpastatin, coexists with calpains in

various tissues (Suzuki et al., 1988), and may play an important role in the regulation of calpain activities.

Other proteolytic enzymes

Other nonlysosomal proteases in the cytosol may also be important components of the myofibril degradation process. Several alkaline or neutral proteinases have been found in muscle cells and include neutral serine proteases and ATP-dependent proteolytic systems. Neutral serine proteases belong to the serine protease family (Kay et al, 1982) and are able to degrade various myofibrillar proteins such as actin, myosin, α-actinin, troponin-I and Z-disks, at neutral pH (Kar and Pearson,1980). It has been shown that the activity of neutral serine proteases in skeletal muscle is accelerated greatly in Duchenne muscular dystrophy, polymyositis and in denervating diseases (Kar and Person, 1980). This suggests that the neutral serine proteases may be important in conditions characterized by accelerated protein degradation in muscle cells (Kar and Pearson, 1980).

THE CALPAIN SYSTEM

Introduction

Calpains (EC 3.4.22.17), also referred to as calcium-activated neutral protease (CANP) or calcium-dependent protease (CDP), were purified from porcine skeletal muscle in 1976 by Dayton and his coworkers (1976a). Recently, calpains in chicken, rabbit, pig, rat, sheep and human have also been found (Ohno et al., 1984; Emori et al., 1986a; Sakihama et al., 1985; Aoki et al., 1986; Ohno et al., 1986). The calpain system contains four known proteins; two isozymes with different calcium sensitivities exist, μ-calpain and m-calpain, active at micromolar and millimolar concentrations of Ca^{++} respectively, a small subunit (30 KDa protein) and a calpain-specific inhibitor, calpastatin. The structures of μ-calpain and m-calpain have been analyzed Figure 1-1). These two isozymes consist of different large (80 KDa) subunits and identical small (30 KDa) subunits. Additionally, several members of the calpain family have been found in humans, rats and chickens. A skeletal muscle-specific calpain (p94) was found at the mRNA level but not the protein level in human and rat muscle (Sorimachi et al., 1989) and a high Ca^{++}-requiring calpain (high m-calpain) was found at the protein level in chicken muscle (Wolfe et al., 1989). In addition, two new calpain isoforms (nCl-2 and nCl-2') are expressed predominantly in the stomach (Sorimachi et al., 1993a).

The muscle-specific calpain (p94) consists of 820 amino acids and shows significant sequence homology with the large subunit of both human μ-calpain

(54%) and m-calpain (51%). However, three unique sequences exist in p94; at the N-terminal of domain I (NS), in the middle of domain II (intervening sequence I; IS1) and at the C-terminal of domain III (intervening sequence; IS2; Sorimachi and Suzuki, 1992). To date, p94 has been detected only in human and rat skeletal muscle by Northern blot analysis (Sorimachi et al., 1989) and was recently shown to have proteolytic activity. Following a recent in vitro study (Sorimachi et al., 1993b) it has been proposed that after translation, p94 moves to the nuclear membrane along the cytoskeleton with the aid of the IS2 region, where it is subjected to rapid autolysis. A small percentage of p94 is imported into the nucleus and regulates the levels of short-lived regulatory proteins such as transcription factors (Sorimachi et al., 1993b). However, the special function of this protease is still under investigation. High-m-calpain has been found in chicken muscle (Wolfe et al., 1989). It requires 3.8 mM Ca^{++} for one half-maximal activity, and only a single peptide (no 30 KDa subunit) of 74-76 kD is labeled on Western blots probed with an anti-calpain polyclonal antibody. Because of the high Ca^{++} required for activation of this proteinase, the physiological function of high-m-calpain remains unclear.

Functions of calpain

The physiological function of the calpain system is not known, although its ubiquitous distribution has led to its being associated with a large number of different, Ca^{++}-related proteolytic changes in cells. In general, in in vitro studies, the calpains cleave a limited number of specific sites in native proteins

and produce large peptide fragments rather than degrading proteins to small peptides and amino acids. The substrates for calpain can be divided into three categories: (1) regulatory enzymes such as protein kinase C, calcineurin, myosin light chain kinase, phosphorylase kinase, c-jun and c-fos; (2) cytoskeletal proteins including desmin, nebulin, troponin T, c-protein, titin, tropomyosin, troponin I, filman, talin, vinculin and tubulin; (3) hormone receptors such as EGF receptor, estrogen receptor, progesterone receptor, glucocorticoid receptor, PDGF receptor and L-type Ca^{++} -channel proteins (Goll et al., 1991). In the case of many kinases and phosphatases, these large fragments retain enzymatic activity but are no longer subject to the controls that regulate the intact enzyme. Although several studies have suggested that calpains are involved in regulation of protein kinase C activity, it is not clear why it would be beneficial for the cell to generate an unregulatable form of enzyme that has widespread effects on cellular metabolism. In addition, calpain also has been reported to rapidly degrade the c-fos and c-jun transcription factors (Hirai et al., 1991). This implies that calpain may regulate gene expression via degradation of transcription factors. Calpains also cleave a limited number of sites in many cytoskeletal proteins. In this instance, however, the large polypeptide remaining after cleavage has frequently lost the ability to cross-link or bind to the proteins with which it is normally associated. For this reason, many investigators believe that calpains also have a role in remodeling or disassembly of the cell cytoskeleton, especially in the area where cytoskeletal filaments attach to the

plasma membrane or to subcellular structures. Calpains also very quickly and specifically remove Z-disks from striated muscle myofibrils, and this property has suggested that calpains initiate metabolic turnover of the myofibrillar proteins by releasing them from their filamentous structure (Goll et al., 1991). Calpains also make specific cleavages in a number of hormone receptors. These cleavages, however, generally either have little effect on or diminish activity of the receptor, and it is not certain whether this function has physiological significance.

Structures and properties of calpains

Both μ-calpain and m-calpain consist of two subunits, the 80 KDa subunit and 30 KDa subunit. The 80 KDa subunit is composed of four distinct domains (Figure 1-1). Domain I is a propeptide domain which is similar to propeptides of various cysteine proteinases in various respects, (i.e. length of region, lack of tryptophan residues and intron break point; Suzuki et al., 1988). However, the sequence of domain I is not homologous to those of propeptides (Suzuki et al., 1988). The function of this domain is not clear.

Domain II is the most important domain of calpains and is responsible for protease activity. Its amino acid sequence is highly homologous to other typical cysteine proteases such as cathepsins B and H and papain (Takio, 1983). Also, the sequence of this domain between m-calpain and μ-calpain is highly homologous compared with the other three domains (Suzuki, 1987; Suzuki et al., 1988).

The function of domain III in the 80 KDa subunit is also not clear. Presumably, it is important for the regulation of protease activity or the interaction with the 30 KDa subunit or calpain's endogenous inhibitor (Murachi, 1983).

The C-terminal fourth domain (IV) is a calmodulin-like Ca^{++}-binding domain with four consecutive "EF hand" structures. Ca^{++} binds to the EF hand region. It is believed that the differences in Ca^{++}-sensitivity which exist between μ-calpain and m- calpain probably arise from differences in domain IV (Suzuki et al., 1988).

The 30 KDa subunit is composed of at least two domains. The N-terminal domain is glycine- and hydrophobic residue-rich. The importance of this domain is that it can interact with phospholipids in the membrane in a Ca^{++}-independent manner (Suzuki et al., 1988). The C-terminal domain of the 30 KDa subunit (domain IV') is also a calmodulin-like Ca^{++}-binding domain homologous to its corresponding C-terminal domain (IV) in the 80 KDa subunit (Emori et al., 1986a; Sakihama et al., 1985). This suggests that this domain (IV') is also responsible for calcium sensitivity of the dimer.

The upstream region of the human m-calpain large (80 KDa) subunit promoter lacks typical promoter elements such as TATA and CAAT boxes. It is characterized by its high GC content (-300 to -20) and contains multiple transcription initiation sites which cluster between -142 and -103 (Hata et al., 1989). The gene encoding the small subunit of calpain also shares similar

structural characteristics of large subunit in its 5'-flanking region (Miyake et al., 1986). Their structural features are common to many genes encoding housekeeping proteins. Since calpain promoters share many features in common to other essential housekeeping genes, this implies that calpains are also involved in essential cellular functions.

Mechanisms of regulation of calpain activity

Several mechanisms have been proposed for the modulation of calpain activity. First, obviously, Ca^{++} concentration is the most important factor for regulation of calpain activities. Native calpains are completely inactive and it is suggested that the native calpains should be called proenzymes or zymogens (Suzuki et al, 1988). In the cytosol, the concentration of Ca^{++} is not sufficient for pro-calpain activation. When Ca^{++} concentrations increase to above 1-2 μM, pro-calpain's conformation changes and causes translocation of the pro-enzymes to the inner cell membrane. Autolysis of these proteases occurs at this site. Membrane phospholipids may play an important role in the autolysis of calpain. Autolysis consists of cleavage of small fragments of the N-terminal of domain I of the large subunit and subsequent cleavage of the N-terminal of domain V of the small subunit. Proteases are then released to the cytosol as soluble, physiologically-active proteases (Pontremoli and Melloni, 1986; Suzuki et al., 1988). Also, autolysis of μ-calpain may occur in the cytosol without participation of the inner cell membrane (Suzuki et al., 1988).

Normally, pro-m-calpain requires millimolar concentrations of Ca^{++} for autolysis. However, the interaction of domain V of the small subunit with membrane phosphotidylinositol reduces the Ca^{++} requirement of m-calpain to μM levels (Suzuki et al., 1988). Activated μ-calpain may also be involved in the activation of m-calpain (Saido, personal communication).

The calpain-specific inhibitor, calpastatin, undoubtedly plays an important role in the regulation of calpain activity. It is the only known proteinaceous inhibitor specific for calpain. Calpastatin co-exists with calpain in the cytosol and possibly also in the nucleus. At least two kinds of calpastatin exist in vertebrates. The most prominent type, found in all tissues and cells except erythrocytes, has molecular weight of 73 (human) to 77 (pig and rabbit) KDa, but migrates on SDS-PAGE with a molecular weight of 107 to 117 KDa. A small form of calpastatin with a molecular mass of 48 KDa (70 KDa on SDS-PAGE) is found in erythrocytes. The large calpastatin molecule contains four internal repeating structures (domains I-IV) of about 140 amino acid residues each that are functional units of the inhibitor and exhibit inhibitory activities independently (Emori et al., 1987; 1988) and an N-terminal sequence, called domain L. Hence, one large molecule of calpastatin can inhibit up to four μ- or m-calpain molecules (Imajoh et al., 1987; Suzuki et al., 1988). The function of domain L is unknown.

Highly conserved sequences (Glu-Lys-Leu-Gly-Glu-Xaa-Ile-Pro-Pro-Xaa-Tyr-Arg) are found in the centers of calpastatin repeating inhibitory sequences.

The sequence around the highly conserved sequence (inhibitory sequence) is one of the sites of interaction with calpain. Other conserved sequences in the four repeats are found in the N- and C-terminal flanking regions of the inhibitory sequence (Kawasaki et al., 1989). Calpastatin inhibits translocation of pro-calpains to the plasma membrane and removes both active and inactive forms of calpains from the cell inner membrane (Suzuki et al., 1988). It inhibits the autolytic activation of pro-calpains and it inhibits actual proteolysis of substrates catalyzed by membrane-bound and cytosolic active enzymes (Suzuki et al., 1988). Ca^{++} is required for calpastatin to bind the calpains (Ishiura et al., 1982; Otsuka and Goll, 1987). Proteolytic activity of calpains occurs at Ca^{++} concentrations which is different from the Ca^{++} requirement for binding of calpain and calpastatin. This suggests that more than one set of Ca^{++}-binding sites exists in calpains. One set of Ca^{++}-binding sites is associated with calpastatin binding. A second set of Ca^{++}-binding sites may be involved in proteolytic activity (Kapprell and Goll, 1989). Recently, Kawasaki et al. (1993) have reported that calpain binds to cell membranes through a site (regulatory site) other than the active site and that calpastatin inhibits the binding of calpain to cell membranes via a site (regulatory inhibition site) other than the inhibitory sequence. Calpain and calpastatin can undergo independent interactions: interaction between the catalytic site of calpain and the inhibitory sequence of calpastatin, and interaction between the regulatory site of calpain and the regulatory inhibition site of calpastatin.

Recently, a 40 KDa protein that lowers the Ca^{++} concentration required for proteolytic activity of m- but not μ-calpain was identified in extracts of muscle, neutrophils and erythrocytes (Pontremoli et al., 1988, 1990; Salamino et al., 1993). This protein is present in association with the plasma membrane and promotes expression of calpain activity at a concentration of Ca^{++} close to physiological concentrations (Salamino et al., 1993). These observations suggest that the natural activating factor is an essential component of the general activating mechanism of calpain. A summary for activation of calpain in the presence of calpastatin and activator is shown in Figure 1-2.

Finally, some low-molecular weight proteins (17-20 KDa) have been found in calf and bovine brain which are capable of stimulating by 2-3 fold the activity of the proteases without changing their affinity for calcium. Furthermore, L-isovalerylcarnitine, a product of the metabolism of L-leucine, is a potent activator of calpains in human neutrophils (Malik et al., 1983; DeMartino and Blumenthal, 1982; Pontremoli et al., 1987). These factors also are proposed as natural activators of calpain.

SUMMARY

In the past two decades, most of the effort in attempting to increase the rate of animal growth in domestic animals has involved studies on protein synthesis in muscle. Little attention has been given to improving the rate of muscle growth by decreasing the rate of muscle protein degradation. Because of the important role that muscle protein degradation has in the rate of muscle growth, it is important to examine what is known about the mechanism of muscle protein degradation and how it is regulated. Current evidence has shown that calpains play an important role in the degradation of myofibrillar proteins. However, the methods used for those studies could not rule out the possibility of involvement by other proteinases. By using modern molecular biology techniques such as specific proteinase inhibitors, over-expression of negative dominant proteinases (mutation in active site), antisense nucleotides and homologous recombination (knock-out gene), researchers should be able to further identify the role of calpains in muscle protein degradation. The understanding of mechanisms of muscle protein degradation could improve the efficiency of animal growth and provide high quality meat.

REFERENCES

Allen, R. E. 1986. Muscle cell growth and development. Designing Foods. Animal Product Options in the Marketplace. National Research Council, National Acad. Press. Wash. D. C. pp 142-162.

Aoki, K., S. Imajoh, S. Ohno, Y. Emori, Y. Koike, G. Kosaki and K. Suzuki. 1986. Complete amino acid sequence of the large subunit of the low-Ca^{++}-requiring form of human Ca^{++}-activated neutral protease (μCANP) deduced from its cDNA sequence. FEBS Lett. 205:313-317.

Benyon, R. J. and J. S. Bond. 1986. Catabolism of intracellular protein: molecular aspects. Am. J. Physiol. 251:C141-C152.

Busch, W. A., M. H. Stromer, D. E. Goll and A. Suzuki. 1972. Ca^{2+}-specific removal of Z-lines from rabbit skeletal muscle. J. Cell Biol. 52:367-381.

Dayton, W. R., D. E. Goll, M. G. Zeece, R,. M. Robson and W. J. Reville. 1976a. A Ca^{2+}-activated protease possibly involved in myofibrillar protein turnover. Purification from porcine muscle. Biochemistry 15:2150-2158.

Dayton, W. R., W. J. Reville, D. E. Goll and M. H. Stromer. 1976b. A Ca^{2+}-activated protease possibly involved in myofibrillar protein turnover. Partial characterization of the purified enzyme. Biochemistry 15:2159-2167.

Dayton W. R. , J. V. Schollmeyer, R. A. Lepley and L. R. Cortes. 1981. A calcium-activated protease possibly involved in myofibrillar protein turnover. Isolation of a low calcium requiring form of the protease. Biochim. Biophys. Acta 659:48-61.

DeMartino, G. N. and D. K. Blumenthal. 1982. Identification and partial purification of a factor that stimulates calcium-dependent proteases. Biochemistry 21:4297-4303.

Driscoll, J. and A. L. Goldberg. 1989. Skeletal muscle proteasome can degrade proteins in an ATP-dependent process that does not require ubiquitin. Proc. Natl. Acad. Sci. USA 86:787-791.

Emori, Y., H. Kawasaki, S. Imajoh, S. Kawashima and K. Suzuki. 1986b. Isolation and sequence analysis of cDNA clones for the small subunit of rabbit calcium-dependent proteinase. J. Biol. Chem. 261:9472-9476.

Emori, Y., H. Kawasaki, S. Imajoh, K. Imahori and K. Suzuki. 1987. Endogenous inhibitor for calcium-dependent cysteine protease contains four internal repeats that could be responsible for its multiple reactive sites. Proc. Natl. Acad. Sci. USA 84:3590-3594.

Emori, Y., H. Kawasaki, S. Imajoh-ohmi, Y. Minami and K. Suzuki. 1988. All four repeating domains of the endogenous inhibitor for calcium-dependent protease independently retain inhibitory activity. J. Biol. Chem. 263:2364-2370.

Emori, Y., H. Kawasaki, H. Sugihara, S. Imajoh, S. Kawashima and K. Suzuki. 1986a. Isolation and sequence analysis of cDNA clones for the large subunits of two isozymes of rabbit calcium-dependent proteinase. J. Biol. Chem. 261:9465-9471.

Etlinger, J. D. and A. L. Goldberg. 1977. A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal protein in reticulocytes. Proc. Natl. Acad. Sci. USA 74:54-58.

Forsberg, N. E., M. A. Ilian, A. Ali-Bar, P. R. Cheeke and N. B. Wehr. 1989. Effects of cimaterol on rabbit growth and myofibrillar protein degradation and on calcium-dependent proteinase and calpastatin activities in skeletal muscle. J. Anim. Sci. 67:3313-3321.

Fujiwara, T., K. Tanaka, A. Kumatori, S. Shin, T. Yoshimura, A. Ichihara, F. Tokunaga, R. Aruga, S. Iwanaga, A. Kakizuka and S. Nakanishi. 1989. Molecular cloning of cDNA for proteasomes (multicatalytic proteinase complexes) from rat liver: primary structure of the largest component (C2). Biochemistry 28:7332-7340.

Furuno, K. and A. L. Goldberg. 1986. The activation of protein degradation in muscle by Ca^{2+} or muscle injury does not involve a lysosomal mechanism. Biochem. J. 237:859-864.

Gerard, K. W. and D. L. Schneider. 1979. Evidence for degradation of myofibrillar proteins in lysosomes. Myofibrillar proteins derivatized by intramuscular injection of N-ethymaleimide are sequestered in lysosomes. J. Biol. Chem. 254:11798-11805.

Goll, D. E. 1991. Role of proteinases and protein turnover in muscle growth and meat quality. Reciprocal Meat Conference Proceeding, 4:25-36.

Goll, D. E., W. R. Dayton, I. Singh, and R. M. Robson. 1991. Studies of the actinin/actin interation in the Z-disk by using calpain. J. Biol. Chem. 266:8501-8510.

Goll, D. E., W. C. Kleese and A. Szpacenko. 1989. Skeletal muscle proteases and protein turnover. Animal Growth Regulation. Plenum Publishing Corporation. pp 141-182.

Goll, D. E., J. D. Shannon, T. Edmunds, S. K. Sathe, W. C. Kleese and P. A. Nagainis. 1983. Properties and regulation of the Ca^{2+}-dependent proteinase. Calcium-Binding Proteins. Elsevier, Amsterdam. pp 19-35.

Gottesman, S. and M.R. Maurizi. 1992. Regulation by proteolysis: enzyme-dependent protease and their targets. Microbiol. Rev. 56:592-621.

Gopalakrishna, R. and S. H. Barsky. 1985. Quantitation of tissue calapin activity after isolation by hydrophobic chromatography. Anal. Biochem. 148:413-423.

Hata, A., S. Ohno, Y. Akita and K. Suzuki. 1989. Tandemly-reiterated negative enhancer-like elements regulate transcription of a human gene for the large subunit of calcium-dependent protease. J. Biol. Chem. 264:6404-6411.

Hershko, A. and A. Ciechanover. 1982. Mechanisms of intracellular protein breakdown. Ann. Rev. Biochem. 51:335-364.

Hirai, S. I., H. Kawasaki, M. Yaniv and K. Suzuki. 1991. Degradation of transcription factors, c-Jun and c-Fos, by calpain. FEBS Lett. 287:57-61.

Imajoh, S.,H. Kawasaki, Y. Emori, S. Ishiura, Y. Minami, H. Sugita, K Imahori and K. Suzuki. 1987. A fragment of an endogenous inhihitor produced in Escherichia coli for calcium-activated neutral protease retains an inhibitory activity. FEBS Lett. 215:274-278.

Ishida, S., Y. Emori and K. Suzuki. 1991. Rat calpastatin has diverged primary sequence from other mammalian calpastatin but retains functionally important sequences. Biochim. Biophys. Acta. 1088:436-438.

Ishiura, S., S. Tsuji, H. Murofushi and K. Suzuki. 1982. Purification of an endogenous 68000-dalton inhibitor of Ca^{++}-activated neutral protease from chicken skeletal muscle. Biochim. Biophys. Acta. 701:216.

Kapprell, H. P. and D. E. Goll. 1989. Effect of Ca^{++} on binding of the calpains to calpastatin. J. Biol. Chem. 264:17888-17896.

Kar, N. C. and C. M. Pearson. 1980. Elevated activity of neutral proteinase in human muscular dystrophy. Biochem. Med. 26:238-243.

Kawasaki, H., Y. Emori, S. Imajoh, Y. Minami and K. Suzuki. 1989. Identification and characterization of inhibitory sequences in four repeating domains of the endogenous inhibitor for calcium-dependent protease. J. Biochem. 106:274-281.

Kawasaki, H., Y. Emori and K. Suzuki. 1993. Calpastatin has two distinct sites for interation with calpain: effect of calpastatin fragments on the binding of calpain to membranes. Arch. Biochem. Biophys. 305:467-472.

Kay, J., L. M. Siemankowski, R. F. Siemankowski, J. A. Greweling and D. E. Goll. 1982. Degradation of myofibrillar protein by trypsin-like serine protease. Biochem. J. 201:279-285.

Kleese, W. C., D. E. Goll, T. Edmunds and J. D. Shannon. 1987. Immunofluorescent localization of the Ca^{2+}-dependent proteinase and its inhibitor in tissue of Crotalus atrox. J. Exp. Zool. 241:277-289.

Koohmaraie, M. 1992. Ovine skeletal muscle multicatalytic proteinase complex (proteasome): purification, characterization, and comparison of its effects on myofibrils with μ-calpains. J. Anim. Sci. 70:3697-3708.

Lewis, S. E. M., P. Anderson and D. F. Goldspink. 1982. The effects of calcium on protein turnover in skeletal muscle of the rats. Biochem. J. 204:257-264.

Libelius, R., J. O. Josefsson and I. Lundquist. 1979. Endocytosis in chronically denervated mouse skeletal muscle. A biochemical and ultrastructural study with horseradish peroxidase. Neuroscience 4:283-290.

Malik, M. N., M. D. Fenko, K. Iqbal and H. M. Wisniewski. 1983. Purification and characterization of two forms of Ca^{2+}-activated neutral protease from calf brain. J. Biol. Chem. 258:8955-8962.

Matsukura, U., A. Okitani, T. Nishimuro and H. Kato. 1981. Mode of degradation of myofibrillar proteins by an endogenous protease, cathepsin L. Biochim. Biophys. Acta 662:41-47.

Miyake, S., Y. Emori and K. Suzuki. 1986. Gene organization of the small of human calcium-activated neutral protease. Nucleic acids Res. 14:8805-8817.

Murakami Y., S. Matsufuji, T. Kameji, S. Hayashi, K Igarashi, T. Tamura, K. Tanaka and A. Ichihara. 1992. Orthnithine decarboxylase is degraded by the 26S proteasome without ubiquitination. Nature 360:597-599.

Murachi, T. 1983. Calpain and calpastatin. Trends Biochem. Sci. 8:167-169.
Obinata, T., K. Maruyama, H. Sugita, K. Kohama and S. Ebashi. 1981. Dynamic aspects of structural proteins in vertebrate skeletal muscle. Muscle Nerve 4: 456-462.

Ohno, S., Y. Emori, S. Imajoh, H. Kawasaki, M. Kisaragi and K. Suzuki. 1984. Evolutionary origin of a calcium-dependent proteinase by fusion of gene for a thiol-protease and calcium-binding protein. Nature 312:566-570.

Ohno, S., Y. Emori and K. Suzuki. 1986. Nucleotide sequence of a cDNA coding for the small subunit of human. Nucleic Acids Res. 14: 5559.

Orlowski, M. 1990. The multicatalytic proteinase complex, a major extralysosomal proteolytic system. Biochemistry. 29:10289-10297.

Otsuka, Y. and D. E. Goll. 1987. Purification of the Ca^{++}-dependent proteinase inhibitor from bovine cardiac muscle and its interaction with the millimolar Ca^{++}-dependent proteinase. J. Biol. Chem. 262:5839-2851.

Pontremoli, S. and E. Melloni. 1986. Extralysosomal protein degradation. Ann. Rev. Biochem. 55:455-481.

Pontremoli, S., E. Melloni, M. Michetti, B. Sparatore, F. Salamino, N. Siliprandi and B. L. Horecker. 1987. Isovalerylcarnitine is a specific activator of calpain of human neutrophils. Biochem. Biophys. Res. Comm. 148:1189-1195.

Pontremoli, S., E. Melloni, M. Michetti, F. Salamino, B. Sparatore and B. L. Horecker. 1988. An endogenous activator of Ca2+-dependent proteinase of human neutrophils that increases its affinity for Ca2+. Proc. Natl. Acad. Sci. USA 85:1740-1743.

Publicover, S.J., C. J. Duncan and J. L. Smith. 1978. The use of A23187 to demonstrate the role of intracellular calcium in causing ultrastructural damage in mammalian muscle. J. Neuropathol. Exp. Neurol. 37:544-550.

Reeds. P. J., S. M. Hay, P. M. Dorwood and R. M. Palmer. 1986. Stimulation of muscle growth by clenbuterol: lack of effect on muscle protein biosynthesis. Br. J. Nutr. 56:249-258.

Sakihama, T., H. Kakidani, K. Zenita, N. Yumoto, T. Kukichi, T. Sasaki, R. Kannagi, S. Nakanishi, M. Ohmori, K. Takio, K. Titani and T. Murachi. 1985. A putative Ca^{2+}-binding protein:structure of the light subunit of porcine calcium elucidated by molecular cloning and protein sequence analysis. Proc. Natl. Acad. Sci. USA 82:6075-6079.

Salamino, F., R. De Tullio, P. Mengotti, P. L. Viotti, E. Melloni and S. Pontremoli. 1993. Site-directed activation of calpain in promoted by membrane-associated natural activator protein. Biochem. J. 290:191-197.

Schwartz, W. N. and J. W. C. Bird. 1977. Degradation of myofibrillar proteins by cathepsins B and D. Biochem. J. 167:811-820.

Sorimachi, H., S. Ishiura and K. Suzuki. 1993a. A novel tissue-specific calpain species expressed predominantly in the stomach comprises two alternative splicing products with and without Ca^{2+}-binding domain. J. Biol. Chem. 268:19476-19482.

Sorimachi, H., S. I. Ohmi, Y. Emori, H. Kawasaki, S. Ohno, Y. Minami and K. Suzuki. 1989. Molecular cloning of a novel mammalian calcium-dependent protease distinct from both m- and μ-type. J. Biol. Chem. 264:20106-20111.

Sorimachi, H. and K. Suzuki. 1992. Sequence comparison among muscle-specific calpain, p94, and calpain subunits. Biochi. Biophys. Acta. 1160:55-62.

Sorimachi, H., N. Toyama-Sorimachi, T. C. Saido, H. Kawasaki, H. Sugita, M. Miyasaka, K. Arahata, S. Ishiura and K. Suzuki. 1993b. Muscle-specific calpain, p94, is degraded by autolysis immediately after translation, resulting in dispearance from muscle. J. Biol. Chem. 268:10593-10605.

Sugita, H., S. Ishiura, K. Suzuki and K. Imahori. 1980. Ca^{2+}-activated neutral protease and its inhibitors: in vitro effect on intact myofibrils. Muscle Nerve 3:335-345.

Suzuki, K. 1987. Calcium-activated neutral protease: domain structure and activity regulation. Trends Biochem. Sci. 12:103-105.

Suzuki, K., S. Imajoh, Y. Kawasaki, Y. Minami and S. Ohno. 1988. Regulation of activity of calcium-activated neutral protease. Adv. Enzyme Reg. 27:153-169.

Suzuki, K. and S. Ohno. 1990. Calcium activated neutral protease--structure-function relationship and functional implications--. Cell Structure and Function 15:1-6.

Takio, K., T. Towatori, N. Katunuma, D. C. Teller and K. Tirani. 1983. Homology of amino acid sequences of rat liver cathepsin B and H with that of papain. Proc. Natl. Acad. Sci. U.S.A. 80:3666-3670.

Tanaka, K., T. Tamura, T. Yoshimura and A. Ichihara. 1992. Proteasomes: protein and gene structures. New Biologist. 4:173-187.

Tanaka, K., T. Yoshimura, A. Ichihara, A. Ikai, M. Nishigai, M. Morimoto, M. Sato, N. Tanaka, Y. Katsube, K. Kameyama and T. Takagi. 1988. Molecular organization of a high molecular weight multi-protease complex from rat liver. J. Mol. Biol. 203:985-996.

Ugai, S. I., T. Tamura, N. Tanahashi, S. Takai, N. Komi, C. H. Chung, K. Tanaka and A. Ichihara. 1993. Purification and characterization of the 26S proteasome complex catalyzing ATP-dependent breakdown of ubiquitin-ligated proteins from rat liver. J. Biochem. 113:754-768.

Van der Westhuyzen, D. R., K. Matsumoto and J. D. Etlinger. 1981. Easily releasable myofibrillar assembly and turnover. J. Biol. Chem. 256:11791-11797.

Vernon, B. G. and P. J. Buttery. 1978. Protein turnover in rats treated with trenbolone acetate. Br. J. Nutr. 36:575-579.

Wolfe, F. H., S. K. Sathe, D. E. Goll, W. C. Kleese, T. Edmunds and S. M. Duperret. 1989. Chicken skeletal muscle has three Ca^{2+}-dependent proteinases. Biochem. Biophys. Acta 998:236-250.

Yoshimura, N., T. Murachi, R. Health, J. Kay, B. Jasani and G. R. Newman. 1986. Immunogold electron-microscopic localization of calpain I in skeletal muscle of rats. Cell Tissue Res. 244:265-270.

Zeman, R. J., T. Kameyama, K. Matsumoto, P. Bernstein and J. D. Etlinger. 1985. Regulation of protein degradation in muscle by calcium. Evidence for enhanced non-lysosomal proteolysis associated with elevated cystolic calcium. J. Biol. Chem. 260:13619-13624.

Figure 1-1. Schematic structures of calpain. 80 KDa and 30KDa subunits contain four (I-IV) and two (IV', V) domains, respectively. The diamond and square show active site Cys and His residues, respectively. The circles in domains IV and IV' are EF hand structures, potential Ca^{++} binding sites (Suzuki and Ohno, 1990).

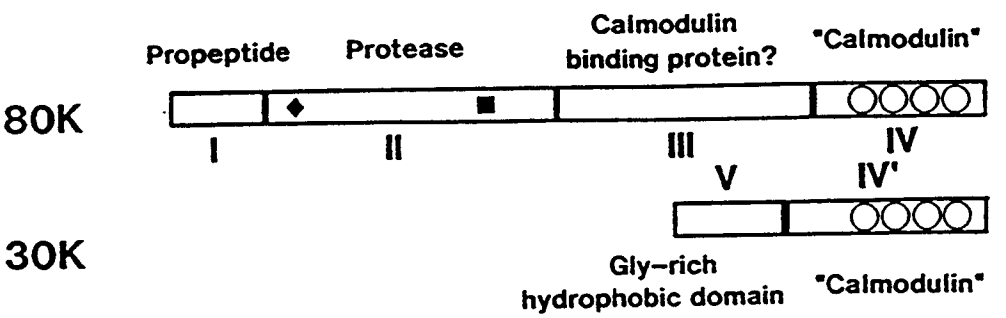
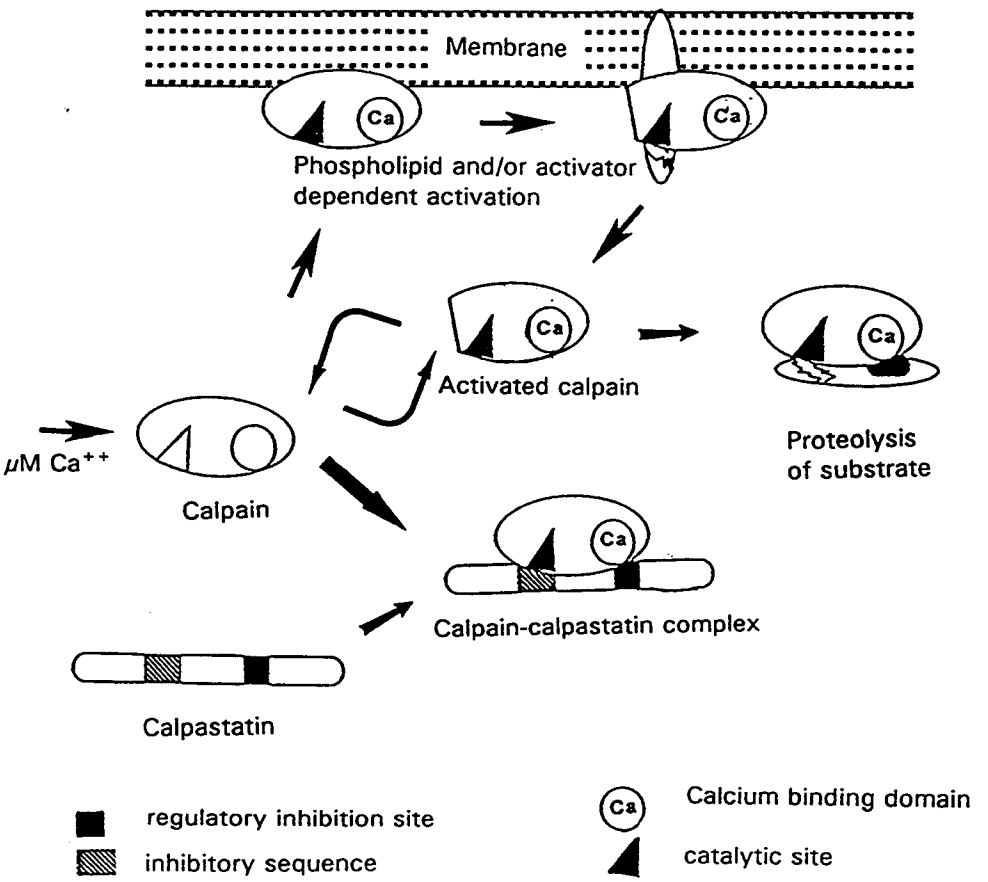


Figure 1-2. Activation of calpain in the presence of calpastatin and activator. Most calpain binds to calpastatin at μM levels of calcium through interaction between the calcium binding domain of calpain and the "regulatory inhibition site" of calpastatin. Some calpain may escape from its complex with calpastatin and be activated on the membrane by activator and/or phospholipid. Activated calpain then released into cytosol. Activated calpain may also partial digest native calpain to activated other calpain in cytosol (Kawasaki et al., 1993).



CHAPTER II

EFFECTS OF ANTISENSE OLIGODEOXYRIBONUCLEOTIDE AND ANTISENSE RNA ON CALPAIN EXPRESSION IN L8 MYOTUBES

ABSTRACT

The objectives of this study were to down-regulate m-calpain protein concentrations in cultured L8 myotubes using antisense oligonucleotide (oligoDNA) or antisense RNA. Initially, addition of antisense DNA directly to muscle cell cultures was assessed. Uptake of 5'-end ^{32}P-labeled m-calpain antisense oligoDNA by L8 myotubes increased over time. A predominant form of radioactivity detected intracellularly at 24 hr was a high molecular weight RNA:oligomer complex. When oligoDNA was incubated with culture medium, shorter oligomers were detected throughout 24 hr of exposure time. L8 myotubes treated with antisense oligoDNA for 36 hours and 72 hours reduced m-calpain concentrations slightly.

It was decided that this level of inhibition of calpain gene expression was insufficient for our needs and in subsquent studies we evaluated the use of antisense RNA as a means of down-regulating muscle m-calpain. Antisense and sense orientations of m-calpain cDNA were inserted into a pMAMneo-inducible eukaryotic expression vector. L8 myoblasts were transfected with these plasmids and selected with G418-containing medium. m-Calpain sense and antisense RNAs were induced by addition of dexamethasone (Dex) to culture

medium. m-Calpain protein concentrations increased in Dex-treated sense-transfected myotubes; however, calpain concentration did not change in antisense-transfected cells. These results imply that the Dex-dependent increase in m-calpain concentrations in normal muscle cells is prevented by the Dex-dependent control of m-calpain antisense RNA expression in L8A cells.

We found that neither the antisense DNA or antisense RNA methods were adequate for down-regulating calpain. However, several interesting function of the biology of calpain were identified in the course of these studies. First, Dex increases m-calpain concentration in myotubes. This may explain Dex ability to increase protein degradation in myotubes which we have reported earlier. Second, the limited ability of two antisense approaches to extensively down-regulate m-calpain mRNA or protein suggests that the calpain system contains abilities to compensate for external manipulation. This possibility is consistent with previous studies of ours where we found that cells were metabolically-geared to maintain calpain at stable levels despite physiological perturbation. Finally, the observation that Dex prevented m-calpain concentration from increasing in m-calpain antisense-expressing cells suggests an alternative mechanism of action of antisense RNA. Specifically, this observations suggest that antisense RNA may complex with target RNA and interfered with normal translation.

INTRODUCTION

Calpains, also referred to as calcium-activated neutral proteases (CANP) or calcium-dependent proteases (CDP), include at least two isoforms which are activated by micromolar and millimolar levels of Ca^{2+} respectively (μ-calpain and m-calpain). These two isozymes consist of different large (80 KDa) subunits and an identical small (30 Kda) subunit (Suzuki et al., 1988). Currently, a mechanism for explaining myofibrillar protein degradation suggests that calpains initiate metabolic turnover of the myofibrillar proteins by releasing thick and thin filaments from the surface of myofibrils; then individual myofibrillar protein can be degraded to free amino acids by lysosomal proteases and proteasome (Allen, 1986; Goll et al., 1992). At present it is not possible to distinguish between roles of μ- and m-calpains in muscle. Both calpains have similar properties and substract requirements. In muscle, m-calpain exists at a two- to three-fold higher concentration than μ-calpain (Ou et al., 1991; Ou and Forsberg, 1991) and possesses an intrinsically higher turnover rate than μ-calpain (Goll et al., 1992) so it may play a more important role in degrading contractile proteins. In order to understand the role of m-calpain in muscle protein degradation, our goal was to develop a method to reduce muscle cell calpain levels. To accomplish this, we undertook two approaches: antisense oligonucleotides and antisense RNA.

Antisense nucleic acids can be introduced into prokaryotic and eukaryotic cells to inhibit various processes such as translation or the replication of viruses

(Sartorius and Franklin, 1991). The blockage of translation by oligoDNA hybridizing to the mRNA is generally possible during translation initiation (Sartorius and Franklin, 1991). The 5' end of mRNA and the sequences around the AUG codon are better targets for hybridization arrest than the coding region. In this case, antisense oligoDNA can either prevent the initiation complex or block its propagation (Maher and Dolnick, 1987). If the coding region is used, a different mechanism for translation inhibition can be employed that involves ribonuclease H (RNase H) activity. RNase H degrades the mRNA after duplex formation with antisense oligoDNA (Zon, 1990). Although the coding region of the mRNA was considered to be an inefficient target for hybridization of antisense oligoDNA for translation inhibition, there is some evidence that blockage of translation may occur within the coding region (Teichman-Wernberg et al., 1988; Wickstorm et al., 1988).

In this study we evaluated two antisense methods for down-regulating m-calpain in muscle cells: antisense DNA and antisense RNA. We attempted the antisense DNA method first because of its simplicity and then antisense RNA second because the antisense DNA did not result in sufficient large reduction in m-calpain levels to detect a phenoltype. Neither method proved adequate as a means to down-regulate calpain; however, in the course of these studies, several interesting biological properties of calpains revealed.

MATERIALS AND METHODS

Materials

Calf serum (CS), fetal calf serum (FCS) and horse serum (HS) were purchased from Hyclone (Logan, UT). Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin solution and trypsin were from GIBCO (Grand Island, NY). Trichloroacetic acid (TCA), glycine, sodium dodecyl sulfate (SDS), dexamethasone, dextran sulfate, sonicated denatured salmon testes DNA, ethidium bromide and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO). The ECL Western blotting analysis system was from Amersham (Arlington Heights, IL). Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG, molecular weight standards and the Bradford protein assay dye reagent were from Bio-Rad (Richmond, CA). Cell culture dishes and plates were from Corning (Corning, New York). [α-^{32}P] dCTP (6,000 Ci/mmol) was from New England Nuclear (Boston, MA). S & S Nytran^{+} (0.45um) membrane was from Schleicher & Schuell (Keene, NH). Denhardt's solution (100 x) was from 5 prime-3 prime Inc. (Boulder, CO). The QIAEX agarose gel extraction kit was from QIAGEN Inc. (Chatsworth, CA). The random-primed DNA labeling kit was from USB (Cleveland, OH). Formamide and formaldehyde were from Mallinckrodt (Paris, KY). Safe II scintillation cocktail was from Research Products International Co. (Mount Prospect, IL). Quick-Spin G-50 and G-25 Sephadex columns were from Boehringer Mannheim

(Indianapolis, IN). m-Calpain polyclonal antibody (Ab) was obtained from Dr. Takaomi Saido (Tokyo Metropolitan Institude of Medical Science, Japan).

Cell culture

L8 rat myoblast cells were obtained from American Type Culture Collection (Rockville, MD). Cells were grown in DMEM with 10% calf serum (CS), 100 units of penicillin/ml, 100 μg of streptomycin/ml, 44 mM sodium bicarbonate in an humidified atmosphere of 5% CO_2 and 95% air at 37°C. The medium was changed every two days. After cells reached confluency, the medium was replaced with 2% horse serum (HS) to induce differentiation. Microscopic examination was used to monitor cell differentiation.

Oligonucleotides

The sequence 5'- GAT GTT GGT CTG CCC -3', which corresponds to 1325-1339 bp (5'- GGG CAG ACC AAC ATC -3') on rat m-calpain cDNA (Deluca et al., 1993), was selected for antisense oligoDNA. For control oligoDNA 5'- CAT GTT CGT GTC CGG -3' was selected. This oligomer maintained base-pair frequency of the antisense m-calpain 15-mer. Both 15-mers met the following criteria :

a. 15-mer was not associated with secondary structure.

b. 15-mer sequence was not found in any entries in GeneBank (NIH) or UEMBL databases.

Uptake and stability of oligomers

L8 myoblasts were cultured in 12-well plates. At confluency, medium was replaced with DMEM supplemented with 2% HS. The anti-sense oligomer was 5′ end-labelled with ^{32}P-ATP using T4 kinase then purified using a Sephadex G-25 Quick Spin column. To each well $1X10^5$ cpm (Experiment 1) or $5X10^5$ cpm (Experiment 2) of 5′-^{32}P-labeled oligoDNA were mixed with unlabeled oligoDNA to a final concentration of 5 μM. This mixture was then added in 1 ml DMEM containing 1% FCS. Following incubation, cells were scraped in 0.4 ml lysis buffer (10 mM Tris (pH 7.4), 10 mM NaCl, 3mM $MgCl_2$, 0.5% NP-40, 0.5% SDS, 0.01% proteinase K) and extracted with phenol. The aqueous phase was removed and re-extracted with a phenol:chloroform:isoamylalcohol (25:24:1) mixture. Aliquots of the aqueous extract, cell lysate, cell wash and medium were assayed by liquid scintillation counting. Aliquots of the aqueous phase (intracellular compartment) and of the medium (extracellular compartment) were lyophilized, dissolved in 50μl of 50% deionized formamide, heated to 90°C for 3 min and electrophoresed on a 20% denaturing gel and then exposed to Kodak X-film at -80°C.

Western blot analysis

Protein samples were subjected to 7.5-15% SDS-PAGE according to the method of Laemmli (1970). The separated proteins were transferred onto nitrocellulose membranes (0.45 μm, S & S) at 4°C overnight using a Bio-Rad transfer apparatus (30 volts) according to the method of Towbin et al. (1979)

in transfer buffer containing 20% methanol, 25 mM Tris-HCl and 192 mM glycine. After transfer, membrane non-specific sites were blocked by 5% skim milk in TTBS (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.05% Tween-20) for 1 hour at room temperature. Membranes were hybridized with primary antibody for 2 hours at room temperature. After three washes (15 min each) in TTBS, the secondary antibody (HRP-conjugated goat anti-rabbit IgG; 1:2000 dilution in 1% skim milk in TTBS) was added and incubated for 1 hour at room temperature. Following this incubation, the membrane was washed four times with TTBS. Specific binding of antibody on the membrane was detected by the ECL detection system (Amersham). Levels of protein were quantified by densitometric scanning of the Western blot using a Model 1650 transmittance/reflectance scanning densitometer (Bio-Rad) and the Hoefer GS 350 data system (Hoefer Scientific Instruments, San Francisco, CO).

Plasmid construction

To construct an expression vector, a 0.9 kb EcoR1 fragment of rat m-calpain cDNA (pCAL II was provided by Dr. John S. Elce, Queen's University) was blunt-end with klenow fragment of DNA polymerase I and cloned into the Xho I site using T4 ligase (Promega). pMAMneo had been previously treated with Xho I, klenow and calf intestinal alkaline phosphatase (CIAP). Plasmids with a correctly oriented insert were selected by restriction analysis with Bgl II. The resulting construct, pMAMA contained an antisense insert and pMAMS contained a sense insert. Inserts were downstream from the Dex-inducible

mouse mammary tumor virus (MMTV) long terminal repeat promoter, linked to the Rous sarcoma virus promoter (RSV), and upstream of the SV40 polyadenylation signal and possessed the neomycin resistance gene, which conferred resistance to the neomycin derivative G418.

Plasmids PMAMA (Figure 2-4) and PMAMS (Figure 2-5) were transformed into E. Coli. HB101 competent cells by using calcium chloride transformation. Large scale plasmid purification was performed using the polyethyleneglycol (PEG) method (Maniatis et al., 1982).

Cell transfection

A rat myoblast cell line (L8 cells) was cultured in Dulbecco's modified Eagles's medium, supplemented with 10% calf serum and antibiotics. Transfection was performed by the calcium-phosphate co-precipitation method as outlined by Maniatis and Sambrook (1982). Cells were cultured to 70% confluency. Before transfection, fresh medium containing 10% CS was replaced. 15 μg of plasmid DNA per plate (10cm plate) were used to transfect cells. After transfection, cells were washed with fresh medium twice, after which G418-containing medium was added. Culture medium was changed every other day. Stable G418-resistant clones were maintained in G418-selecting medium. L8A cells are those transfected with pMAMA plasmid, L8S cells are those transfected with pMAMS plasmid and L8P cells are those transfected with parental pMAMneo plasmid.

Extraction of total RNA

Extraction of total RNA has been described previously (Chomczynski and Sacchi, 1987). Briefly, myotube cultures grown in 10-cm diameter dishes were washed three times with ice-cold phosphate buffered saline (PBS) and lysed directly on the dishes using 2 ml of Solution A (4 M guanidium isothionate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 10 mM ß-mercaptoethanol). The monolayer was scraped with a rubber policeman to ensure that all cells were released from the dishes. The lysate was transferred to a 15 ml polypropylene tube. To this, 0.2 ml of 2 M sodium acetate (pH 4.0), 2 ml of phenol and 0.4 ml of a chloroform-isoamyl alcohol mixture (49:1) were added sequentially. The lysate was mixed by inversion after each addition. After brief vortexing, the mixture was kept on ice for 15 min and was separated by centrifugation (12,000g) for 30 min at 4°C. The upper aqueous phase was transferred to a new tube and an equal volume of ice-cold isopropanol was added. The samples were mixed and stored at -20°C overnight. RNA was collected by centrifugation (12,000g, 20 min at 4°C). The supernatant was discarded and the RNA pellet was resuspended in 0.4 ml of Solution A. One volume of isopropanol was added, and the mixture was stored at -20°C overnight. The RNA pellet was recovered by centrifugation at 12,000g for 15 min at 4°C, washed twice with 70% ethanol and dried under a vacuum. The dried RNA pellet was then dissolved in diethylpyrocarbonate (DEPC)-treated water and was quantitated by spectrophotometry at a wavelength of 260 nm.

cDNA probes

m-Calpain cDNA (900 bp EcoRI fragment) and neo cDNA (900 bp Hind III and Bgl II fragment) were prepared from pCAL II and pMAMneo, respectively. After restriction enzyme digestion, cDNA fragments were separated by electrophoresis and were recovered using a QIAEX agarose gel extraction kit. cDNA fragments (25 ng) were labeled with [α-^{32}P] dCTP (6000 Ci/mmol) using a random-prime labeling kit. A labeled cDNA probe was purified using a Quick Spin G-50 Sephadex column (Boehringer Mannheim).

Northern blot hybridization

RNA samples (25 μg) were denatured at 55°C for 15 min, applied to a 1.2% agarose gel containing 2.2 M formaldehyde, then electrophoresed at 30 V for 12 h. RNA was transferred to Nytran^{+} membranes and immobilized by baking at 70°C for 40 min. Membranes were prehybridized at 42°C overnight in prehybridization buffer (5 x SSPE, 0.2% SDS, 5 x Denhardt's solution, 100 μg/ml sonicated salmon testes DNA and 50% formamide). Following prehybridization, the membranes were hybridized for 36 hours at 42°C in prehybridization buffer containing 10% dextran sulfate and [^{32}P] cDNA (1,000,000 cpm/ml). After hybridization, membranes were washed three times with 1 x SSPE and 0.1% SDS for 15 min at room temperature and twice with 0.1 x SSPE and 0.1% SDS for 15 min at 50 °C. Membranes were exposed to Kodak X-Omat or Fuji RX film with intensifying screens for 6-12 hours at -80 °C. Quantitation of exposures on autoradiographic films was performed using

a Bio-Rad model 1650 scanning densitometer and a Hoefer GS-350H scanning program.

Preparation of genomic DNA

Genomic DNA was prepared from monolayers grown in 10 cm tissue culture dishes. Cells were rinsed once with PBS, and 10 ml of DNA lysis buffer (10mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% SDS, 50μg/ml proteinase K) were added and the petri dish was incubated at 37°C overnight. NaCl was added to the lysate to 250 mM. The solution was then extracted twice with phenol/chloroform (1:1, v/v). The DNA pellet was dissolved in TE buffer (1 μg/μl; Maniatis et al., 1982). DNA was digested with EcoR1 and transferred from 0.8% agarose gels to Nytran (S & S) membranes in 0.4 M NaOH. Membranes were immobilized at 70°C for 45 min and then prehybridized at 37°C for 24 hours in prehybridization buffer. Denatured α-^{32}PdCTP-labeled cDNA probes were used for hybridization. After hybridization, membranes were washed three times with 1 x SSPE and 0.1% SDS for 15 min at room temperature and twice with 0.1 x SSPE and 0.1% SDS for 15 min at 50 °C. Membranes were exposed to Kodak X-Omat film with intensifying screens for 6-12 hours at -80°C.

RESULTS

Part I

Goals of the first study were to evaluate utility of antisense oligonucleotide treatment as a means of down-regulating calpain levels in cultured muscle cells.

Stability and uptake of oligoDNAs

Uptake of the labeled 15-mer by myotubes increased linearly over time (Figure 2-1). The lower uptake noted in Experiment 2 may be due to the washing step. Myotubes in Experiment 1 were washed three times whereas myotubes in Experiment 2 were washed five times. The 15-mer in the extracellular compartment was degraded (Figure 2-2; Panel A). A progressive yield of shorter oligmers was detected throughout 24 hr of exposure of the labelled oligomer to culture conditions. A predominant form of radioactivity intracellularly at 24 hr was a high MW RNA:oligomer complex (Figure 2-2; Panel B) which was reported previously (Thinakaran and Bag, 1991). After 24 hr, intact 15-mer was the predominant intracelluar form of free oligoDNA. The oligoDNA:RNA complex is clearly visible at the top of the 24 hr lane. Intermediate-sized fragments, which were detected in the extracellular compartment, were not detected intracellularly. m-Calpain oligomer totally disappeared after 1 hour incubation with 100% FCS; however, little effect was seen in 1% FCS culture medium (Figure 2-2; Panel C).

Regulation of m-calpain concentration by using antisense oligoDNA

Effects of antisense and control oligoDNAs on m-calpain concentration are shown in Figure 2-3. To maintain intact antisense oligomer concentrations in culture media, cultures were changed every 8 hr and fresh oligoDNA was added. The antisense sequence caused slight reduction in m-calpain concentration following exposure of myotubes for 36 and 72 hr. It has been reported that high concentrations of oligoDNA caused cytotoxicity (Zon, 1990). In this study, the control oligoDNA (50μM) did not affect m-calpain concentration or cell viability.

Part II

Goals of the second group of studies were to evaluate the utility of the antisense RNA approach as a means of down-regulating m-calpain levels in muscle cells.

Construction of plasmids

pMAMA (pMAMneo containing antisense orientation of m-calpain cDNA; Figure 2-4) and pMAMS (pMAMneo containing sense orientation of m-calpain cDNA; Figure 2-5) were constructed and identified by Bgl II restriction enzyme digestion (Figure 2-6). An asymmetric Bgl II site in the m-calpain insert allowed us to distinguish between sense and antisense clones. Following Bgl II digestion, pMAMA yielded two fragments (7.54 kbp and 1.66 kbp) and pMAMS yielded 7.09 kbp and 2.11 kbp fragments (Figure 2-6).

Analysis of genomic DNA in transfected myoblasts

Figure 2-7 shows a Southern blot of genomic DNA from antisense- and sense-transfected cells digested with EcoR1 and probed with a ^{32}P -labeled neomycin-resistance gene fragment probe. As expected, a 5.8 kb band was detected in pMAMneo transfected cells, a 6.7 kb band was detected in pMAMS- and pMAMA- transfected cells and not detected in the control cells. These results suggest that the plasmid DNA integrated into host genomic DNA. The multiple signals in the same sample may be due to multiple copy number and/or integration of the neo gene at different sites in the host cell genome.

Expression of antisense RNA in transfected myotubes

Figure 2-8 shows a Northern blot of total RNA from L8, L8P, L8S and L8A cells. All four clones expressed intact m-calpain mRNA (left, 7-day differentiated cells; right, 10-day differentiated cells); however, only the clones with the m-calpain cDNA insert expressed the 900 bp m-calpain mRNA. This result proved that the sense and antisense m-calpain mRNA were expressed in transfected cells. Surprisingly, the expression of m-calpain cDNA was not affected by Dex. The higher level of expression of antisense mRNA in L8A (Figure 2-8) may be due to the multiple integration of plasmids. Despite the expression of m-calpain antisense RNA, the native m-calpain mRNA concentrations were not changed in 10-day differentiated L8A myotubes (Figure 2-8).

Regulation of m-calpain concentration in transfected myotubes

Effects of Dex (0, 1 ,5, and 10 μM) on m-calpain concentrations in L8P, L8A and L8S cells were examined (Figure 2-9) using Western blot analysis. Despite the fact that Dex-dependent antisense RNA expression was not sensitive to Dex, Dex increased m-calpain concentrations in L8S and L8P cells. However, Dex only slightly increased m-calpain levels in L8A myotubes (Figure 2-10).

DISCUSSION

Roles of calpains in muscle protein degradation

Calpains are believed to regulate myofibrillar protein degradation. Hence, control of their activities may be key to control of muscle growth. To assess the true role of calpains, an obvious approach is to down-regulate calpains and examine the phenotype. In this study, we examined the utility of the antisense approach as a means of down-regulating calpain levels in muscle cells.

Antisense OligoDNA

The use of antisense oligonucleotides for control of genetic expression has received an increasing amount of attention in the past decade. The antisense concept suggests that inhibition of gene expression may result from the Watson-Crick base pair binding (complementary hybridization) of oligonucleotides to single-stranded RNA or DNA such that the normal, essential functions of the nucleic acid (gene expression) are disrupted. Structurally unmodified oligonucleotides, which are now readily obtained by the use of automated DNA synthesizers, have been more or less successfully employed for inhibition of specific protein synthesis in vitro, both acellularly and with whole-cell cultures (Zon, 1990). Although it has been reported that unmodified oligoDNA was degraded more easily than modified oligoDNA such as phosphorothioates and methylphosphonates, the advantage of using unmodified oligoDNA was confirmed by the high rate of its uptake by cells (Boiziau and Toulme, 1991; Reed et al., 1990). Oligonucleotides are readily degraded by 5'

and 3' exonucleases and also by endonucleases (Holt et al., 1988). In our study, the unmodified intact oligoDNA can be detected in culture medium with 1% FCS for at least 12 hr (Figure 2-2). The increase in shorter oligoDNA in the medium presumably was due to the degradation of oligoDNA by serum nucleases. The stability of oligomers has been found to vary between cell lines. In HL60 cells, oligomers have been found to be stable for up to 3 days (Holt et al., 1988), whereas in PC12 cells, oligomers were stable for only 6-12 hr (Teichman et al., 1988). We have detected significant levels of undegraded labeled oligomer after incubation for 6 hr. Uptake of the labeled 15-mer by myotubes increased over time (Figure 2-1). Rates of uptake and percentage uptake agree well with published reports for myoblasts and myotubes (Florini and Ewton, 1990; Thinakaran and Bag, 1991). The mechanism of oligonucleotide uptake by cells is still not clear. Loke et al (1989) reported that oligoDNA is taken up in a saturable, size-dependent manner compatible with receptor-mediated endocytosis. Using oligo (dT)-cellulose for affinity purification, Loke et al. (1989) identified an 80 kDa membrane protein that may mediate oligoDNA transport. Similar results have been reported by Vlassov and co-workers (Yakubov et al., 1989).

It has been found that the best site for antisense inhibition is preferably at the 5' translation initiation region (Liebhaber et al., 1991), and that truncated polypeptides are produced at the sites within the coding region (Haeuptle et al., 1986). The m-calpain antisense oligoDNA which was used in this study was

within the coding region. We chose this region because the sequence of the 5' region had not been published. To maintain levels of oligoDNA, fresh oligoDNA was added periodically to culture media. The down-regulation of m-calpain protein concentration was detected after 36 hr incubation. Increasing the incubation period to 72 hr with additions of fresh culture medium containing 50 μM antisense oligoDNA every 8 hr failed to produce any further reduction of m-calpain protein concentrations. Similar results have been reported for troponin C (Thinakaran and Bag, 1991). The reason why m-calpain protein concentrations were maintained in antisense oligoDNA-treated cells is still not clear. Thinakaran and Bag (1991) reported that the transcription rate of the target gene was increased in antisense oligoDNA-treated myotubes. It is possible that cells can either increase m-calpain mRNA transcription, increase the stability of m-calpain mRNA or increase the m-calpain translation rate to maintain appropriate protein concentrations.

The limitations of using antisense oligoDNA to regulate m-calpain activity are (1) antisense oligoDNA did not down-regulate m-calpain concentrations in large magnitute, (2) the down-regulation of m-calpain is only a temporal effect and (3) synthesis of oligoDNA is expensive. To obviate the limitations of using antisense oligoDNA, antisense RNA was tried as an alternative to down-regulate m-calpain in muscle cells.

Antisense RNA

The antisense RNA approach was chosen because, in the past ten years, there have been increasing numbers of reports that expression of specific genes can be inhibited or down-regulated by the presence of antisense RNA. Compared to the use of antisense oligoDNA, the advantages of using antisense RNA are (1) serum nucleases are no longer a major concern, and (2) it could be controlled by specific promoters to turn on and turn off antisense expression at appropriate times. pMAMneo is a mammalian expression vector. It has been successfully used to express eukaryotic gene in transfected cells (Murakami et al., 1992). This plasmid contains an MMTV-inducible promoter for control of gene expression (Lee et al., 1981) and a neomycin resistance gene for selection of mammalian cell transfection. We subcloned rat m-calpain cDNA into the multiple cloning sites of pMAMneo which placed its expression under control of the MMTV promoter. The MMTV promoter is a Dex inducible promoter (Lee et al., 1981). The sense or antisense orientations of the cDNA insert were confirmed by Bgl II restriction enzyme digestion mapping. To determine whether transfected cells were G418 resistant, genomic DNA and neo resistance gene expression were analyzed by using Southern blot and Northern blot analysis. Genomic DNA analysis showed that the integration of plasmid DNA could be detected in transfected L8P, L8S and L8A cells. Possibily due to multiple copies and different sites of integration, multiple bands could be seen in the Southern blot analysis. Neomycin resistance gene expression

was confirmed using Northern blot analysis probed with neo cDNA. The expression of neo mRNA can be detected in all transfected cells except the control cells.

Native m-calpain mRNA was detected by Northern blot analysis of L8, L8S, L8P and L8A cells and antisense m-calpain RNA (0.9 kb) was detected in L8A and L8S cell lines (Figure 2-8). However, Dex (5 μM) did not increase m-calpain cDNA expression.

It has been reported that the MMTV promoter only needs 1 μM concentration to induce transcription (Lee et al., 1981). Possible explanations for the inability of Dex to alter calpain mRNA concentration are that either L8 cells do not have enough glucocorticoid receptor, the RSV promoter was too strong, (i.e., the expression of m-calpain cDNA was driven by pMAMneo's RSV promoter directly) or serum contained high levels of glucocorticoids. It has been reported that Dex increases protein degradation in L8 myotubes (Hong and Forsberg, 1994). Therefore, it is unlikely that L8 myotubes would lack the Dex receptor. Calf serum contains glucocorticoids such as cortisol and corticosterone. In this study, according to our calculations, the concentration of cortisol was 1.9 nM and corticosterone was 1 nM. These concentrations should not be enough to induce the MMTV promoter. To ensure low concentrations of glucocorticoids in serum, charcoal-adsorbed serum was used. m-Calpain cDNA expression was still detected in DMEM supplemented with 2% charcoal-adsorbed serum.

Although we detected expression of m-calpain antisense RNA in L8A cells, native m-calpain mRNA concentrations did not change in 10-day differentiated L8A myotubes (Figure 2-8; i.e., the antisense RNA did not reduce native m-calpain mRNA). It is believed that antisense RNA may produce its effects in a variety of ways. The m-calpain antisense RNA presumably acts by base pairing with the coding region of endogenous sense mRNA; then the RNA:RNA duplex may be rapidly degraded by nucleases (Steel and Harris, 1989). However, in this study, we did not see a change in the m-calpain mRNA concentrations. It is unclear why m-calpain mRNA levels were not reduced despite expression of antisense m-calpain RNA. Perhaps secondary structures of the antisense RNA and native mRNA prevented interaction and down-regulation. Alternatively, muscle cells may contain mechanisms to compensate for the antisense-dependent down-regulation of m-calpain mRNA. For example, in the presence of antisense RNA, calpain gene transcription or calpain mRNA stability may be enhanced. These possibilities are not unexpected as in several other studies we have determined that in the presence of many physiological or endocrine challenges that extensive regulation exists to maintain calpain levels appropriate to the need of the cells.

In additional studies, we found that Dex increased m-calpain concentrations in L8P and L8S cells; however, Dex only slightly increased m-calpain concentrations in L8A cells. This implies that Dex is a regulator of m-calpain concentration in normal muscle cells and that some aspect of the

antisense tranfection prevented Dex from exerting this action. It is possible that the hybridization of the antisense message in L8A cells with native m-calpain mRNA prevented the ability of Dex to increase m-calpain concentration. It has been reported that Dex can increase protein degradation in L8 myotubes (Hong and Forsberg, 1994). In this study, we inadvertently demonstrated that the increase in protein degradation in L8 myotubes may be due to increased calpain concentrations.

Conclusion

The upstream region of the human m-calpain large subunit lacks typical promoter elements such as TATA and CAAT boxes; it is characterized by its high GC content (-300 to -20) and contains multiple transcription initiation sites which cluster between -142 and -103 (Hata et al., 1989). Their structural features are common to many genes encoding for housekeeping proteins. These features imply that calpains are involved in essential cellular functions. In the past several years, several studies have been done in our lab showing that the regulation of m-calpain activity is not at the transcription level. For instance, in a fasting study, m-calpain mRNA increased significantly in fasting animals; however, calpain concentrations were not affected by fasting (Ilian and Forsberg, 1991). In an aging study, m-calpain activity decreased with aging, and the mRNA level did not change as much as the m-calpain activity (Ou and Forsberg, 1991). These results imply that m-calpain activity is regulated at post-transcription level such as autolysis or translocation. Suzuki et al. (1988)

proposed a membrane activation model for the calpains. In this model, an increase in cytosolic Ca^{++} concentration induces a conformational change in calpain which causes their association via Domain V with membrane lipids. Association with lipids reduces Ca^{++} required for autolysis of N-termini of the 80 KDa and 30 KDa subunits. Autolysis reduces the Ca^{++} concentration required for calpain activity to "near-physiological" levels. According to this model, the regulation of m-calpain activity occurs primarily at the post-translation level.

Objectives of this study were to develop a means of down-regulating m-calpain levels in muscle cells. The rationale for this was that we believe calpain function could be elucidated in m-calpain-deficient myotubes. To accomplish this, two approaches were used: antisense DNA and antisense RNA. With antisense DNA, we achieved small to moderate reduction in m-calpain concentrations; however, it was judged that this reduction was not sufficient to elucidate calpain function. Hence, we tried an alternative approach (antisense RNA). Expression of antisense calpain RNA did not effectively down-regulate calpain concentrations; however, through this approach several interesting facts of the biology of calpains and of the antisense approach emerged. First, we found that Dex increased m-calpain concentrations in muscle cells. In earlier studies we reported that Dex increased protein degradation in cultured L8 myotubes (Hong, 1993). Hence, it is possible that the Dex-dependent increased in muscle protein degradation is mediated by an increase m-calpain

concentrations. Second, the calpain system appears to be able to counter any attempt to affect it. This may underscore of calpains to cells and that several points of regulation exert. Specifically, there seem to be a limit to which antisense DNA could reduce m-calpain concentrations. Increasing duration of exposure of cells to antisense oligoDNA or increasing antisense oligoDNA concentrations were ineffective to further reducing m-calpain levels. In the antisense RNA studies, m-calpain concentrations remain unchanged despite expression of the antisense RNA. Finally, this studies may have identified a means by which antisense RNA exerts action. In our study the antisense message was detected in L8A cells; however, native m-calpain mRNA was unaffected. Yet, Dex reduced the ability of itself to increase m-calpain in L8A cells without changing m-calpain mRNA, this implies that the antisense RNA interfered with translation of the native message. Perhaps the native m-calpain:antisense complex is translated at lower efficiency than intact m-calpain mRNA. Hence, we proposed that antisense RNA, in addition to inducing the RNaseH-dependent degradation of nucleotide may also interfere with translation of their target message.

REFERENCES

Allen, R. E. 1986. Muscle cell growth and development. Designing Foods. Animal Product Options in the Marketplace. National Research Council, National Acad. Press. Wash. D. C. pp 142-162.

Boiziau, C. and J. J. Toulme. 1991. Modified oligonucleotides in rabbit reticulocytes: uptake, stability and antisense properties. Biochimie 73:1403-1408.

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156-159.

Cohen, J. S. 1991. Oilgonucleotides as therapeutic agents. Pharmac. Ther. 52:211-225.

Cook, P. D. 1991. Medicinal chemistry of antisense oligonucleotides - future opportunities. Anti-cancer Drug Design 6:585-607.

Deluca, C. L., P. L. Davies, J. A. Samis, J. S. Elce. 1993. Molecular cloning and bacterial expression of cDNA for rat calpain II 80 kDa subnunit. Biochim. Biophys. Acta. 1216:81-93.

Florini, J. R. and D. Z. Ewton. 1990. Highly specific inhibition of IGF-1-stimulated differentiation by an antisense oligonucleotide to myogenin mRNA. J. Biol. Chem. 265:13435-13437.

Goll, D. E., V. F. Thompson, R. G. Taylor, J. A. Christiansen. 1992. Role of the calpain system in muscle growth. Biochimie 74:225-237.

Haeuptle, M., R. Frank and B. Dobberstein. 1986. Translation arrest by oligonucleotides complementary to mRNA coding sequences yields polypeptides of predetermined length. Nucl. Acids Res. 14:1427-1448.

Hata, A., S. Ohno, Y. Akita and K. Suzuki. 1989. Tandemly-reiterated negative enhancer-like elements regulate transcription of a human gene for the large subunit of calcium-dependent protease. J. Biol. Chem. 264:6404-6411.

Hong, D. H. and N. E. Forsberg. 1994. Effects of serum and insulin-like growth factor-1 on protein degradation and protease gene expression in rat L8 myotubes. J. Anim. Sci. in press.

Holt, J. F., R. L. Redner and A. W. Nienhuis. 1988. An oligomer complementary to c-myc mRNA inhibits proliferation of HL60 promyelocytic cells and induces differentiation. Mol. Cell. Biol. 8:963-973.

Ilian, M. A. and N. E. Forsberg. 1992. Gene expression of calpains and their specific endogenous inhibitor, calpastatin, in skeletal muscle of fed and fasted rabbits. Biochem. J. 287:163-171

Laemmli U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lee, F., R. Mulligan, P. Berg and G. Ringold. 1981. Glucocorticoids regulate expression of dihydrofolate reductase cDNA in mouse mammary tumour virus chimaeric plasmids. Nature 294:228-234.

Liebhaber, S. A., J. E. Russell, F. Cash and S. S. Eshelman. 1991. Intramolecular duplexes in eukaryotic mRNA supress translation in a position-dependent manner. J. Cell. Biochem. 15D:CD 007.

Loke, S. L., C. A. Stein, X. H. Zhang, M. Nakanishi, C. Subasinghe, J. S. Cohen, L. M. Neckers. 1989. Characterization of oligonucleotide transport into living cells. Proc. Natl. Acad. Sci. USA 86:3473-3478.

Maher, L. J. and B. J. Dolnick. 1987. Specific hybridization arrest of dihydrofolate reductase mRNA in vitro using anti-sense RNA or anti-sense oligonucleotides. Arch. Biochem. Biophys. 253:214.

Maniatis, T. E., F. Frisch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor.

Murakami, Y., S. Matsufuji, Y. Miyazaki and S. Hayashi. 1992. Destabilization of ornithine decarboxylase by transfected antizyme gene expression in hepatoma tissue culture cells. J. Biol. Chem. 267:13138-14141.

Ou, B. R. and N. E. Forsberg. 1991. Determination of skeletal muscle calpain and calpastatin activities during maturation. Am. J. Physiol. 261:E677-E683.

Ou, B. R., H. H. Meyer and N. E. Forsberg. 1991. Effects of age and castration on activities of calpains and calpastatin in sheep skeletal muscle. J. Anim. Sci. 69:1919-1924.

Reed, J. C., C. A. Stein, C. Subasinghe, S. Haldar, C. M. Croce, S. Yum and J. S. Cohen. 1990. Antisense-mediated inhibition of BCL2 proto-oncogene expression and leukemic cell growth; comparisons of phosphorothioate oligonucleotides. Cancer Res. 50:6565-6570.

Sartorius, C. and R. M. Franklin. 1991. The use of antisense oligonucleotides as chemotherapeutic agents for parasites. Paras. Today 7:90-93.

Smidt, P., T. L. Doan, S. Falco and T. Berkel. 1991. Association of antisense oligonucleotides with lipoproteins prolong the plasma half-life and modifies the tissue distribution. Nucl. Acids Res. 19:4695-4700.

Sorimachi, H., S. I. Ohmi, Y. Emori, H. Kawasaki, S. Ohno, Y. Minami, K. Suzuki. 1989. Molecular cloning of a novel mammalian calcium-dependent protease distinct from both m- and μ-type. J. Biol.Chem. 264:20106-20111.

Steel D. M. and H. Harris. 1989. The effect of antisense RNA to fibronectin on the malignancy of hybrids between melanoma cells and normal fibroblasts. J Cell Sci. 93:515-524.

Suzuki, K. 1987. Calcium-activated neutral protease: domain structure and activity regulation. Trends Biochem. Sci. 12:103-105.

Suzuki, K., S. Imajoh, Y. Kawasaki, Y. Minami, S. Ohno. 1988. Regulation of activity of calcium-activated neutral protease. Adv. Enzyme Reg. 27:153.

Teichman-Weinberg, A., U. Z. Littauer, I. Ginzburg. 1988. The inhibition of neurite outgrowth in PC12 cells by tubulin antisense oligodeoxyribonucleotides. Gene 72:297.

Thinakaran, G. and J. Bag. 1991. Alterations in the expression of muscle-specific genes mediated by troponin C antisense oligonucleotide. Exp. Cell Res. 192:227-235.

Towbin, H., T. Staehelin and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamine gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350-4354.

Wickstorm, E. L., T. A. Bacon, A. Gonzalez, D. L. Freeman, G. H. Lyman. 1988. Human promyelocytic leukemia HL-60 cell proliferation and c-myc protein expression are inhibited by an antisense pentadecadeoxynucleotide targeted against c-myc mRNA. Proc. Natl. Acad. Sci. USA 85:1028.

Yakubov, L. A., E. A. Deeva, V. F. Zarytova, A. S. Ryte, L. V. Yurchenko and V. V. Vlassov. 1989. Mechanism of oligonucleotide uptake by cells: involvement of specific receptor? Proc. Natl. Acad. Sci. USA 86:6454-6458.

Zon, G. 1990. Innovations in the use of antisense oligonucleotides. Aids: Anti-HIV Agents, Therapies, and Vaccines 616:161-172.

Figure 2-1. Uptake of m-calpain antisense oligomer. L8 myotubes were incubated with 50μM antisense m-calpain oligomer in DMEM with 1% FCS. At different times, cells were harvested and oligomer uptake was determined. Detailed methods are described in Materials and Methods.

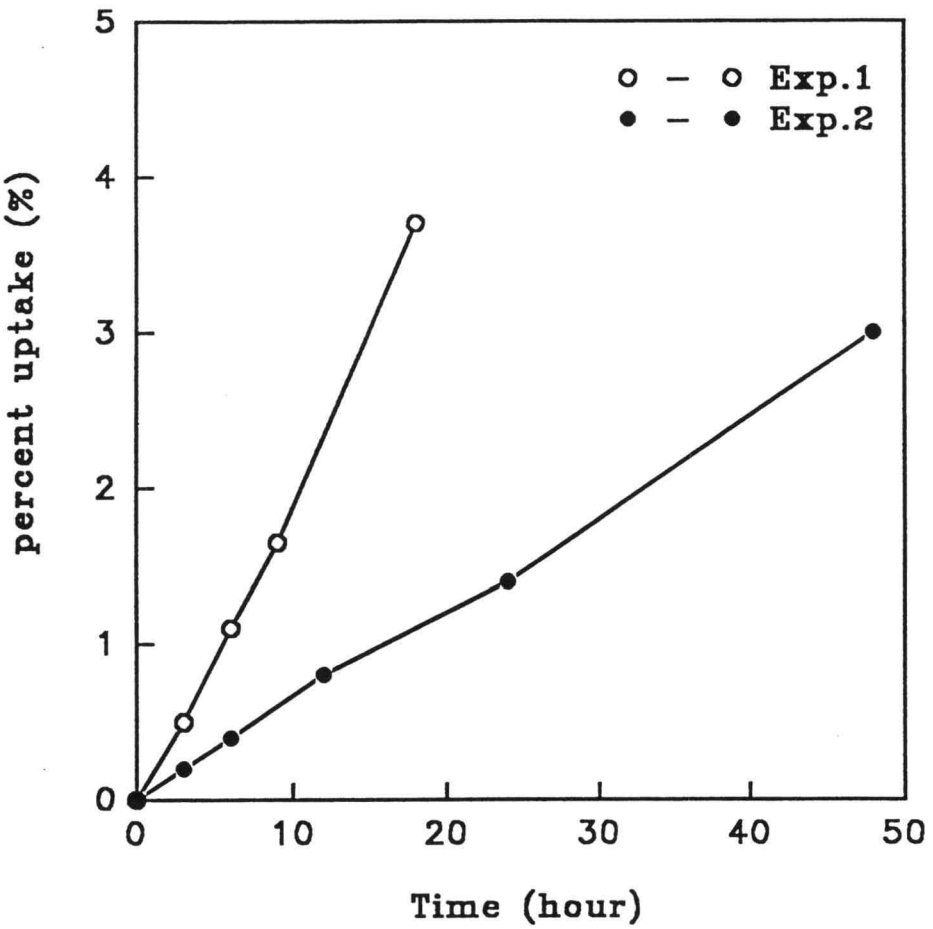


Figure 2-2. Stability of m-calpain antisense oligomer. Labeled oligomers were incubated in culture medium (Panel A), in the intracelluar compartment (Panel B) and in the presence of 100% FCS and 1% FCS (Lanes "a" and "b" in Panel C, respectively). In Panel A, the lanes represent a control (C), which corresponded to the quantity of oligomer added to medium, zero-time control (0) and to the medium samples following 3, 6, 12 and 24 hr of culture. In Panel B lanes correspond to aqueous extracts (intracelluar compartment) of myotubes exposed to oligomer for 3, 6, 12 and 24 hr.

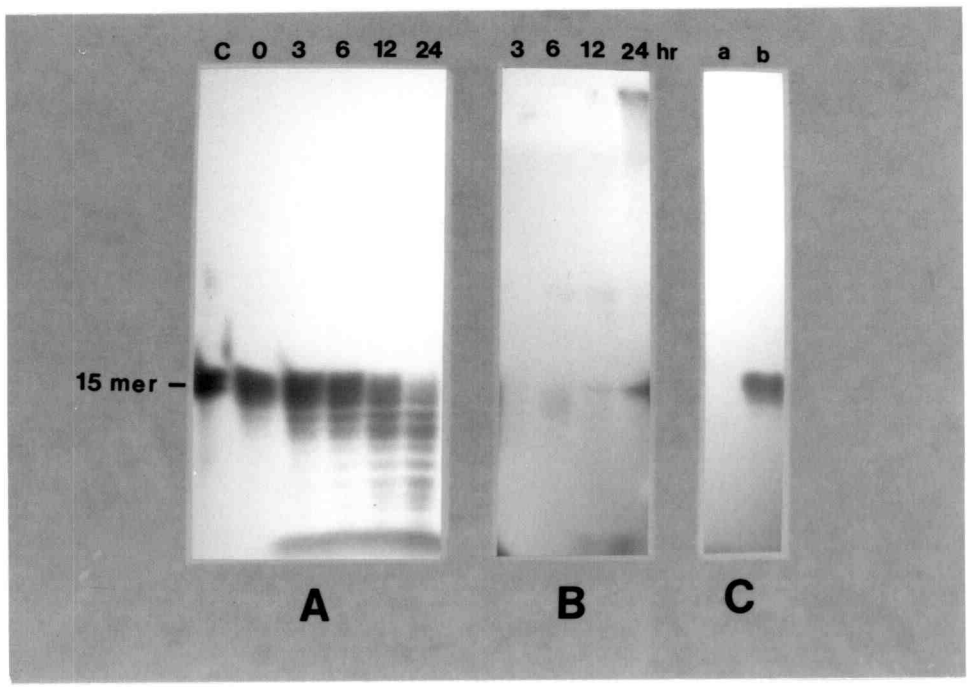


Figure 2-3. Effects of oligoDNA in L8 cells on m-calpain concentrations. L8 myotubes were incubated without oligoDNA (C) and with m-calpain antisense (A) oligoDNA (50μM), and control (S) oligoDNA (50μM) for 36 and 72 hr. During the incubation time, culture medium was changed every 8 hr and fresh oligoDNA was added. Cells were harvested and Western blot analysis was performed to determine m-calpain protein concentrations.

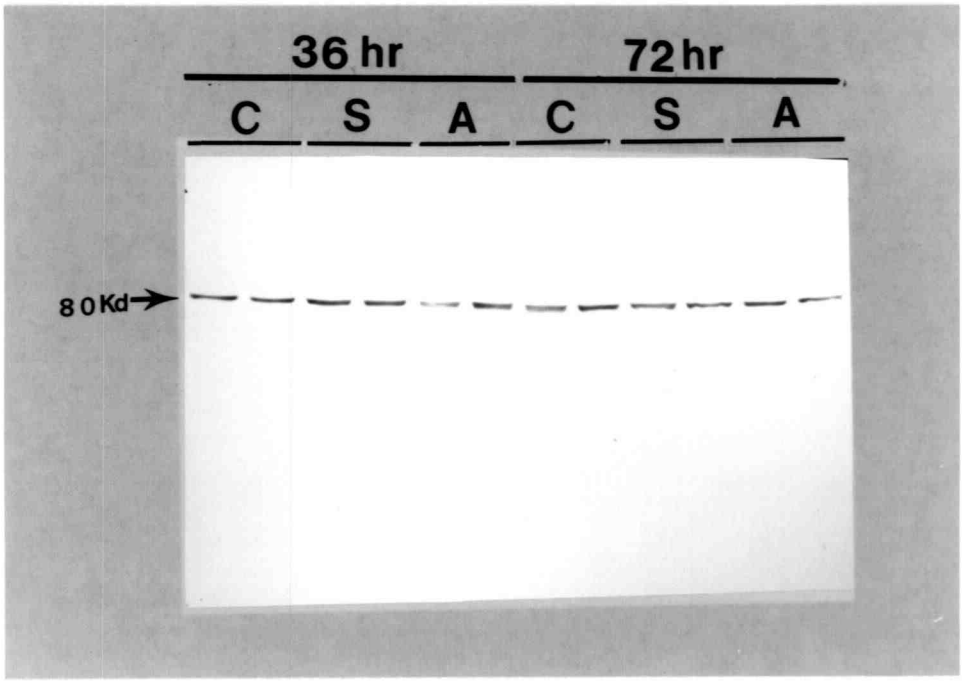


Figure 2-4. Plasmid map of pMAMA. Antisense orientation m-calpain cDNA (CAL II) was inserted into the Xho I site of pMAMneo eukaryotic cell expression vector. Arrow indicates the orientation of m-calpain cDNA.

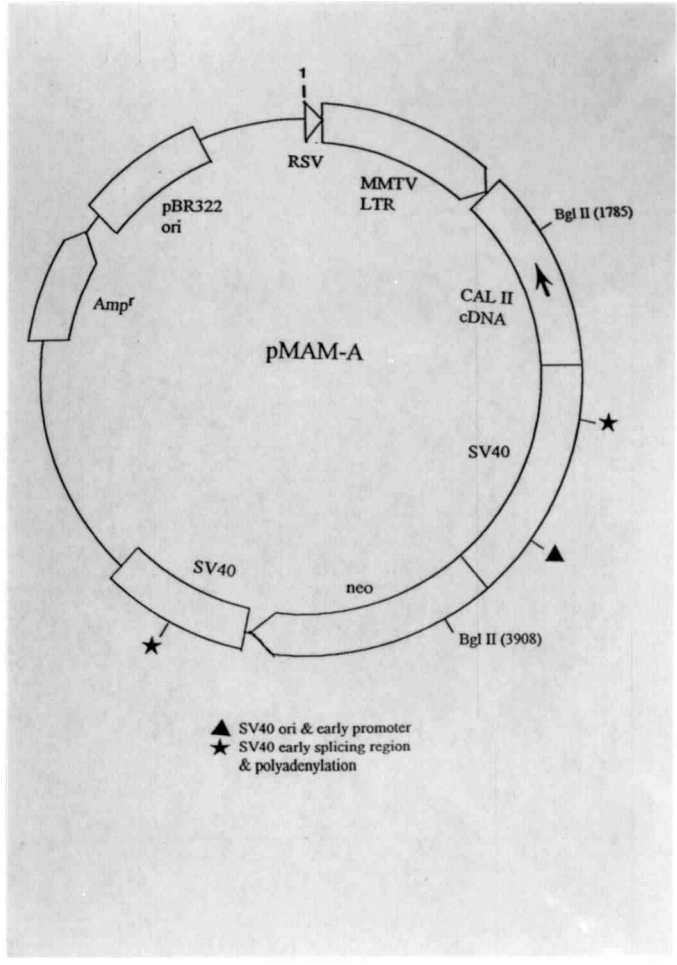


Figure 2-5. Plasmid map of pMAMS. Sense orientation m-calpain cDNA (CAL II) was inserted into the Xho I site of pMAMneo eukaryotic cell expression vector. Arrow indicates the orientation of m-calpain cDNA .

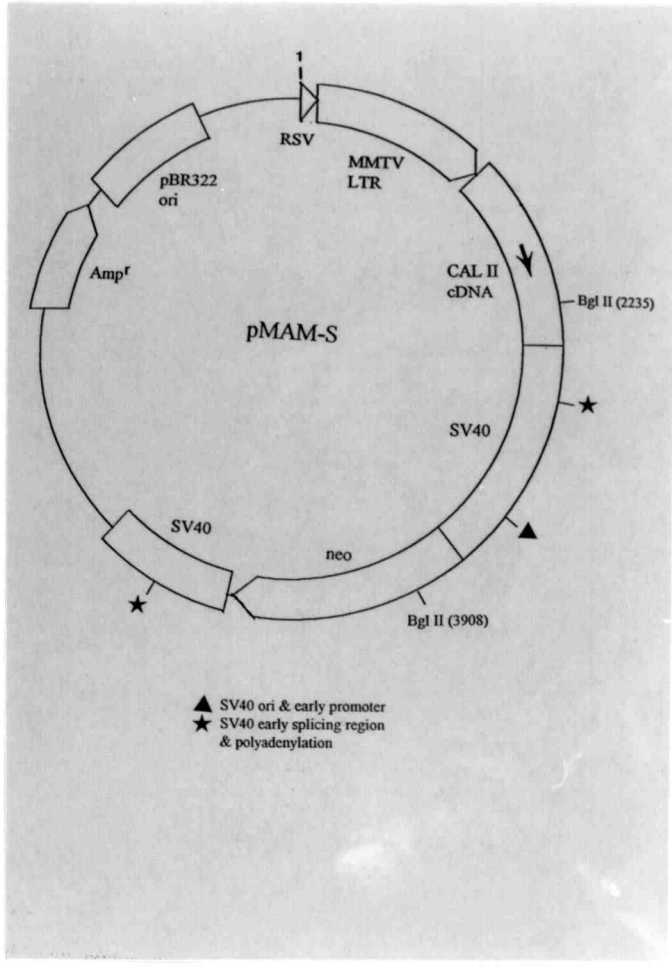


Figure 2-6. Restriction enzyme digestion of pMAMneo, pMAMS and pMAMA. pMAMneo (lane B), pMAMS (lane C) and pMAMA (lane D) were digested with Bgl II. An asymmetric Bgl II site in the m-calpain insert allowed us to distinguish between sense and antisense clones. Lanes A and E contain molecular weight markers.

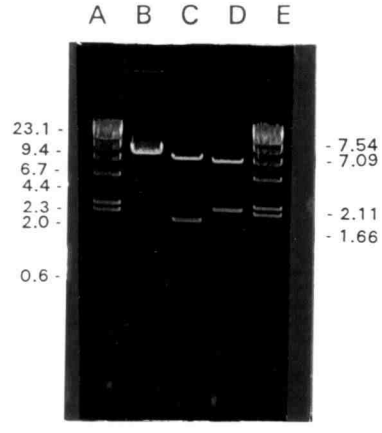


Figure 2-7. Southern blot analysis of transfected and control cells. Genomic DNA samples were isolated from L8 cells, L8 cells transfected with pMAMneo (L8P), L8 cells transfected with pMAMS (L8S) and L8 cells transfected with pMAMA (L8A) and digested with EcoR1, electrophoresed through a 0.8% agarose gel and blotted onto a Nytran^{+} membrane. The blot was probed with an α-^{32}P-labeled 900 bp neo fragment derived from pMAMneo.

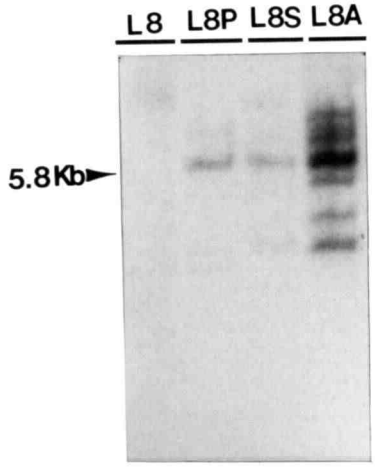


Figure 2-8. Northern blot analysis of transfected and control cells. Total RNA was isolated from 7-day (left) and 10-day (right) differentiated L8 cells, L8 cells transfected with pMAMneo (L8P), L8 cells transfected with pMAMS (L8S) and L8 transfected with pMAMA (L8A) and electrophoresed through a 1.0% formaldehyde denaturing gel (25μg per lane). RNA was transferred onto a Nytran^{+} membrane. The blot was probed with an α-^{32}P-labeled 900bp m-calpain cDNA fragment. The bottom panel shows the ethidium bromide stain. Arrows indicate positions of native and transfected calpain RNA.

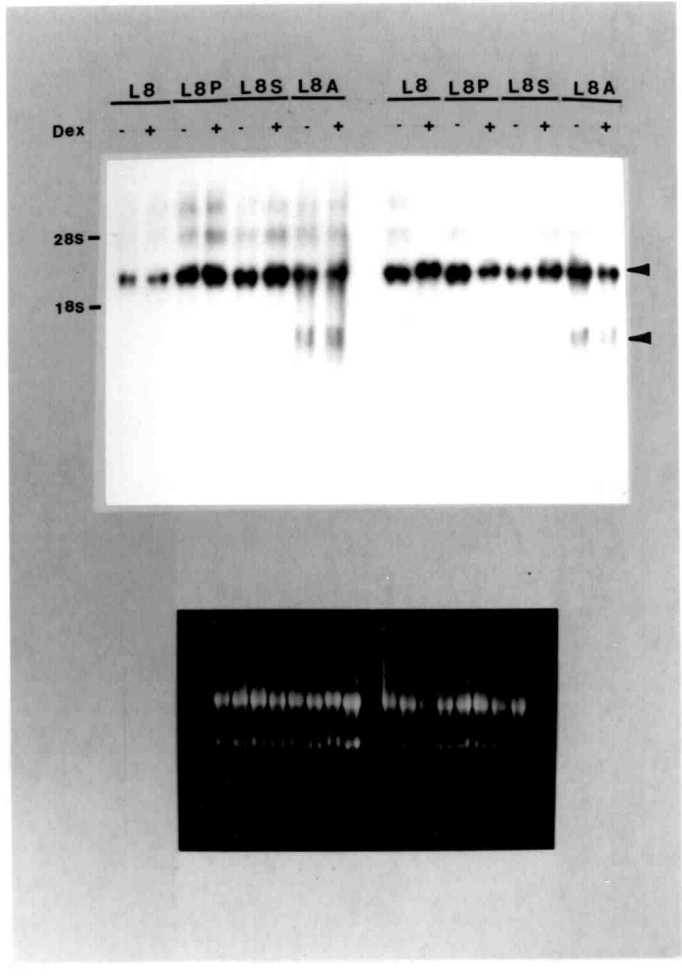


Figure 2-9. Western blot analysis of transfected cells. Transfected L8 myotubes (L8P, L8A and L8S) were treated with various concentrations of Dex (0, 1, 5 and 10 μM) for 48 hr; then the cells were collected in homogenate buffer and subjected to 10% SDS-PAGE. After protein was transferred onto a nitrocellulose membrane, the blot was hybridized with an m-calpain-specific polyclonal Ab. The ECL system was used to detect the m-calpain-specific signal.

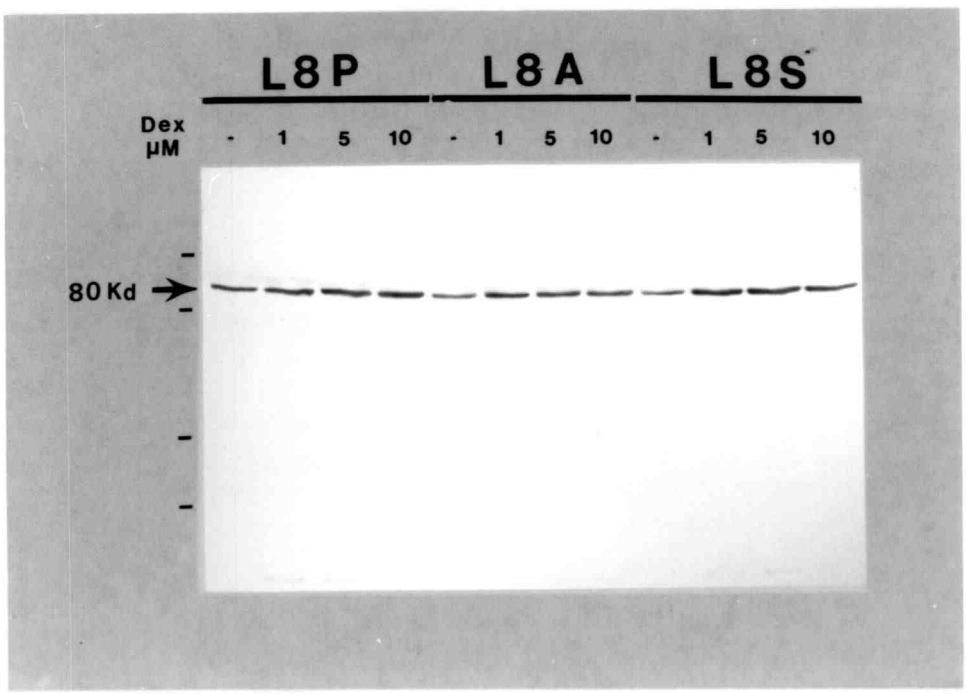
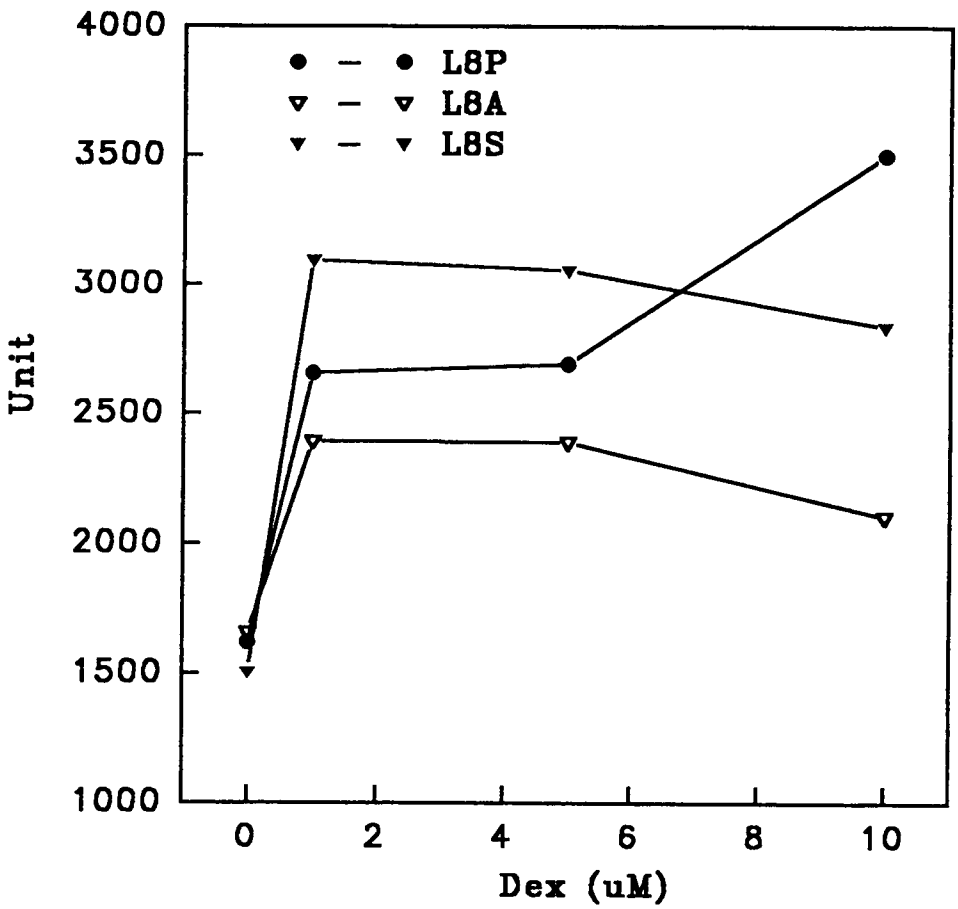


Figure 2-10. Scanning densitometry of Western blot analysis shown in Figure 2-9.



CHAPTER III

EFFECTS OF CALPAIN INHIBITOR II, CHLOROQUINE AND IGF-1 ON MUSCLE PROTEIN DEGRADATION

ABSTRACT

Objectives were to investigate the role of calpains and lysosomal proteinases in myofibrillar and non-myofibrillar protein degradation and to elucidate the mechanism by which IGF-1 reduces muscle protein degradation. L8 myotube proteins were labeled with ^{3}H-tyrosine and then incubated with calpain inhibitor II (CI-II, 30 μg/ml), chloroquine (CQ, 25 μM) or IGF-1 (200 ng/ml) for 24 hours. Protein degradation was determined by release of free ^{3}H-tyrosine into incubation media. Total protein degradation was reduced 40%, 15% and 21% by CI-II, CQ and IGF-1, respectively. Differential centrifugation was used to separate non-myofibrillar and myofibrillar protein-rich fractions. CI-II reduced myofibrillar protein degradation by 29%; however, CQ and IGF-1 did not alter degradation of this pool. In contrast, CQ and IGF-1, but not CI-II, tended to reduce degradation of the non-myofibrillar protein pool. Interestingly, CI-II increased myofibrillar protein synthesis but had no effect in non-myofibrillar protein synthesis. Concomitant with these effects, CI-II, but not CQ or IGF-1, caused accumulation of myofibrillar proteins. In addition, SDS-PAGE autoradiography showed that some individual myofibrillar proteins were stabilized by CI-II. CQ and IGF-1 did not affect the stability of individual

myofibrillar proteins. These results demonstrate that the decrease in total protein degradation caused by CI-II was primarily mediated by the reduction in myofibrillar protein breakdown and the reduction in total protein degradation caused by CQ and IGF-1 was mediated by a reduction in non-myofibrillar protein degradation. We conclude that calpains play a crucial role in myofibrillar protein degradation, possibly in the initiation step of myofibrillar protein degradation and that lysosomal enzymes are important to non-myofibrillar protein degradation. IGF-1 regulates degradation of the non-myofibrillar protein pool via a lysosomal mechanism. Finally, this study documents validity of the concept that protein accretion will occur if myofibrillar protein degradation is reduced and protein synthesis is enhanced.

INTRODUCTION

Skeletal muscle consists of two discrete pools of proteins: a rapidly turning-over non-myofibrillar component and a more stable myofibrillar component (Widnell and Pfenninger, 1989). The non-myofibrillar proteins consist of sarcoplasmic, membrane and organellar proteins while the myofibrillar components consist of the contractile, regulatory and structural proteins of the sarcomere. It has been reported that the degradation of myofibrillar versus non-myofibrillar proteins is regulated independently (Goodman et al., 1987; Kadowaki et al., 1989).

The mechanism by which the myofibrillar degradative process is coordinated in skeletal muscle has been controversial for several years. In fact, this is a complex process; not only is protein degradation involved, but degradation also requires the initial disassembly of a complex highly-organized structure. Lysosomes (Gerard et al., 1988), four calpain isoforms (μ-calpain, m-calpain, high m-calpain (Dayton et al., 1976; Wolfe et al., 1989) and p94 (Sorimachi et al., 1989)) and at least two ATP-dependent proteolytic systems (Dahlman et al., 1985; Fagan et al., 1987) have been identified in skeletal muscle. Despite evidence that an ATP-dependent proteolytic system (proteasome; Furuno et al., 1990) and lysosomal proteinases (Allen, 1986) play a role in myofibrillar protein degradation, other evidence suggests that calpains initiate the process (Goll et al., 1989; Goll et al., 1992).

Skeletal muscle cells can be cultured in vitro. Myoblasts can be induced to differentiate into multi-nucleated myotubes (Wakelam, 1985; Richler and Yaffe, 1970). Myotubes express muscle-specific proteins such as myosin, α-actin, tropomyosin and creatine kinase. Thus, cultured muscle cells provide a unique model for studying muscle protein degradation (Nadel-Ginard, 1978).

Several growth factors and hormones have long-established and well accepted effects on muscle protein degradation. Insulin and insulin-like growth factor 1 (IGF-1) are anabolic (Harper et al., 1987, Roeder et al., 1986; 1988) while dexamethasone (Yeh et al., 1994) and thyroid hormones (Forsberg and Wehr, 1990) are catabolic. It is still not clear that these hormones specifically regulate the myofibrillar protein pool, and if so, whether their effects are generalized or whether all myofibrillar proteins are affected. In this study, we developed methods for the determination of myofibrillar protein degradation and non-myofibrillar protein degradation. Using calpain inhibitor II, chloroquine and IGF-1, we investigated the possible role of calpains and lysosomal enzymes in myofibrillar protein and non-myofibrillar protein degradation and the target of IGF-1 in control of muscle protein degradation.

MATERIALS AND METHODS

Materials

Calf serum (CS) and horse serum (HS) were purchased from Hyclone (Logan, UT). Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin solution and trypsin were from GIBCO (Grand Island, NY). Human recombinant insulin-like growth factor-1 (IGF-1) was from Interogen (Purchase, NY). Trichloroacetic acid (TCA), glycine, chloroquine, sodium dodecyl sulfate (SDS), mouse anti-skeletal muscle α-actinin, α-actin and tropomyosin monoclonal antibodies and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO). The ECL Western blotting analysis system was from Amersham (Arlington Heights, IL). Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG and molecular weight standards were from Bio-Rad (Richmond, CA). Cell culture dishes and plates were from Corning (Corning, New York). Calpain inhibitor II and Quick-spin G-50 Sephadex column were from Boehringer Mannheim (Indianapolis, IN). The QIAEX agarose gel extraction kit was from QIAGEN Inc. (Chatsworth, CA). The random-primer DNA labeling kit was from USB (Cleveland, OH). Entensify solution and Solvable were from Dupont (Boston, MA). Nytran$^+$ membrane and nitrocellulose membrane were from Schleicher & Schuell (Keene, NH). α-32P-dCTP (6000 Ci/mmol) was from New England Nuclear (Boston, MA).

Cell culture

L8 rat myoblast cells were obtained from American Type Culture Collection (Rockville, MD). Cells were grown in DMEM with 10% CS, 100 units of penicillin/ml, 100 μg of streptomycin/ml and 44 mM sodium bicarbonate in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The medium was changed every two days. After cells reached confluence, the medium was replaced with DMEM containing 2% horse serum (HS) to induce differentiation. Microscopic examination was used to monitor cell differentiation.

Extraction of total RNA

Extraction of total RNA has been described previously (Chomczynski and Sacchi, 1987). Briefly, myotube cultures grown in 10-cm diameter dishes were washed three times with ice-cold phosphate buffered saline (PBS) and lysed directly on the dishes using 2 ml of Solution A (4 M guanidium isothionate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 10 mM ß-mercaptoethanol). The monolayer was scraped with a rubber policeman to ensure that all cells were released from the dishes. The lysate was transferred to a 15 ml polypropylene tube. To this, 0.2 ml of 2 M sodium acetate (pH 4.0), 2 ml of phenol and 0.4 ml of a chloroform-isoamyl alcohol mixture (49:1) were added sequentially. The lysate was mixed by inversion after each addition. After brief vortexing, the mixture was kept on ice for 15 min and was separated by centrifugation (12,000g) for 30 min at 4°C. The upper aqueous phase was transferred to a new tube and then an equal volume of ice-cold isopropanol was

added. The samples were mixed and stored at -20°C overnight. RNA was collected by centrifugation (12,000 g, 20 min at 4°C). The supernatant was discarded and the RNA pellet was resuspended in 0.4 ml of solution A. One volume of isopropanol was added, and the mixture was stored at -20°C overnight. The RNA pellet was recovered by centrifugation at 12,000Xg for 15 min at 4°C, washed twice with 70% ethanol and dried under a vacuum. The dried RNA pellet was then dissolved in diethylpyrocarbonate (DEPC)-treated water and was quantitated by spectrophotometry at a wavelength of 260 nm.

cDNA probes

Mouse myogenin cDNA was prepared from EMSV-Myo8 plasmid which was a gift from Dr. Eric N. Olson, TX. After EcoR1 digestion, the cDNA fragment (1000 bp) was separated by electrophoresis and was recovered using a QIAEX agarose gel extraction kit. cDNA fragments (25ng) were labeled with [α-^{32}P] dCTP (6000 Ci/mmol) using a random-primer labeling kit (USB). A labeled cDNA probe was purified using a Quick Spin G-50 Sephadex column (Boehringer Mannheim).

Northern blot hybridization

RNA samples (25 μg) were denatured at 55°C for 15 min, applied to a 1.2% agarose gel containing 2.2 M formaldehyde, then electrophoresed at 30 V for 12 h. RNA was transferred to Nytran^{+} membranes (Schleicher & Schuell) and immobilized by baking at 70°C for 40 min. Membranes were prehybridized at 42°C overnight in prehybridization buffer (5 x SSPE, 0.2% SDS, 5 x

Denhardt's solution, 100 μg/ml sonicated salmon testes DNA and 50% formamide). Following prehybridization, the membranes were hybridized for 36 hours at 42°C in prehybridization buffer containing 10% dextran sulfate and [^{32}P] cDNA (1,000,000 cpm/ml). After hybridization, membranes were washed three times with 1 x SSPE and 0.1% SDS for 15 min at room temperature and twice with 0.1 x SSPE and 0.1% SDS for 15 min at 50 °C. Membranes were exposed to Kodak X-Omat film with intensifying screens for 6-12 hours at -80 °C.

Measurement of total protein degradation

Rates of total protein degradation were determined by measuring the release of free ^{3}H-tyrosine into the medium at various incubation times after labeling myotubular proteins with ^{3}H-tyrosine. After cells completed differentiation (4-5 days), cells were labeled with ^{3}H-tyrosine (5 μCi/ml) for 24-36 hours in DMEM containing 2% HS. After labeling, cells were washed once with DMEM and then placed in DMEM containing 2% HS and 2mM non-radioactive tyrosine for 24 hours. At the end of the experiment, cell culture medium was transferred to a microcentrifuge tube and trichloroacetic acid (TCA) was added to a final concentration of 10% (w/v). After incubation at 4°C for at least 1 hour, samples were centrifuged for 10 min at 14000g. The supernatants were collected to determine free ^{3}H-tyrosine radioactivity. Cell monolayers were washed with ice-cold PBS and solubilized with 0.5N NaOH containing 0.1% Triton X-100. A measured volume was neutralized with acetic

acid before determination of radioactivity in a Beckmann LS6000 SE scintillation counter.

Measurement of protein synthesis

Rates of protein synthesis were measured by monitoring the incorporation of ^{3}H-tyrosine into acid-insoluble material. After cells completed differentiation, the culture medium was replaced with experimental medium containing ^{3}H-tyrosine (1 μCi/ml). Myotubes were incubated with ^{3}H-tyrosine for 1 hr, the radiolabelling medium was removed and the monolayers were rinsed 3 times with ice-cold PBS containing 2 mM non-radioactive tyrosine. Cells were scraped from the dishes in 1 ml homogenization buffer (40 mM NaCl, 1 mM dithiothreitol, 0.1 mM EGTA, 0.1% Triton X-100 in 5 mM sodium phosphate, pH 6.8) and separation of myofibrillar and non-myofibrillar protein-rich fractions was performed (outlined below). After separation, proteins were precipitated with 10% TCA, chilled at least 1 hr at 4°C and centrifuged for 10 min at 14000xg. The pellets were washed once with 10% TCA and dissolved in 1.0 ml of 0.5N NaOH with 0.1% Triton X-100. A small portion was taken for protein analysis with bovine serum albumin (BSA) as a standard. A measured volume was neutralized with acetic acid before determination of radioactivity in a Beckmann LS6000 SE scintillation counter.

Separation of myofibrillar protein-rich and non-myofibrillar protein-rich fractions

Cells were washed 3 times with ice-cold PBS, pH7.4, then scraped from the dish with a rubber policeman and suspended in 0.5 ml low salt buffer (40

mM NaCl, 1 mM dithiothreitol, 0.1 mM EGTA, 0.1% Triton X-100 in 5 mM sodium phosphate, pH 6.8). Cells were then homogenized using a Dounce homogenizer with 15 strokes of a tight fitting A pestle. The homogenate was centrifuged at 1000Xg for 5 min. The supernatant was thereafter referred to as the non-myofibrillar protein-rich fraction. The pellet which contained the myofibrillar protein-rich fraction, was dissolved in 2.3% SDS, 5% 2-mercaptoethanol and 62.5 mM Tris buffer, pH 6.8, in 10% glycerol for SDS-PAGE analysis and protein determination.

Purification of myofibrillar proteins

L8 myotubes were washed twice with ice-cold PBS and then added to 1 ml homogenate buffer (40 mM NaCl, 1 mM dithiothreitol, 0.1 mM EGTA, 0.1% Triton X-100 in 5 mM sodium phosphate, pH 6.8). Cell monolayers were scraped with a rubber policeman and homogenized using a Dounce homogenizer. Cell homogenates were centrifuged at 1000xg for 10 min at 4°C. The pellets were washed with homogenization buffer an additional three times. After washing, pellets were resuspended in homogenization buffer with 1% Triton X-100. The pellets were washed three times and transferred to 100 mM KCl buffer. After washing twice in 100 mM KCl buffer, pellets were resuspended in 100 mM NaCl for protein analysis. For long term storage, the pellets were resuspended in 50% glycerol, 50 mM KCl, 20 mM K_2PO_4, 2mM EGTA and 1mM NaN_3 (pH 6.8). This method provided myofibrils free of membrane proteins.

SDS polyacrylamide gel electrophoresis

Myofibrillar protein-rich and non-myofibrillar protein-rich fractions were analyzed by electrophoresis on 7.5% to 15% polyacrylamide gradient gels according the method of Laemmli (1970). Gels were fixed in 10% acetic acid and amplified in ENTENSIFY solution (DuPont). They were then heat-dried under vacuum and exposed to Kodak X-Omat film for 3 weeks. Proteins were excised from gels and incubated in 0.5 ml Solvable (DuPont) at 55°C overnight and radioactivity was then counted in a liquid scintillation counter.

Western blot analysis

Protein samples were subjected to 7.5-15% gradient SDS-PAGE according to the method of Laemmli (1970) or to two-dimensional gel electrophoresis according to the method of O'Farrell (1975). The separated proteins were transferred onto nitrocellulose membranes (0.45 micron; Schleicher & Schuell) at 4°C overnight using a Bio-Rad transfer apparatus (30 volts) by the method of Towbin et al. (1979) in transfer buffer containing 20% methanol, 25 mM Tris-HCl and 192 mM glycine. After transfer, membrane non-specific sites were blocked by incubating 5% non-fat skim milk in TTBS (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.05% Tween-20) for 1 hour at room temperature. Membranes were hybridized with primary antibody for 2 hours at room temperature. After three washes (15 min each) with TTBS, secondary antibody (HRP-conjugated goat anti rabbit IgG, Bio-Rad, diluted 1:2000 in TTBS containing 1% non-fat skim milk) was added and incubated for 1 hour at room

temperature. Following this incubation, the membrane was washed four times with TTBS at room temperature. Specific binding of antibody on the membrane was detected by the ECL detection system (Amersham). Levels of protein were quantified by densitometric scanning of the Western blot analysis using a Model 1650 transmittance/reflectance scanning densitometer (Bio-Rad) and a Hoefer GS 350 data system (Hoefer Scientific Instruments, San Francisco, CA).

Two-dimensional gel electrophoresis

Purified myofibrillar proteins (100μg) were dissolved in a 50 μl solution containing 9.5 M urea, 20% Nonidet P-40, 2% Ampholine (1.6%, pH range 5 to 7: and 0.4%, pH range 3.5 to 10; Bio-Rad) and 5% ß-mercaptoethanol. The samples were applied to a two-dimensional gel electrophoresis system as reported by O'Farrell (1975). Isoelectric focusing in the first dimension was carried out for 1400Vh in 3% polyacrylamide, containing 0.18% N,N'-methylenebisacrylamide, 8 M urea, 2% carrier ampholytes (pH3-10), and 1.7% Nonidet P-40. The gels were cast in glass tubes. At the end of the isoelectric focusing step, the gels were extruded from the glass tubes and equilibrated in Tris buffer (62.5 mM) containing 10% glycerol, 2.3% SDS, 5% ß-ME and bromophenol blue, pH 6.8. The second dimension of electrophoresis was carried out on slab gels consisting of 7.5% to 15% polyacrylamide. Broad range molecular weight standards (Bio-Rad) were used to determine the protein molecular weights. Individual myofibrillar proteins were identified by electrophoresis with purified rat skeletal myofibrillar proteins using two-

dimensional gel immunoblotting analysis. Monoclonal antibodies against skeletal muscle α-actinin, α-actin and tropomyosin were obtained from Sigma.

Statistical analysis

Values are presented as means ± SE. Mean values were compared by analysis of variance (ANOVA) with Fisher's least-significant difference (LSD) method for comparing groups (Steel and Torrie, 1980). In all cases, data were examined for equal variance and for a normal distribution prior to statistical analysis. A level of significance of 5% was adopted for all comparisons.

RESULTS

Part I

Preliminary experiments

The L8 muscle cell line was originally established in 1969 by serial passaging of myoblasts isolated from primary rat skeletal muscle cultures prepared from newborn Wistar rats (Yaffe, 1969). Undifferentiated myoblasts were grown in 10% CS until they reached confluency. At this point, the medium was replaced with 2% horse serum. This procedure minimized differentiation until the cells were confluent and synchronized fusion. Usually, it took 4 to 5 days for cells to complete differentiation following addition of 2% HS.

Myogenin, which is a skeletal muscle myogenic factor, was not expressed in L8 myoblasts (Figure 3-1, lane M); however, its expression was detected in the 2-day differentiated cells (Figure 3-1, lanes 1-5). Skeletal muscle-specific contractile proteins such as α-actin, α-actinin and tropomyosin, can be seen in the following 4 days of differentiation using Western blot analysis (Figures 3-2 and 3-3). Again, none of these skeletal muscle-specific contractile proteins were expressed in L8 myoblasts. Of interest, tropomyosin and α-actin were expressed at highst concentrations following 4-days of differentiation, whereas myogenin was early detectable 1 day following differentiation.

Effects of various proteinase inhibitors on total protein degradation in L8 myotubes are shown in Figure 3-4. Calpain inhibitor II (CI-II) had the greatest effect on total protein degradation, followed by CQ and E64d. IGF-1 also decreased total protein degradation. Leupeptin did not alter total protein degradation in myotubes. The maximum dose of CI-II was 50 μg/ml and reduced total protein degradation by 50% (Figure 3-5). Concentrations above 50 μg/ml caused cell death. The maximum inhibition of protein degradation by CQ was 25% (Figure 3-6). Increasing CQ concentration to 50 μM did not further reduce protein degradation in L8 myotubes (data not shown). CQ concentrations over 50 μM killed the cells. Leupeptin and E64d did not affect cell viability at concentrations of 20 nM and 0.5 μM respectively. The effects of IGF-1 concentration on total protein degradation are shown in Figure 3-7. Protein degradation was reduced by IGF-1 in a dose-dependent manner as IGF-1 concentration increased from 50 to 200 ng/ml.

Differential centrifugation was used to separate the myofibrillar protein- and non-myofibrillar protein-rich fractions. Skeletal muscle-specific proteins (α-actin, α-actinin and tropomyosin) were identified in each fraction using Western blot analysis. Seventy percent of total α-actin and tropomyosin were detected in the myofibrillar protein-rich fraction and α-actinin was undetectable in non-myofibrillar protein rich-fraction (Figure 3-8). The procedure used for further purifying myofibrillar protein (Goll et al., 1974) provided evidence that this

method is sufficient for the partial separation of myofibrillar protein- and non-myofibrillar protein-rich fractions.

Part II

Effects of CI-II, CQ and IGF-1 on muscle protein degradation

Rates of total protein degradation were determined by measuring the release of free ^{3}H-tyrosine into medium at various incubation times after labeling myotubular proteins with ^{3}H-tyrosine (Gulve et al. 1991). Effects of CI-II, CQ and IGF-1 are shown in Figure 3-9. Total protein degradation was reduced 40%, 15% and 20% by CI-II (30 ng/ml), CQ (25 μM) and IGF-1 (200 ng/ml), respectively. These treatments had no effect on cell viability or morphology. However, IGF-1-treated cells tended to detach more easily after 24 hours of incubation. Increased concentrations of CI-II (50 μg/ml) and CQ (50 μM) caused cell death. To distinguish between myofibrillar protein degradation and non-myofibrillar protein degradation, differential centrifugation was used to separate myofibrillar and non-myofibrillar pools. CI-II increased the remaining radioactivity (i.e., reduced protein degradation) in the myofibrillar protein-rich fraction by 29% after 24 hours of incubation (Figure 3-10). CQ and IGF-1 did not alter degradation of the myofibrillar protein pool (Figure 3-10). These results indicate that while CI-II reduced myofibrillar protein degradation, CQ and IGF-1 did not change stability of this pool. CI-II, CQ and IGF-1 did not significantly alter non-myofibrillar protein degradation (Figure 3-11), although CQ and IGF-1 tended to increase the radioactivity remaining in this pool.

Effects of CI-II, CQ and IGF-1 in muscle protein synthesis

Fractional rates of myofibrillar and non-myofibrillar protein synthesis were determined by monitoring the incorporation of ^{3}H-tyrosine into acid-insoluble material (Gulve and Dice, 1989). The time course of labeling of myofibrillar and non-myofibrillar protein in myotubes is shown in Figure 3-12. Protein synthesis in the non-myofibrillar protein-rich fraction was slightly higher than in the myofibrillar protein pool; however, no significant difference in rate of incorporation of labeled tyrosine into these two pools was found in the first 24 hours of incubation. Hence, in subsequent studies we evaluated effects of inhibitors on protein synthesis in a range of 8 to 24 hours.

Effects of inhibitors on myofibrillar protein synthesis are shown in Figure 3-13. Irrespective of the duration of the study (8, 16 or 24 hours), CI-II stimulated protein synthesis in the myofibrillar protein pool. IGF-1 had the same effect on this pool. However, CQ did not alter protein synthesis in this pool (Figure 3-13).

Effects of the inhibitors on synthesis of non-myofibrillar proteins were also investigated. In the non-myofibrillar protein pool, the protein synthesis rates were not changed by CI-II, CQ or IGF-1 treatment. Further, the rate of protein synthesis was constant during the 24 hour incubation period (Figure 3-14).

Effects of CI-II, CQ and IGF-1 in muscle protein accumulation

Effects of the inhibitors on accretion of myofibrillar and non-myofibrillar proteins were investigated. After 24 hours of exposure, CI-II caused accumulation of myofibrillar proteins; however, CQ and IGF-1 did not affect the accretion of myofibrillar proteins (Figure 3-15). CI-II and CQ did not increase accretion of non-myofibrillar proteins; however, compared to control myotubes, IGF-1 increased the non-myofibrillar protein accretion significantly (Figure 3-16). Total protein accretion (myofibrillar + non-myofibrillar proteins) was increased in CI-II-treated myotubes by 13.2 % (Figure 3-17). However, CQ and IGF-1 did not alter total protein accretion in L8 myotubes.

Effects of CI-II, CQ and IGF-1 on degradation of individual myofibrillar and non-myofibrillar proteins

Effects of CI-II, CQ and IGF-1 on degradation of individual myofibrillar proteins are shown in Figure 3-18. After labeling, myotubes were exposed to CI-II, CQ or IGF-1 for 24 hours. The myofibrillar protein fraction was recovered and separated on a 7.5-15% gradient SDS-PAGE gel. Autoradiography was obtained after a 3 week exposure to X-Omat film. Autoradiography results indicated that several myofibrillar proteins were stabilized in CI-II-treated myotubes. These included myosin heavy chain (MHC), α-actin, a 25 kDa protein and a 21 kDa protein. To prevent the contamination by non-myofibrillar proteins during the separation, myofibrillar proteins from each sample were further purified using the method of Goll et al (1974) and myofibrillar proteins

processed as outlined previously. The results were similar to the previous results (Figure 3-19). The same group of myofibrillar proteins were stabilized in CI-II treated cells. Specifically, CI-II stabilized MHC, α-actin and the unknown 25kDa and 21 kDa proteins.

To enhance the sensitivity of the analysis, we cut various protein bands from the gels and analyzed radioactivity associated with the specific proteins. Base on this analysis, CI-II and IGF-1 increased MHC radioactivity (i.e., reduced protein degradation) by 16% and 20% respectively; however, no difference was found in CQ-treated cells (Figure 3-20). The inhibitors had a similar effect on α-actin degradation (Figure 3-20). CI-II reduced degradation of the unknown 25 kDa and 21 kDa proteins by a larger magnitude (56% and 60%, respectively; Figure 3-20). Autoradiography of non-myofibrillar protein fractions indicated that the inhibitors did not stabilize specific proteins (Figure 3-21).

DISCUSSION

Part I

Preliminary experiments

Skeletal myotubes express characteristics which are predominately embryonic or neonatal in nature (Whalen et al. , 1979; Blau et al., 1985). Skeletal muscle cells can be cultured in vitro as primary culture or established cell lines (Konigsberg, 1979; Yaffe, 1969). Myoblasts in culture can be induced to fuse into multi-nucleated myotubes, which accumulate muscle-specific proteins (Allen et al., 1979). The L8 myoblast cell line can be induced to express skeletal-muscle specific myogenic factors such as myogenin (Figure 3-1). Myogenin is a member of the MyoD myogenic factor family which includes MyoD, myogenin, Myf-5 and MRF4. It is expressed in the early stage of muscle development (Edmondson and Olson, 1993). In our cell culture system, the expression of this gene occurred as early as after the second day of differentiation (Figure 3-1). Some muscle-specific contractile proteins were used to monitor L8 cell differentiation. Tropomyosin (38 kDa) and α-actin (51 kDa) were detected in 4 day-differentiated cells (Figure 3-2). Two-dimensional Western blot analysis showed that these contractile protein accumulated in 4 day-differentiated L8 myotubes (Figure 3-3). Myotube cultures have been used as a model for protein turnover in fetal/neonatal muscle (Silver and Etlinger, 1985; Gulve and Dice, 1989; Gulve et al., 1991). In this preliminary experiment, we demonstrated that: (1) L8 cells can be induced to fuse into

multinucleated myotubes, (2) myotubes expressed a muscle-specific transcription factor (myogenin) and (3) myotubes accumulated muscle-specific contractile proteins such as tropomyosin, α-actin and α-actinin. Therefore, we believe that L8 cells are a useful model for studying muscle protein metabolism in vitro.

It has been reported that total protein degradation does not reflect myofibrillar protein degradation (Kadowaki et al., 1989). Studies of myofibrillar protein turnover revealed that degradation of myofibrillar and non-myofibrillar fractions can vary widely under certain circumstances. In vivo, myofibrillar protein degradation may be estimated as the release of N^{τ}-methylhistidine (NMH) in urine (Forsberg et al., 1989). In vitro, degradation has been estimated by NMH, tyrosine or phenylalanine (Goodman, 1987: Kayali et al., 1987) release from biopsied muscle bundles or from cultured muscle cells. A limitation of these studies is that NMH release from muscle cells or bundle is the last event arising from a long series of degradative events which start with disassembly and terminate with NMH release across the muscle cell membrane into the medium (Goll et al., 1992). The primary disadvantage of using tyrosine and phenylalanine release as an index of proteolysis is that their release primarily reflects the degradation of the more rapidly turning-over sarcoplasmic protein pool. In recent years, several studies have been conducted to study long-lived proteins (myofibrillar proteins) and short-lived proteins (sarcoplasmic proteins) in cultured muscle cells (Gulve and Dice, 1989; Gulve et al., 1991).

However, it has been reported that there was a broad spectrum of half-lives in myofibrillar components (Wolitzky et al., 1984). This method cannot be used to reliably predict the degradation of myofibrillar proteins. Therefore, it was important for us to develop a method which allowed us to determine myofibrillar protein degradation and non-myofibrillar protein degradation separately. Using differential centrifugation (Wolitzky et al., 1984), we were able to divide muscle protein into two different pools: a myofibrillar protein-rich fraction and a non-myofibrillar protein-rich fraction. About 70% of tropomyosin and α-actin were detected in the myofibrillar protein-rich fraction; α-actinin was not found in the non-myofibrillar protein-rich fraction. These results suggest that the efficiency of this myofibrillar protein separation is at least 70%. The major advantage of this method is that it allowed us to study protein turnover in different pools.

Several proteolytic systems have been identified in skeletal muscle. These include three major proteolytic systems: (1) lysosomal proteinases, which include cathepsins B, D, H and L, (2) proteasome and (3) calcium-dependent proteinases which include μ- and m-calpains. To investigate the roles of these proteinases in muscle protein degradation, we needed to establish a model to regulate various proteinase activities. Proteinase inhibitors and hormones are the most common means researchers have used to study the function of proteinases. In the previous chapter we evaluated the antisense oligoDNA and RNA approaches as means of regulating calpain levels. However, neither

technique was adequate. In this chapter we used a wide variety of inhibitors and endocrine factors to enable us to understand muscle protein degradation.

Calpain inhibitor II (CI-II; N-acetyl-leucyl-leucyl-methioninal) is a commercially-available cell-penetrating cysteine proteinase inhibitor which inhibits both μ- and m-calpain but it also inhibits cathepsin B (Fuller et al. 1992). Chloroquine (CQ) is a lysosomal enzyme inhibitor. It is a weak base known to accumulate in lysosomes immediately after its administration in vivo (De Duve et al., 1974). Short-term administration of chloroquine inhibits lysosomal enzymes and catabolic pathways in culture cells by elevating the intralysosomal pH (Homewood et al., 1972; Wibo and Poole, 1974). Leupeptin is a tripeptide aldehyde with the structure N-acetyl-leucyl-leucyl-arginal. Leupeptin is a broad-base serine and cysteine proteinase inhibitor that inhibits a variety of proteinases including plasmin, trypsin, papain, cathepsin B and calpains (Fuller et al., 1992). The structure of E64d is N-{N-(L-3-trans-ethoxycarbonyloxirane-2-carbonyl)-L-leucyl}-3-methylbutylamine. E64d is an irreversible inhibitor of cysteine proteinases (Wilcox and Mason, 1992). IGF-1 is an anabolic hormone which has been reported to decrease muscle protein degradation both in vivo and in vitro (Tomas et al., 1991: Gulve and Dice, 1989). By screening these inhibitors and hormones, we were able to select a system to study the role of individual proteinases in muscle protein degradation. Among these inhibitors, CI-II showed the greatest effect on muscle protein degradation. CQ , E64d and IGF-1 showed moderate effects in muscle protein degradation. Leupeptin had

little or no effect on muscle protein degradation (Figure 3-4). Wilcox and Mason (1992) reported that leupeptin does not readily permeate membranes; therefore, the efficiency of inhibition in whole cells was low when compared to a cell-penetrating inhibitor such as CI-II.

Base on these preliminary studies, CI-II and CQ were selected to further investigate the role of calpains and lysosomal proteinases in muscle protein degradation. CI-II decreased total protein degradation by as much as 50% without killing the cells. Fuller et al. (1992) have reported similar results in cultured chick myotubes. The maximum inhibition of protein degradation by CQ is 25%. Increased CQ concentration caused cell death. Obviously, myotubes can tolerate more inhibition of protein degradation from CI-II than from CQ. It is not clear why myotubes have these different responses from CI-II and CQ. It is possible that calpains play a major role in degrading non-essential proteins such as myofibrillar proteins and the accumulation of these proteins did not immediately affect cell survival. However, lysosomal enzymes may play an important role in turnover more essential proteins such as sarcoplasmic proteins. Accumulation of these proteins may have caused cytotoxicity.

Part II

<u>Effects of CI-II, CQ and IGF-1 in muscle protein degradation</u>

A controversy in the area of animal growth biology is whether improvements in muscle growth may be achieved through regulation of myofibrillar protein degradation. An enhancement of protein synthesis coupled

with a decrease in breakdown may account for the dramatic increase in fetal muscle protein content (Johnson and Wetmore, 1984). Goll et al. (1989) provided calculations which showed that small reductions (i.e., 10%) in muscle protein degradation in beef cattle would theoretically increase rates of muscle protein accretion by two-fold. However, other investigators hold the belief that compensatory changes in protein synthesis may accompany the exogenous control of muscle protein degradation, and that protein accretion, therefore, could not be efficiently regulated through manipulation of degradation.

From preliminary experiments, we demonstrated that the L8 cell culture system provided a unique model system for studying muscle protein metabolism under a variety of culture conditions. In this study, we used cultured muscle cells to investigate the roles of different proteolytic systems in muscle protein degradation and to assess the concept that accretion may be manipulated via control of muscle protein degradation.

CI-II is a cysteine proteinase inhibitor and CQ is a lysosomal enzyme inhibitor. Both CI-II and CQ inhibited total protein degradation in L8 myotubes (Figure 3-9). By using differential centrifugation, we were able to partially separate myofibrillar protein-rich and non-myofibrillar protein-rich fractions (Wolitzky et al., 1984). CI-II reduced protein degradation in the myofibrillar protein-rich fraction by 29% in 24 hours of incubation. In other words, CI-II specifically inhibited myofibrillar protein degradation. The inhibition of total protein degradation by CI-II was primarily in myofibrillar protein pool whereas

CQ and IGF-1 did not affect degradation of this pool (Figure 3-10). These results suggest that the degradation of myofibrillar proteins and non-myofibrillar proteins were mediated by different proteolytic systems and may be regulated independently. Calpains may be involved in myofibrillar protein degradation and lysosomal enzymes may degrade non-myofibrillar proteins.

Although CI-II is not fully selective, we believe that its inhibition of myofibrillar protein degradation in this study is mediated by the control of calpain activities and not by the control of lysosomal proteolytic enzymes because CQ had no effect on the myofibrillar protein pool. In contrast, CI-II exerted its effects specifically in the myofibrillar protein pool.

IGF-1 reduces proteolysis in muscle cells (Gulve and Dice, 1989; Hong, 1993). Our results also indicated that IGF-1 inhibits total protein degradation in L8 myotubes. However, the mechanism by which IGF-1 inhibits protein degradation is not known. Hong (1993) reported that expression of cathepsin D was reduced by IGF-1 and IGF-1 slightly up-regulated m-calpain gene expression. In this study, we found that IGF-1 did not alter degradation of the myofibrillar protein pool. Instead, it tended to reduce degradation of non-myofibrillar proteins. The effects of IGF-1 are similar to the effects of CQ in protein degradation. These results suggest that IGF-1 exerted control of protein degradation via lysosomal proteolytic system.

It is believed that protein synthesis may change to compensate for the exogenous control of muscle protein degradation. In our study, the effects of

CI-II, CQ and IGF-1 were not compensated for by antagonistic changes in protein synthesis. In fact, both CI-II and IGF-1 enhanced myofibrillar protein synthesis. It is not clear why CI-II, a proteinase inhibitor, would increase protein synthesis. A possible explantion is that CI-II may stabilize some specific proteins which are involved in protein synthesis. This, in turn, may increase overall protein translation. IGF-1 has been reported to increase total protein synthesis in muscle cells (Roeder et al., 1988). Our results showed that IGF-1 increased protein synthesis and that this was primarily mediated by increased myofibrillar protein synthesis. The effects of CQ in protein synthesis have been studied in isolated soleus muscle (Kumamoto et al., 1984). Their results showed that CQ did not influence muscle protein synthesis. In our study, CQ also did not affect myofibrillar and non-myofibrillar protein synthesis.

Muscle protein accretion reflects the balance between rates of muscle protein synthesis and protein degradation. It is believed that reduction of muscle protein degradation could increase the rates of muscle protein accretion. Accumulations of myofibrillar proteins were observed in CI-II-treated L8 myotubes. Presumably, this accretion of myofibrillar proteins was due to the CI-II-dependent inhibition of myofibrillar protein degradation and increased in myofibrillar protein synthesis. Accretion of total protein increased in CI-II-treated L8 myotubes. We believed that the accretion of total protein is primarily contributed by accumulations of myofibrillar proteins.

To further investigate the effects of CI-II, CQ and IGF-1 on degradation of individual myofibrillar proteins, we developed a method to examine stabilities of individual myofibrillar proteins. Our results showed that several myofibrillar proteins were stabilized by CI-II and IGF-1. These included MHC, α-actin and unknown 25 kDa and 21 kDa proteins. The molecular weight of the 25 kDa protein is close to that of myosin light chain-1. The differential effects of CI-II on the degradative rates of the myofibrillar proteins suggest that calpains might exert selective degradation of individual proteins from the myofilaments, and/or some degree of localized myofibrillar disassembly. Specifically, the differential stabilization of myofibrillar proteins by CI-II suggests that some proteins (eg, the 25 kDa and 21 kDa proteins) may be direct substrates of calpain. Perhaps their degradation precedes myofibrillar disassembly. Their stabilization related to other myofibrillar proteins indicates that these proteins either turnover very rapidly or that their degradation precedes the release and degradation of other myofibrillar proteins. Perhaps the 21 and 25 kDa proteins are direct substrates of calpain and other substrates for other proteolytic systems.

From this study it is now clear that calpains and lysosomal proteinases have distinct roles in muscle. Specifically, the stabilization of myofibrillar proteins by CI-II and of the non-myofibrillar proteins by CQ demonstrates that calpains likely degrade myofibrillar and lysosomal proteinases degrade non-myofibrillar protein pools. Additional evidence of different function are that small inhibitions of degradation by CQ were much more toxic than similar

reduction by CI-II. This, again, suggests that the proteins degraded by lysosomes are more critical to muscle than those degraded by calpains. The final evidence for distinct function was that CI-II, but not CQ increased protein synthesis. Evidently calpains normally degrade some factors involved in protein synthesis. Perhaps the benefits of inhibiting proteolysis by calpain arise not only from stabilization of myofibrillar proteins but from stabilization of key proteins involved in protein synthesis.

Conclusion

Muscle protein degradation is an important determinant of muscle growth. Calpains are proposed to initiate myofibrillar protein degradation by release of actin filaments from the Z-disk (Goll et al. 1992). In this study, we used calpain inhibitor II (cysteine proteinase inhibitor) and chloroquine (lysosomal proteinase inhibitor) to investigate the role of calpains and lysosomal proteinases in non-myofibrillar protein degradation and myofibrillar protein degradation. CI-II and CQ inhibited total protein degradation in L8 myotubes. The major contribution to the reduction of total protein degradation in CI-II-treated myotubes was the inhibition of myofibrillar protein proteins. CQ inhibited non-myofibrillar protein degradation. This result implies that calpains play important roles in myofibrillar protein degradation and the lysosomal proteolytic system is primarily responsible for non-myofibrillar protein degradation.

Future studies

In this study, we demonstrated that in vitro, protein accretion will occur if myofibrillar protein degradation is reduced and protein synthesis is enhanced. To improve the efficiency of animal growth, it is important to test this concept in vivo. Administration of calpain inhibitor II to animals would be a possible next step to prove that decrease of protein degradation will improve the efficiency of animal growth.

Due to the limitations of using calpain inhibitor II, it is not possible to totally rule out the possibility of involvement by other proteinases and it is not possible to distinguish which calpain is important to myofibrillar protein degradation. Some modern molecular biology techniques should be used to further clarify the roles of individual proteinase in muscle cells. For example, over-expression of calpain-specific inhibitor (calpastatin) or over-expression of negative dominant calpains in muscle cells will be helpful to further understand the role of calpains in muscle protein degradation. In addition, transgenic animals (muscle-specific over-expression of calpastatin or muscle-specific over-expression of negative dominant calpains) could be used to increase the rate of animal growth.

REFERENCES

Allen, R. E. 1986. Muscle cell growth and development. Designing Foods Animal Product Options in the marketplace. National Research Council, National Acad. Press. Wash. D. C. pp 142-162.

Allen, R. E., M. H. Stromer, D. E. Goll and R. M. Robson. 1979. Accumulation of myosin, actin, tropomyosin, and α-actinin in cultured muscle cells. Dev. Biol. 69:655-660.

Blau, H. M., G. K. Pavlath, E. C. Hardeman, C. P. Chiu, L. Silberstein, S. G. Webster, S. C. Miller and C. Webster. 1985. Plasticity of the differentiated states. Science 230:758-766.

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156-159.

Dahlman, B., L. Kuehn, M. Rutschmann and H. Reinauer. 1985. Purification and characterization of a multicatalytic high molecular mass proteinase from rat skeletal muscle. Biochem. J. 228:161-170.

Dayton, W. R., W. J. Reville, D. E. Goll and M. H. Stromer. 1976. A Ca^{2+}-activated protease possibly involved in myofibrillar protein turnover. Partial characterization of the purified enzyme. Biochemistry 15:2159-2167.

De Duve, C., T. De Barsy, B. Poole, A. Trouet, P. Tulkens and F. Van Hoof. 1974. Lysosomotropic agents. Biochem. Pharmacol. 23:2495-2531.

Edmondson, D. G. and E. N. Olson. 1993. Helix-loop-helix proteins as regulators of muscle-specific transcription. J. Biol. Chem. 268:755-758.

Fagan, J. M., L. Waxman and A. L. Goldberg. 1987. Skeletal muscle and liver contain a soluble ATP + ubiquitin-dependent proteolytic system. Biochem. J. 243:335-343.

Forsberg, N. E., M. A. Ilian, A. Ali-Bar, P. R. Cheeke and N. B. Wehr. 1989. Effects of cimaterol on rabbit growth and myofibrillar protein degradation and on calcium-dependent proteinase and calpastatin activities in skeletal muscle. J. Anim. Sci. 67:3313-3321.

Forsberg, N. E. and N. B. Wehr. 1990. Effects of cimaterol on muscle protein metabolism and its actions in hypothyroid and hyperthyroid rats. Domestic Anim. Endocrinol. 7:149-164.

Fuller, J. C., G. A. Link., T. W. Huiatt, J. L. Sell and S. Nissen. 1992. Disparate responses of cultured skeletal muscle cells and growing chicks to tripeptide aldehyde protease inhibitors and an in vivo interaction with ethanol. J. Nutr. Biochem. 3:291-297.

Furuno, K., M. N. Goodman, and A. L. Goldberg. 1990. Role of different proteolytic systems in the degradation of muscle proteins during denervation atrophy. J. Biol. Chem. 265:8550-8557.

Gerard, K. W., A. R. Hipkiss and D. L. Schneider. 1988. Degradation of intracellular protein in muscle. Lysosomal response to modified proteins and chloroquine. J. Biol. Chem. 263:18886-18890.

Goll, D. E., W. C. Kleese and A. Szpacenko. 1989. Skeletal muscle proteinases and protein turnover. In Animal Growth Regulation. Ed. D. R. Campion, G. J. Hausman, R. J. Martin. Plenum Publishing, pp 141-182.

Goll, D. E., V. F. Thompson and R. G. Taylor. 1992. Role of the calpain system in muscle growth. Biochimie 74:225-237.

Goll, D. E., R. B. Young and M. H. Stromer. 1974. Separation of subcellular organelles by differential and density gradient centrifugation. Reciprocal Meat Conference (27) pp250.

Goodman, M. N. 1987. Acute alterations in sodium flux in vitro leads to decreased myofibrillar protein breakdown in rat skeletal muscle. Biochem. J. 247:151-156.

Gulve, E. A., K. Mabuchi and J. F. Dice. 1991. Regulation of myosin and overall protein degradation in mouse C2 skeletal myotubes. J. Cell. Physiol. 147:37-45.

Gulve, E. A. and J. F. Dice. 1989. Regulation of protein synthesis and degradation in L8 myotubes. Biochem. J. 260: 377-387.

Harper, J. M. M., J. B. Soar and P. J. Buttery. 1987. Changes in protein metabolism of ovine primary muscle cultures on treatment with growth hormone, insulin, insulin-like growth factor I or epidermal growth factor. J. Endocrinol. 112:87-96.

Homewood C. A., D. C. Warhurst, W. Peters and V. C. Baggaley. 1972. Lysosomes, pH and the anti-malarial action of chloroquine. Nature. 235:50-52.

Hong, D. Y. 1993. Endocrine control of proteolysis in cultured muscle cells. Ph.D. Thesis, Oregon State University.

Johnson, J. D. and D. L. Wetmore. 1984. Protein turnover in heart and diaphragm of rat fetus. Am. J. Physiol. 247:E781-E785.

Kadowaki, M., N. Harada, S. Takahashi, T. Noguchi and H. Naito. 1989. Differential regulation of myofibrillar and total protein in skeletal muscle of rats: effects of streptozotocin-induces diabetes, dietary protein and starvation. J. Nutr. 119:471-477.

Kayali, A., V. R. Young, and M. N. Goodman. 1987. Sensitivity of myofibrillar proteins to glucocorticoid-induced muscle proteolysis. Am. J. Physiol. 252:E621-E626

Konigsberg, I. R. 1979. Skeletal myoblasts in culture. Methods Enzymol. 58:511-522.

Kumamoto, T., S. Araki, I. Toyoshima and N. Fukuara. 1984. Experimental chloroquine myopathy-morphological and biochemical studies. Clin. Neutrol. 24:505-512.

Laemmli U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Miner, J. H. and B. J. Wold. 1991. c-myc inhibition of MyoD and myogenin-initiated myogenic differentiation. Mol. Cell. Biol. 11:2842-2851.

Nadal-Ginard, B. 1978. Commitment, fusion and biochemical differentiation of a myogenic cell line in the absence of DNA synthesis. Cell 15:855-864.

O'Farrell P. H. 1975. High resolution two-dimentional electrophoresis of proteins. J. Biol. Chem. 250:4007-4021.

Richler, C. and D. Yaffe. 1970. The in vitro cultivation and differentiation capacities of myogenic cell lines. Devel. Biol. 23:1-22.

Roeder, R. A., K. L. Hossner, R. G. Sasser and J. M. Gunn. 1988. Regulation of protein turnover by recombinant human insulin-like grwoth factor-I in L6 myotube cultures. Horm. Metabol. Res. 20:698-700.

Roeder, R. A., S. D. Thorpe, F. M. Byers, G. T. Schelling, and J. M. Gunn. 1986. Influence of anabolic agents on protein synthesis and degradation in muscle cells grown in culture. Growth 50:485-495.

Sorimachi, H., S. Imajoh, T. Ohmi, Y. Emori, H. Kawasaki, S. Ohno, Y. Minami and K. Suzuki. 1989. Molecular cloning of a novel mammalian calcium-dependent protease distinct from both μ and m types. J. Biol. Chem. 264:20106-20111.

Silver, G., and J. D. Etlinger. 1985. Regulation of myofibrillar accumulation in chick muscle cultures: evidence for the involvement of calcium and lysosomes in non-uniform turnover of contractile proteins. J. Cell Biol. 101:2383-2391.

Steel, R. G. D. and L. H. Torrie. 1980. Principles and Procedures of Statistics. Mcgraw-Hill Book Co., Inc., New York.

Tomas, F. M., S. E. Knowles, P. C. Owens, L. C. Read, C. S. Chandler, S. E. Gargosky and F. J. Ballard. 1991. Increased weight gain, nitrogen retention and muscle protein synthesis following treatment of diabetic rats with insulin-like growth factor (IGF)-1 and des(1-3)IGF-1. Biochem. J. 276:547-554.

Towbin, H., T. Staehelin and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamine gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci., U. S. A. 76:4350-4354.

Wakelam, M. J. O. The fusion of myoblasts. Biochem. J. 228:1-12, 1985.

Whalen, R. C., K. Schwartz, P. Bouveret, S. M. Sell and F. Gros. 1979. Contractile muscle isozymes in muscle development: identification of an embryonic form of myosin heavy chain. Proc. Natl. Acad. Sci. USA 76:5194-5201.

Wibo, M. and B. Poole. 1974. Protein degradation in cultures cells. II. The uptake of chloroquine by rat fibroblasts and inhibition of cellular protein degradation and cathepsin B. J. Cell. Biol. 63:430-440.

Widnell, C. C. and K. H. Pfenninger. 1989. Essential Cell Biology. Williams and Wilkins, Baltimore.

Wilcox, D. and R. W. Mason. 1992. Inhibition of cysteine proteinases in lysosomes and whole cells. Biochem. J. 285:4995-502.

Wolfe, F. H., S. K. Sathe, D. E. Goll, W. C. Kleese, T. Edmunds and S. M. Duperret. 1989. Chicken skeletal muscle has three Ca^{2+}-dependent proteinases. Biochim. Biophys. Acta 998:236-250.

Wolitzky, B. A., M. S. Hudecki and H. L. Segal. 1984. Turnover of myofibrillar proteins in cultured muscles from normal and dystrophic chick embryos. Biochim. Biophys. Acta 803:106-114

Yaffe, D. 1968. Retention of differentiation potentialities during prolonged cultivation of myogenic cells. Proc. Natl. Acad. Sci. USA 61:477-483.

Yeh, J. Y., B. R, Ou and N. E. Forsberg. 1994. Effects of dexamethasone on muscle protein homeostasis and on calpain and calpastatin activities and gene expression in rabbit. J. Endocrinol. 141:209-217.

Figure 3-1. Concentrations of myogenin mRNA in various differentiation stages of L8 cells. L8 myoblasts were cultured in DMEM + 10%CS. After cells reached confluence, the medium was replaced with DMEM + 2%HS to induce differentiation. Total RNA was extracted from myoblasts (M), and following 1, 2, 3, 4, and 5 days of differentiation. Northern blot analysis was performed to detect myogenin gene expression.

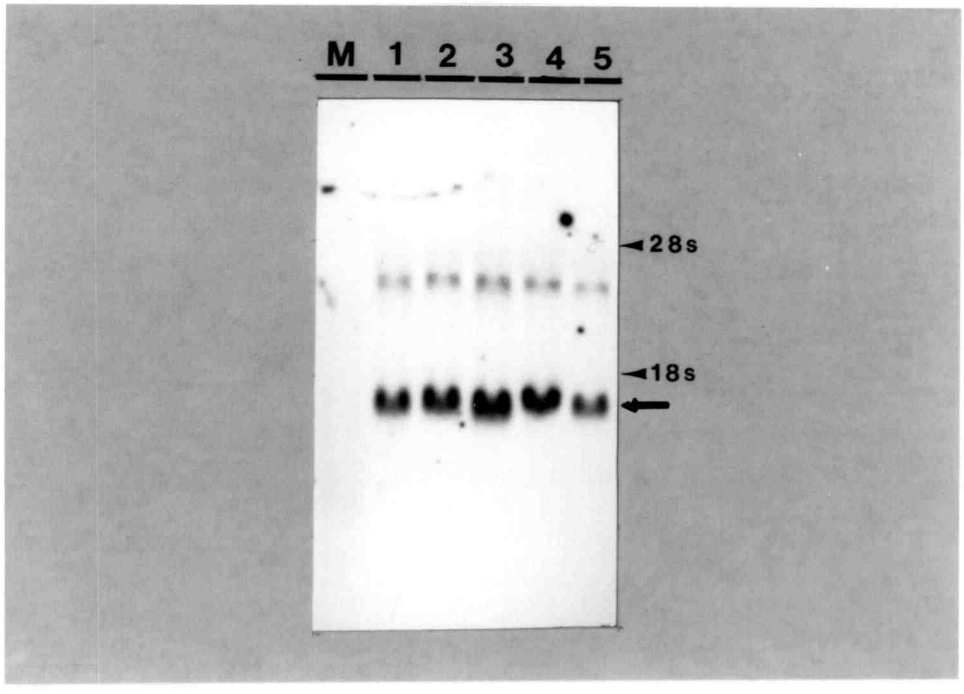


Figure 3-2. Accumulation of muscle-specific contractile proteins in various differentiation stages of L8 cells. L8 myoblasts were cultured in 10% CS DMEM. After cells reached confluence, the medium was replaced with 2% HS DMEM to induce differentiation (lane 1:myoblasts; lane 2: 1-day of differentiation; lane 3: 2-days of differentiation; lane 4: 4-days of differentiation). Western blot analysis was performed to examine the accumulation of muscle specific contractile proteins, tropomyosin (Panel A) and α-actin (Panel B) in L8 cells.

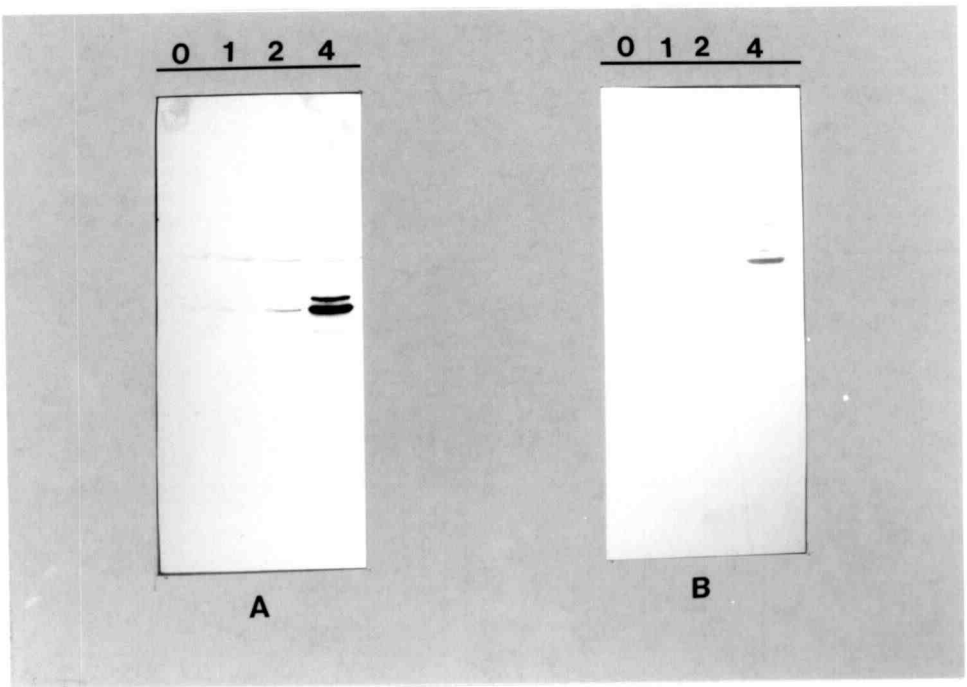


Figure 3-3. Two dimensional Western blot analysis of muscle-specific contractile proteins in L8 myotubes. Myofibrillar proteins were purified from L8 myotubes and then separated on a 2-dimensional gel. Tropomyosin (Tm), α-actin (Ac) and α-actinin (An) monoclonal antibodies were used to detect these muscle-specific contractile proteins.

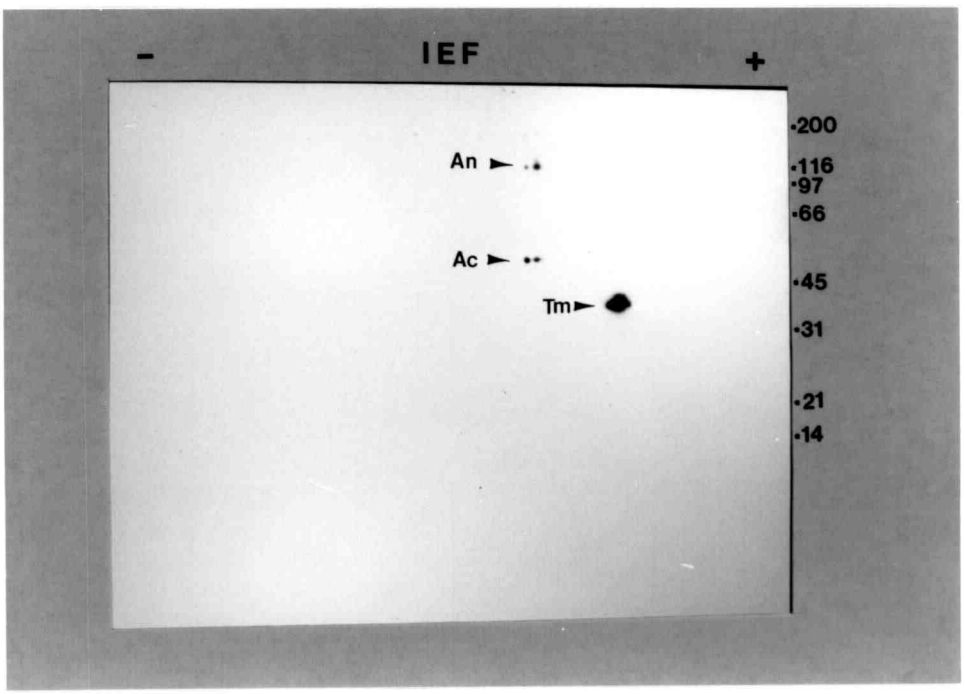


Figure 3-4. Effects of various proteinase inhibitors on total protein degradation in L8 myotubes. L8 myotubes were labeled with 1μCi/ml ^{3}H-tyrosine for 24-36 hours. Cells were then rinsed and exposed to calpain inhibitor II (CI-II; 30 μg/ml), chloroquine (CQ; 25 μM), insulin-like growth factor-1 (IGF-1; 200 ng/ml), E64d (0.5 μM) or leupeptin (Leu; 20 nM) for 24 hours in degradation medium. Degradation medium consisted of DMEM supplemented with 2mM tyrosine. Total protein degradation is expressed as percentage (mean ± SE) of labeled protein broken down in 24 hours. Treatments which do not share a common superscript (above bar) differ significantly ($p<0.05$).

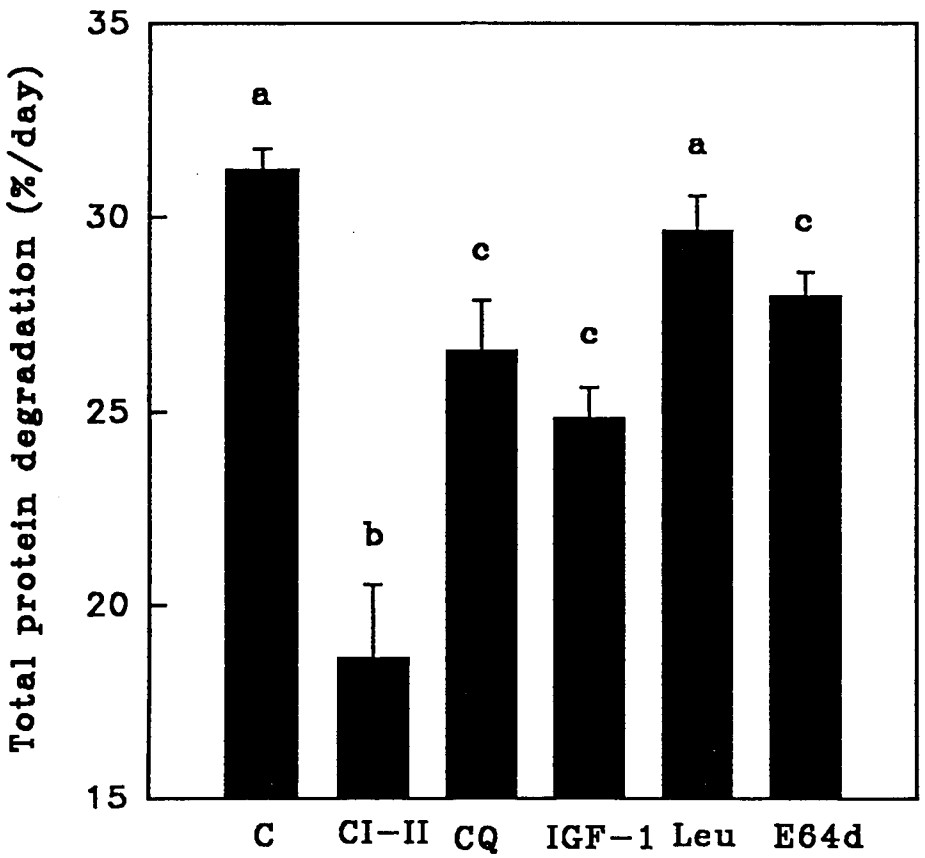


Figure 3-5. Effects of calpain inhibitor II (CI-II) on total protein degradation in L8 myotubes. L8 myotubes were labeled with 1μCi/ml ^{3}H-tyrosine for 24-36 hours. Cells were then rinsed and exposed to CI-II for 24 hours in degradation medium. Degradation medium consisted of DMEM + 2% HS supplemented with 2mM tyrosine. Total protein degradation is expressed as a percentage (mean ± SE) of labeled protein broken down in 24 hours.

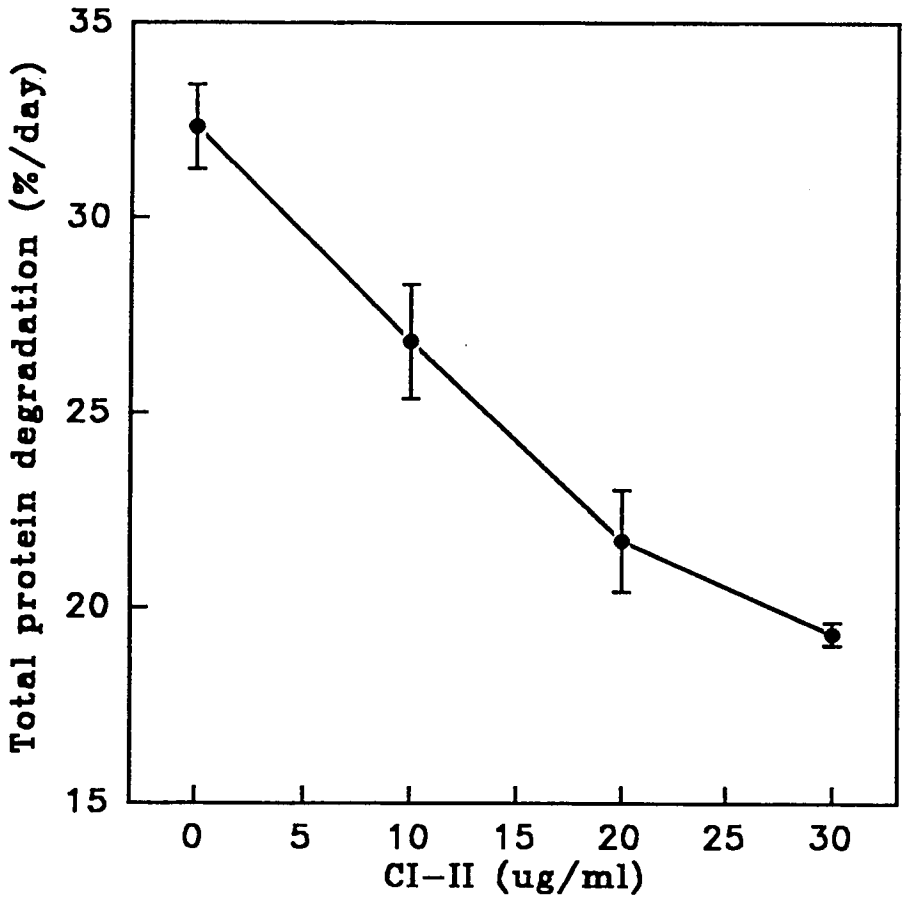


Figure 3-6. Effects of chloroquine (CQ) on total protein degradation in L8 myotubes. L8 myotubes were labeled with 1 μCi/ml ^{3}H-tyrosine for 24-36 hours. Cells were then rinsed and exposed to CQ for 24 hours in degradation medium. Degradation medium consisted of 2% HS DMEM supplemented with 2mM tyrosine. Total protein degradation is expressed as a percentage (mean ± SE) of labeled protein broken down in 24 hours.

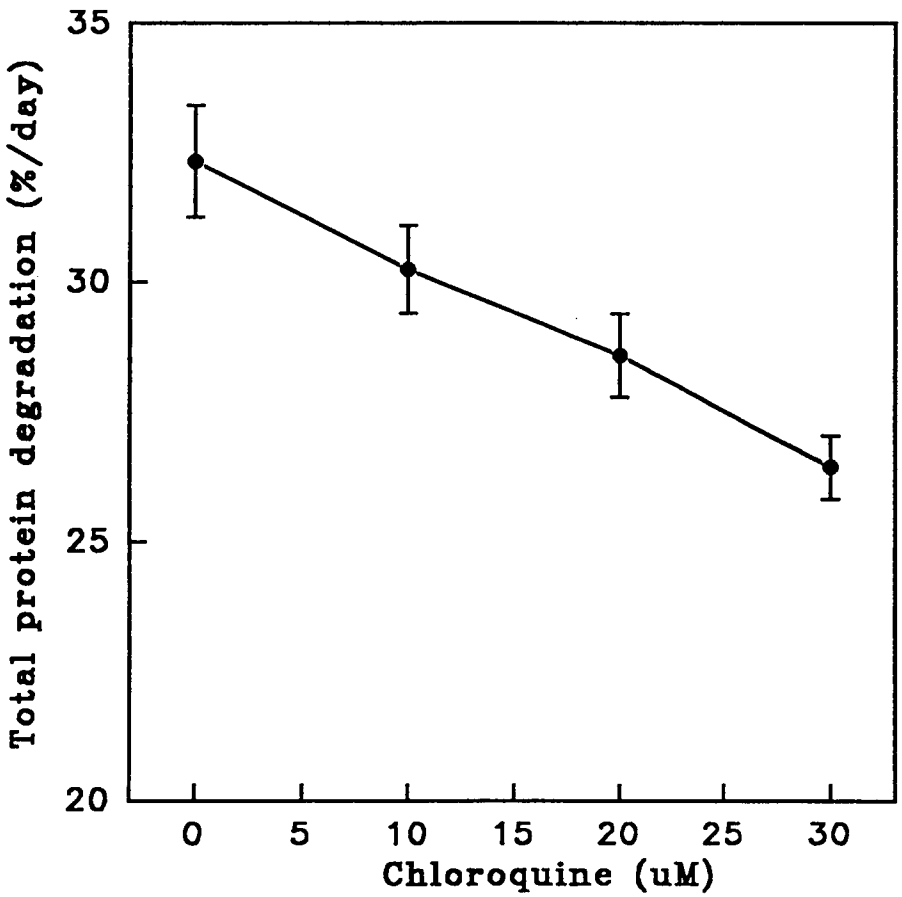


Figure 3-7. Effects of IGF-1 on total protein degradation in L8 myotubes. L8 myotubes were labeled with 1 μCi/ml ^{3}H tyrosine for 24-36 hours. Cells were then rinsed and exposed to IGF-1 for 24 hours in degradation medium. Degradation medium consisted of 2% HS DMEM supplemented with 2mM tyrosine. Total protein degradation is expressed as a percentage (mean ± SE) of labeled protein broken down in 24 hours.

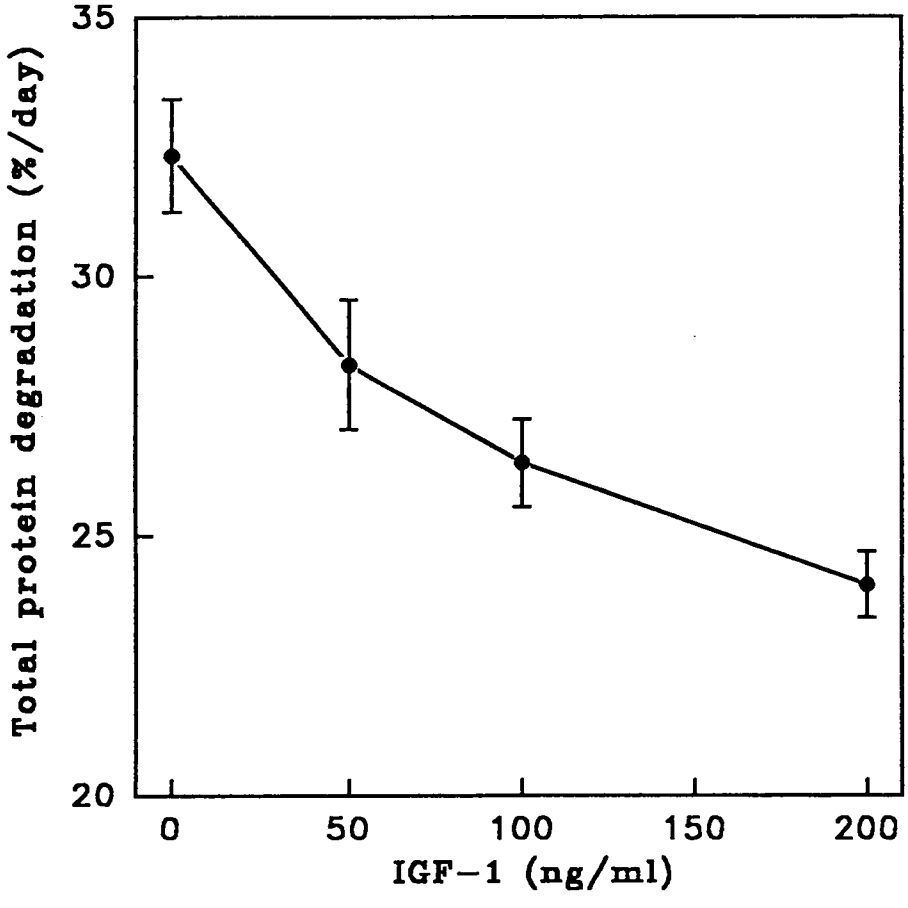


Figure 3-8. Identification of tropomyosin, α-actin and α-actinin in myofibrillar and non-myofibrillar fractions of muscle cells. L8 myotubes were scraped from dishes. Non-myofibrillar proteins (lane 1) and myofibrillar proteins (Lane 2) were separated as described in Materials and Methods. Purified myofibrillar proteins (Lane 3) were purified according to Goll et al. (1974). Western blot analysis was performed to examine the separation efficiency. Tropomyosin (Panel A), α-actin (Panel B) and α-actinin (Panel C) antibodies were used to identify muscle-specific myofibrillar proteins.

Figure 3-9. Effects of calpain inhibitor II (CI-II), chloroquine (CQ) and IGF-1 on total protein degradation in L8 myotubes. L8 myotubes were labeled with 1 μCi/ml ^{3}H- tyrosine for 24-36 hours. Cells were then rinsed and exposed to these treatments (CI-II, 30 μg/ml; CQ, 25 μM; IGF-1, 200 ng/ml) for 24 hours in degradation medium. Degradation medium consisted of 2% HS in DMEM supplemented with 2mM tyrosine. Total protein degradation is expressed as a percentage (mean ± SE) of labeled protein broken down in 24 hours. Treatments which do not share a common superscript (above bar) differ significantly ($p<0.05$).

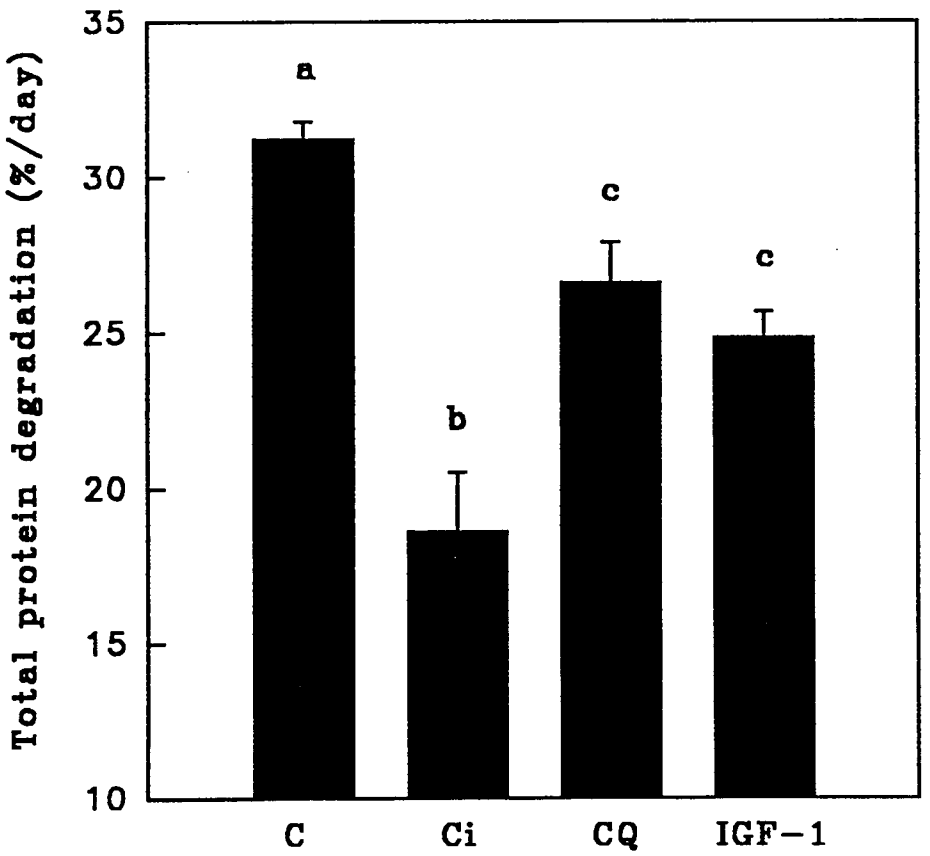


Figure 3-10. Effects of CI-II, CQ and IGF-1 on decay of radioactivity associated with myofibrillar proteins in L8 myotubes. L8 myotubes were exposed to these treatments (CI-II, 30 μg/ml; CQ, 25 μM; IGF-1, 200 ng/ml) for a period of 24 hours. Myofibrillar proteins were then separated and radioactivity associated with this fraction was determined. Treatments which do not share a common superscript (above bar) differ significantly ($p<0.05$).

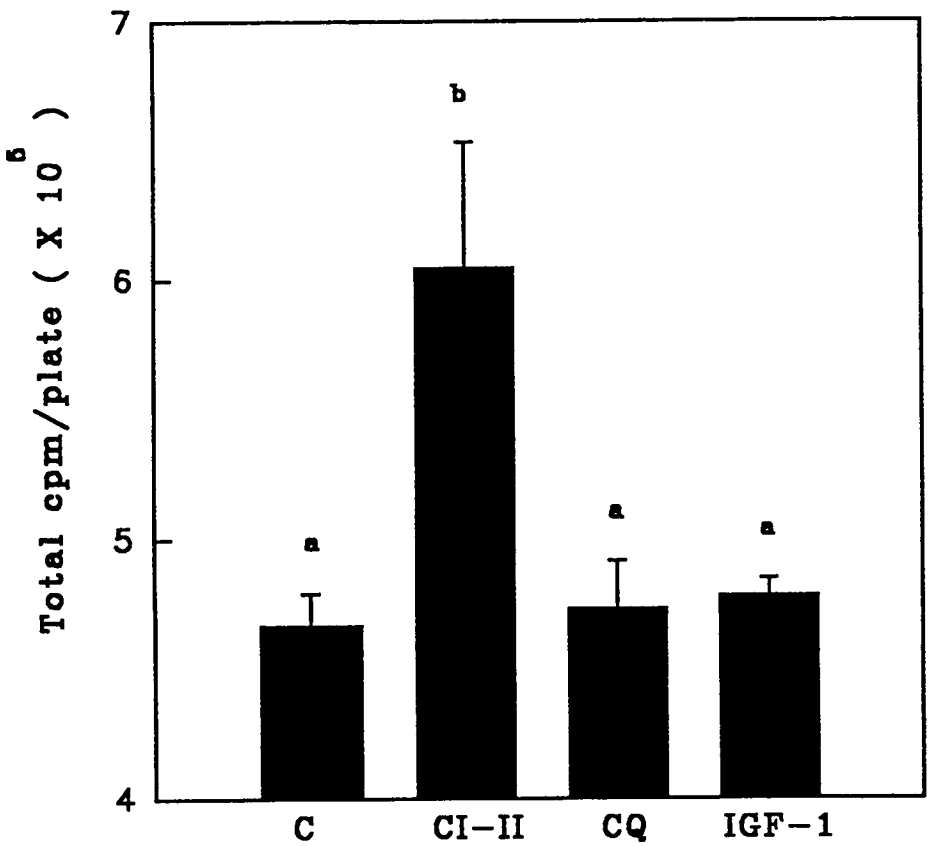


Figure 3-11. Effects of CI-II, CQ and IGF-1 on decay of radioactivity associated with non-myofibrillar proteins in L8 myotubes. L8 myotubes were exposed to these treatments (CI-II, 30 μg/ml; CQ, 25 μM; IGF-1, 200 ng/ml) for a period of 24 hours. Non-myofibrillar proteins were then separated and radioactivity associated with this fraction was determined. Treatments which do not share a common superscript (above bar) differ significantly ($p<0.05$).

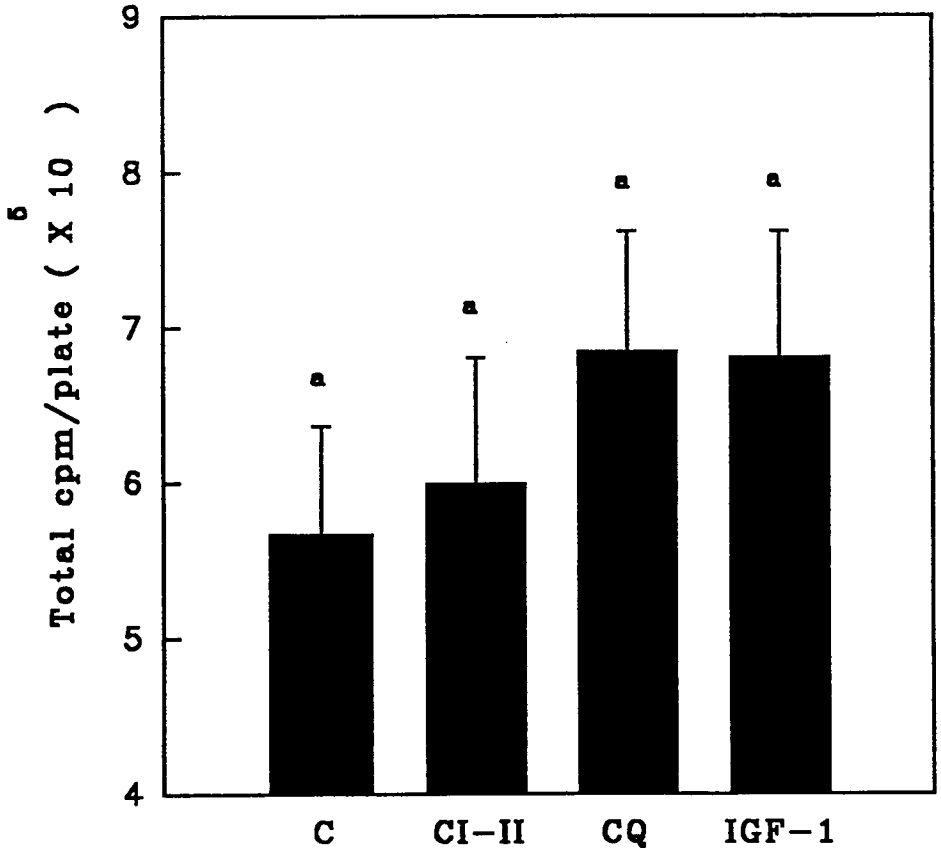


Figure 3-12. Time course of labeling of myofibrillar and non-myofibrillar proteins in L8 myotubes. L8 myotubes were labeled with ^{3}H-tyrosine (1 μCi/ml) for various times. Cells were then scraped and myofibrillar protein and non-myofibrillar protein were separated. The radioactivity associated with each fraction was then determined.

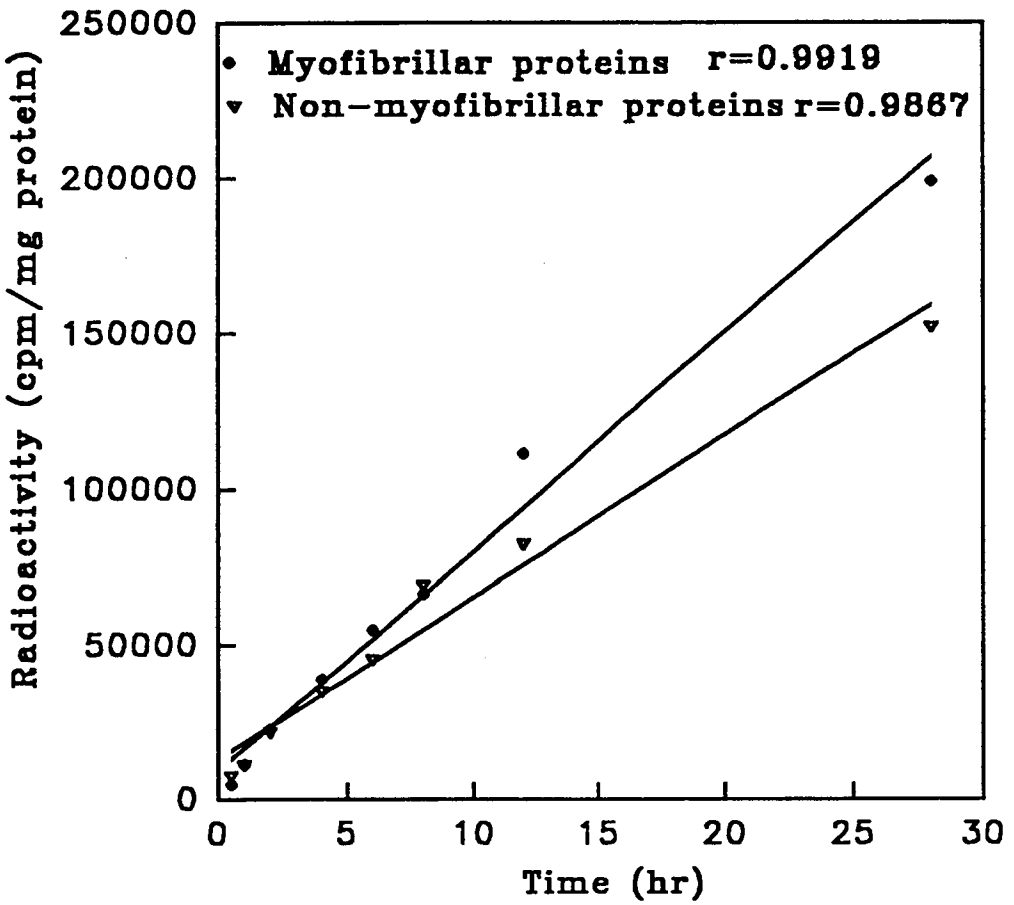


Figure 3-13. Effects of CI-II, CQ and IGF-1 on myofibrillar protein synthesis in L8 myotubes. L8 myotubes were exposed to various treatments (CI-II, 30 μg/ml; CQ, 25 μM; IGF-1, 200 ng/ml) for periods of 8, 16 or 24 hours. One μCi/ml ^{3}H-tyrosine was then added into cell cultures during the last hour of 8, 16 or 24 hours of incubation. Myofibrillar proteins were then separated and radioactivity associated with this fraction was determined. Treatments which do not share a common superscript (above bar) differ significantly ($p<0.05$).

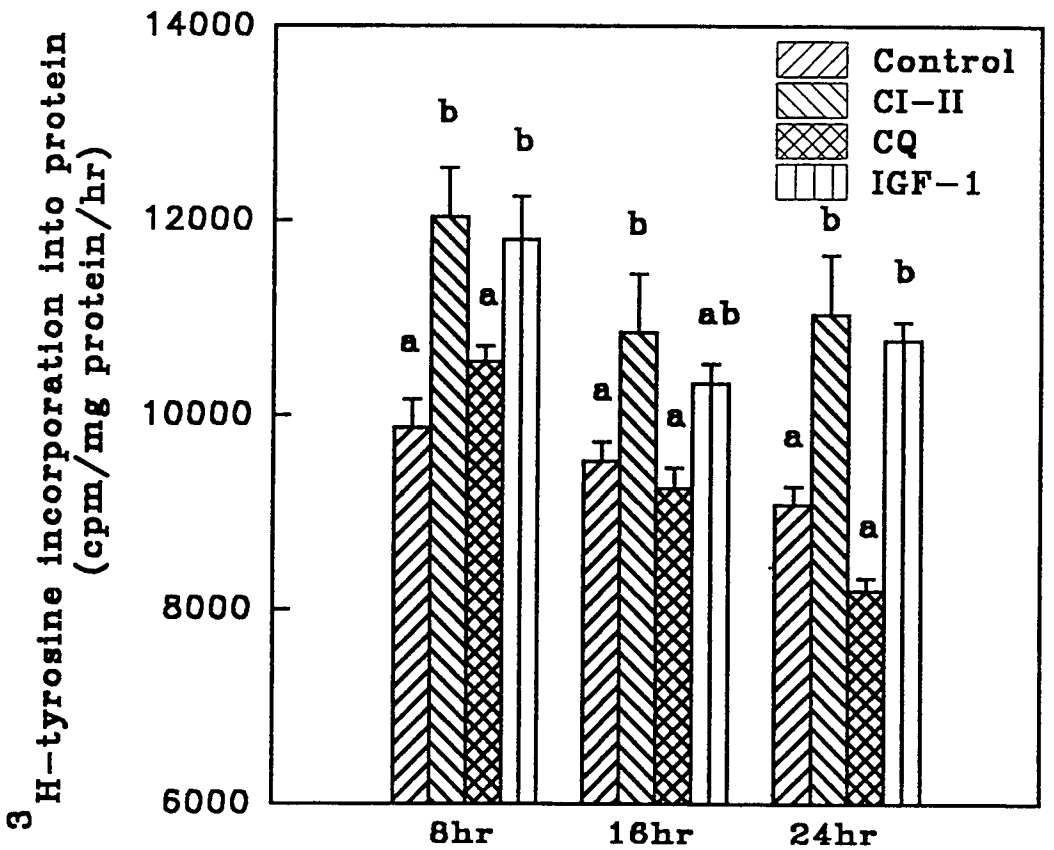


Figure 3-14. Effects of CI-II, CQ and IGF-1 on non-myofibrillar protein synthesis in L8 myotubes. L8 myotubes were exposed to various treatments (CI-II, 30 μg/ml; CQ, 25 μM; IGF-1, 200 ng/ml) for periods of 8, 16 or 24 hours. One μCi/ml ^{3}H-tyrosine was added into cell cultures during the last hour of 8, 16 or 24 hours incubation. Non-myofibrillar proteins were then separated and radioactivity associated with this fraction was determined. Treatments which do not share a common superscript (above bar) differ significantly ($p<0.05$).

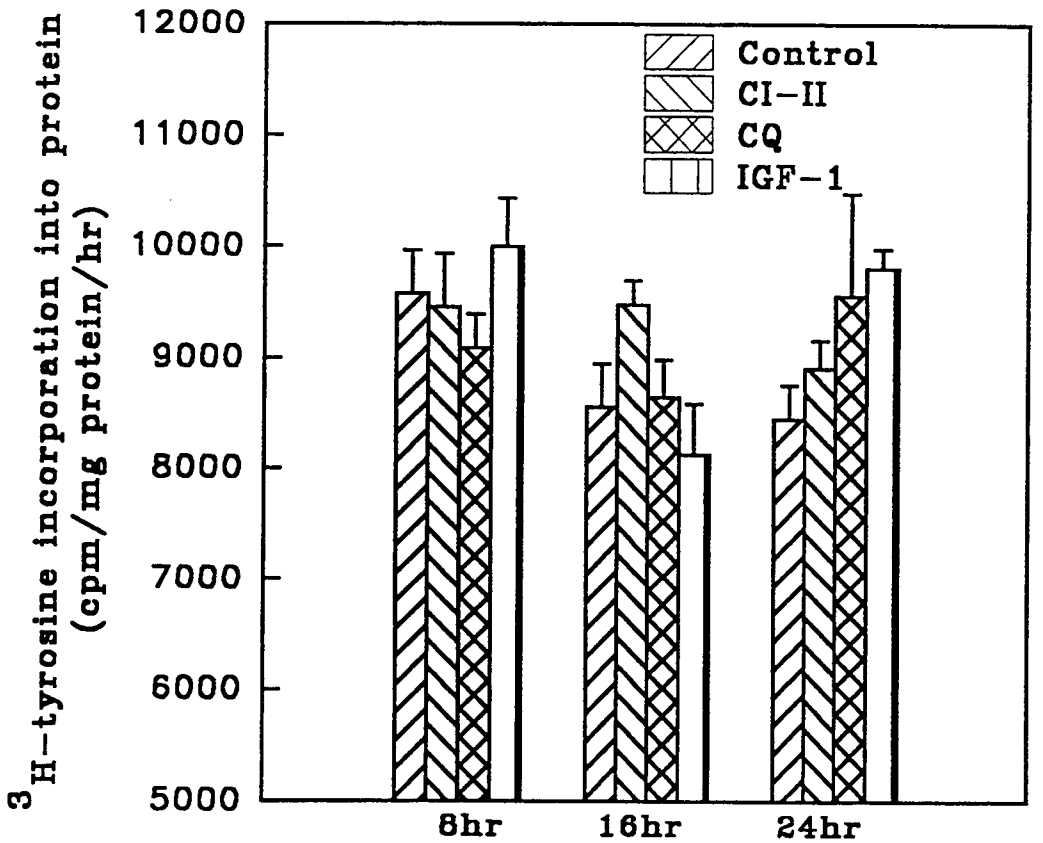


Figure 3-15. Effects of CI-II, CQ and IGF-1 on myofibrillar protein accretion in L8 myotubes. L8 myotubes were exposed to various treatments (CI-II, 30 μg/ml; CQ, 25 μM; IGF-1, 200 ng/ml) for a period of 24 hours. Myofibrillar proteins were then separated and radioactivity associated with this fraction was determined. Treatments which do not share a common superscript (above bar) differ significantly ($p<0.05$).

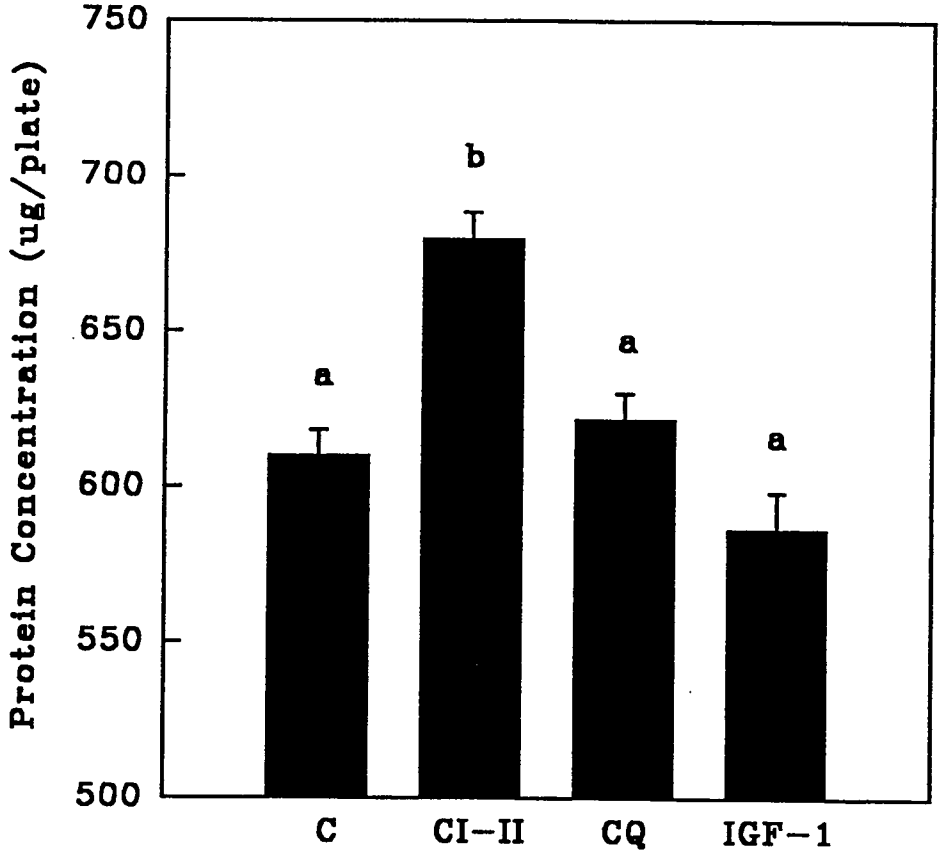


Figure 3-16. Effects of CI-II, CQ and IGF-1 on non-myofibrillar protein accretion in L8 myotubes. L8 myotubes were exposed to various treatments (CI-II, 30 μg/ml; CQ, 25 μM; IGF-1, 200 ng/ml) for a period of 24 hours. Non-myofibrillar proteins were then separated and radioactivity associated with this fraction was determined. Treatments which do not share a common superscript (above bar) differ significantly ($p < 0.05$).

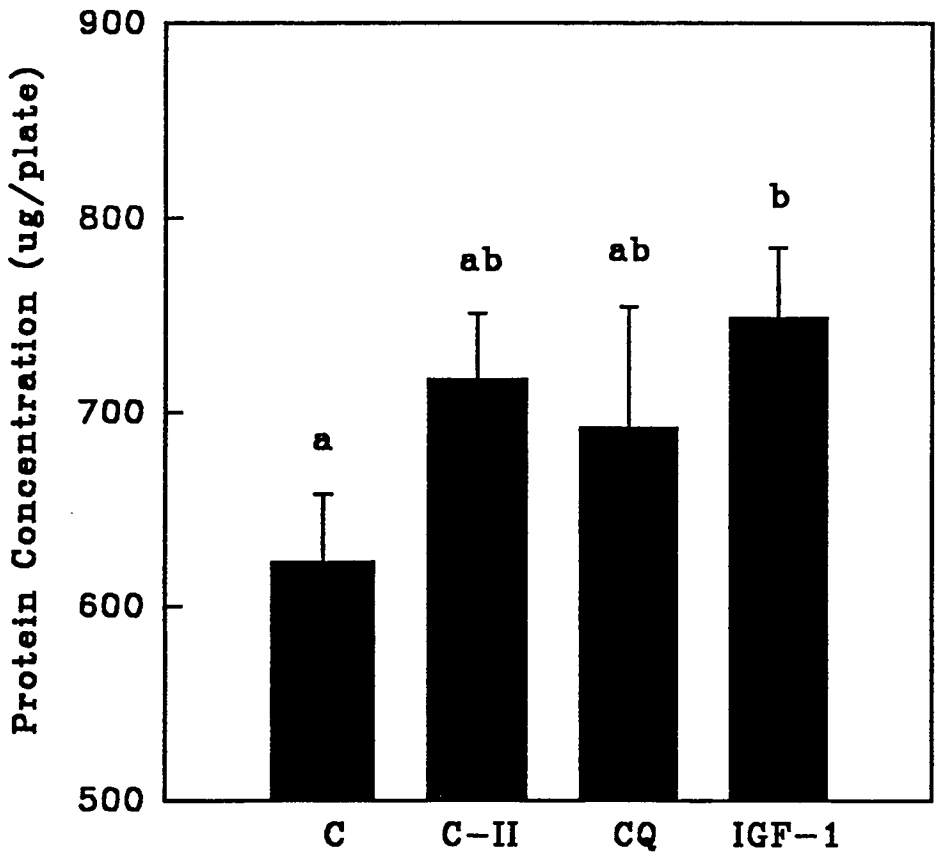


Figure 3-17. Effects of CI-II, CQ and IGF-1 on total protein accretion in L8 myotubes. Treatments which do not share a common superscript (above bar) differ significantly ($p<0.05$).

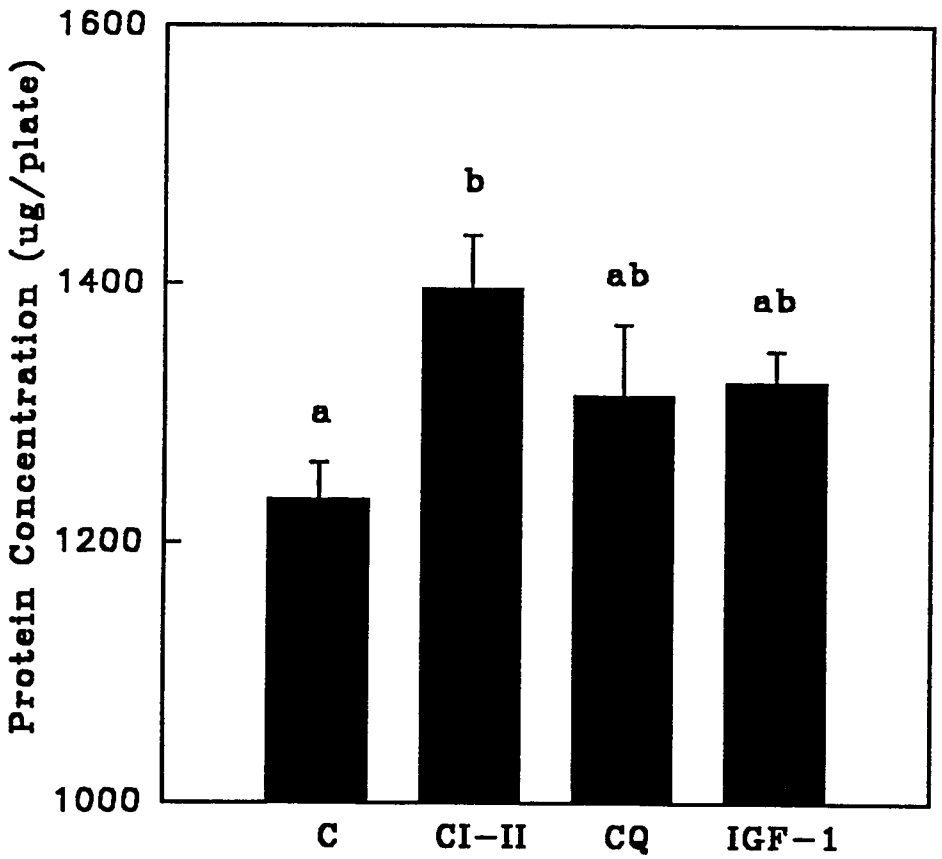


Figure 3-18. Effects of CI-II, CQ and IGF-1 on radioactivity associated with myofibrillar proteins in L8 myotubes. L8 myotubes were exposed to 5 μCi/ml ^{3}H-tyrosine for 24 hours after which they were treated with CI-II (30 μg/ml), CQ (25 μM) or IGF-1 (200 ng/ml) for an additional 24 hours. After this, myofibrillar proteins (100 μg) were recovered and separated on a 7.5-15% gradient SDS-PAGE gel. The gel was dried then exposed to X-Omat film for 3 weeks.

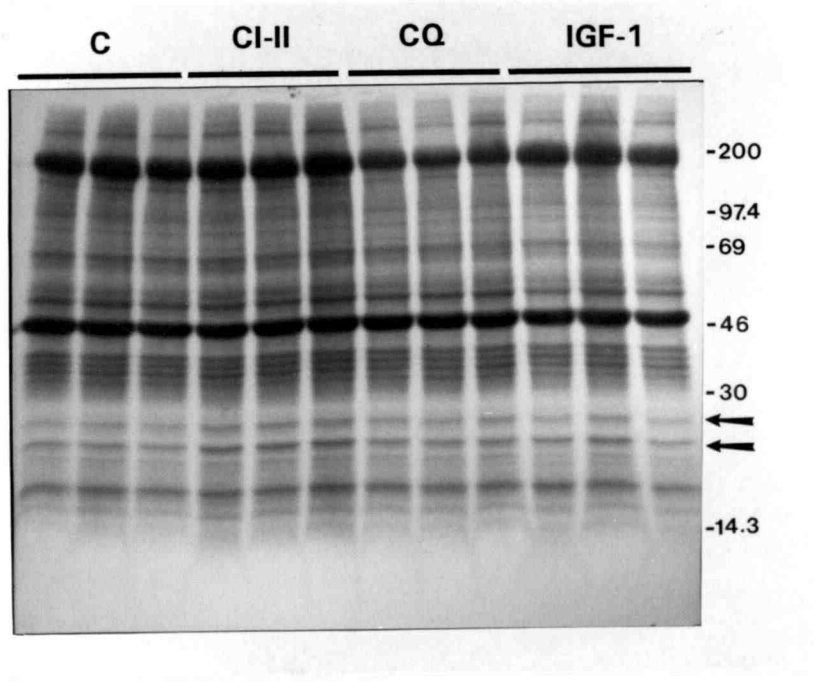


Figure 3-19. Effects of CI-II, CQ and IGF-1 on radioactivity associated with purified myofibrillar proteins in L8 myotubes. L8 myotubes were exposed to ^{3}H-tyrosine for 24 hours after which they were treated with CI-II (30 μg/ml), CQ (25 μM) or IGF-1 (200 ng/ml) for an additional 24 hours. After this, myofibrillar proteins were purified as described in Material and Methods. 100 μg myofibrillar proteins were separated on a 7.5-15% gradient SDS-PAGE gel. The gel was dried then exposed to X-Omat film for 3 weeks.

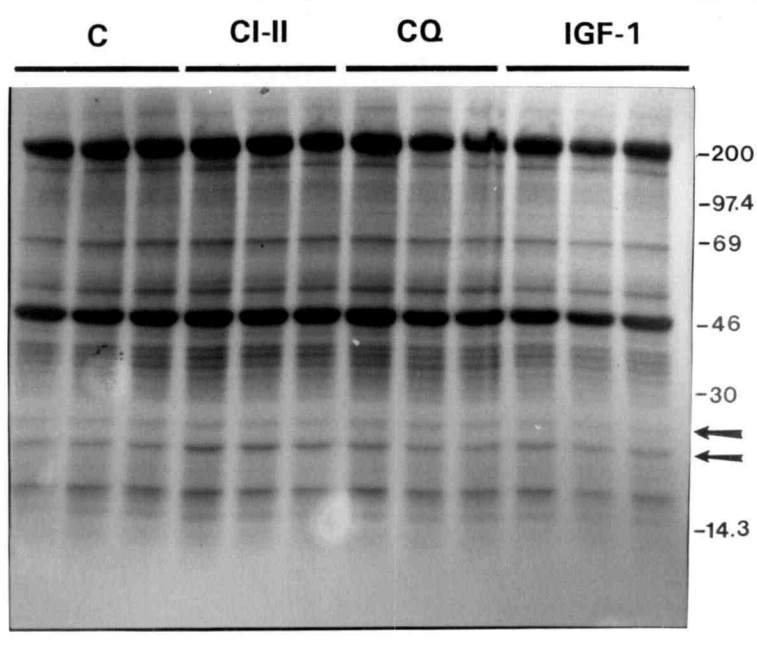


Figure 3-20. Effects of CI-II, CQ and IGF-1 on radioactivity associated with individual myofibrillar proteins in L8 myotubes. L8 myotubes were exposed to 5 μCi/ml ^{3}H-tyrosine for 24 hours after which they were treated with CI-II (30 μg/ml), CQ (25 μM) or IGF-1 (200 ng/ml) for an additional 24 hours. After this, myofibrillar proteins were recovered and separated on a 7.5-15% gradient SDS-PAGE gel. The gel was dried then individual proteins (MHC, α-actin, 25 kDa and 21 kDa proteins) were excised from the gel. After dissolving in Solvable solution, radioactivity was determined. Treatments which do not share a common superscript (above bar) differ significantly ($p<0.05$).

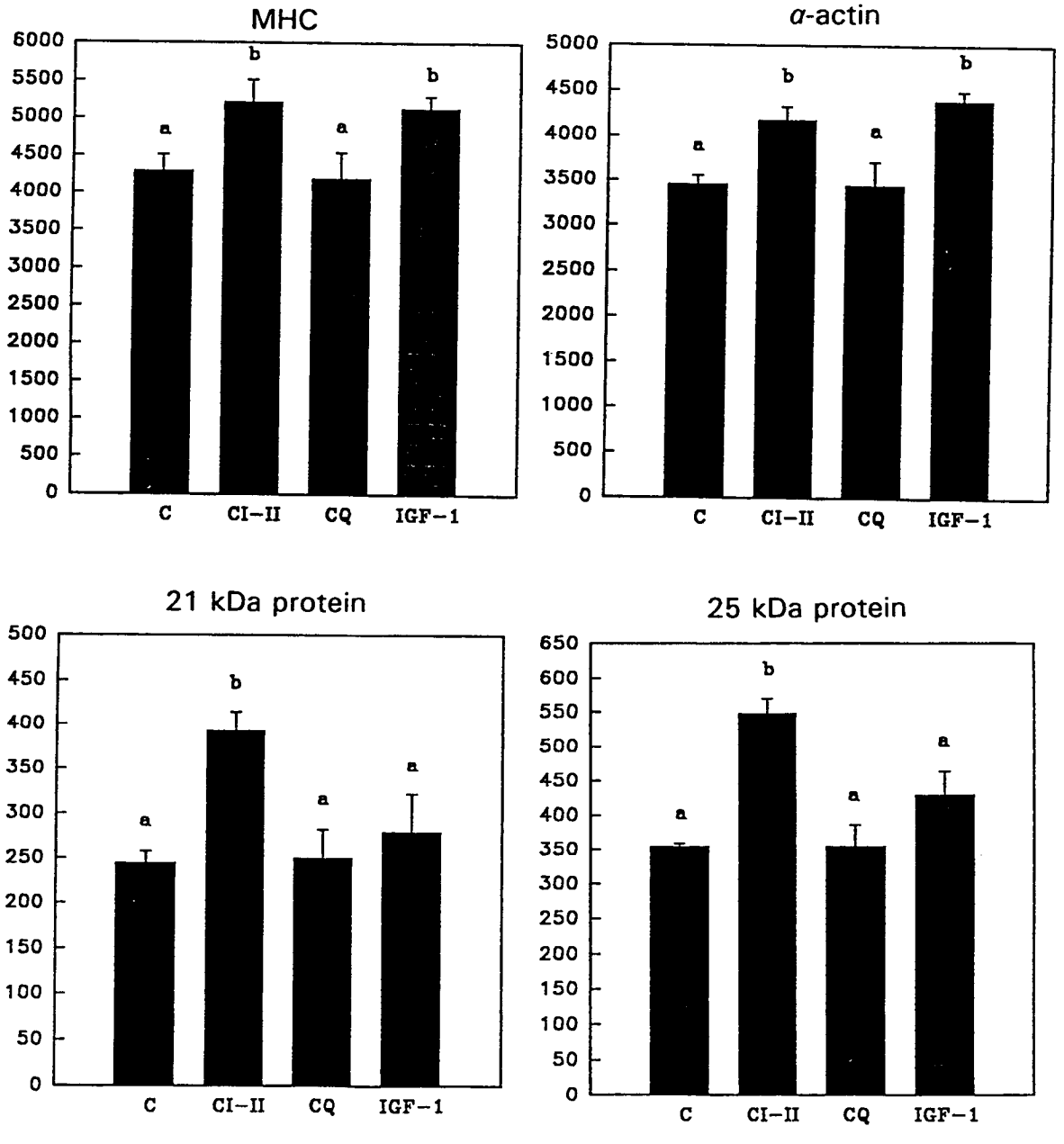
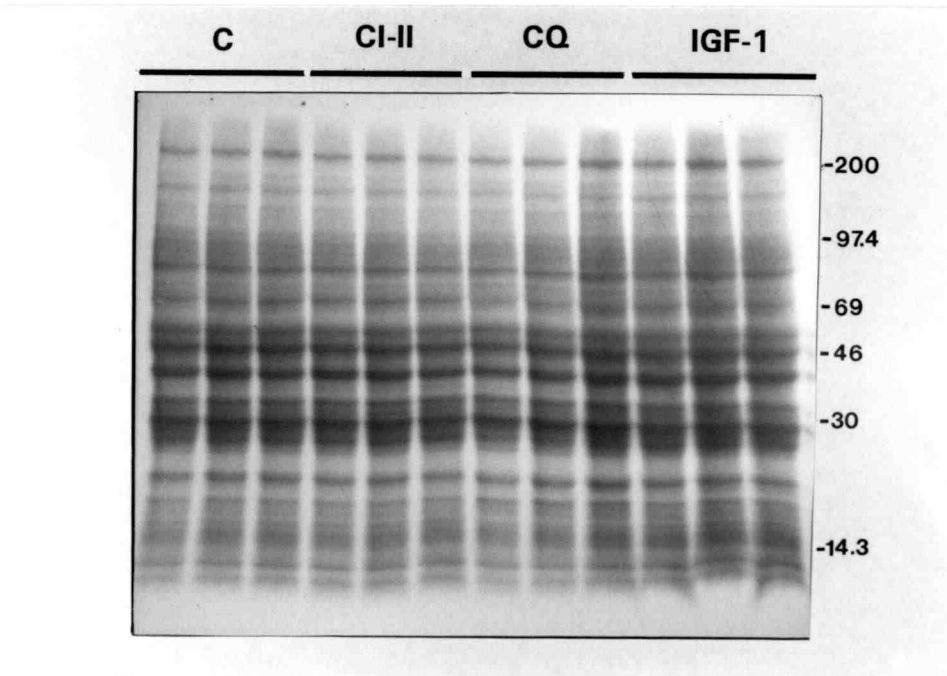


Figure 3-21. Effects of CI-II, CQ and IGF-1 on radioactivity associated with non-myofibrillar proteins in L8 myotubes. L8 myotubes were exposed to 5 μCi/ml ^{3}H-tyrosine for 24 hours after which they were treated with CI-II (30 μg/ml), CQ (25 μM) or IGF-1 (200 ng/ml) for an additional 24 hours. After this, myofibrillar proteins (100 μg) were recovered and separated on a 7.5-15% gradient SDS-PAGE gel. The gel was dried then exposed to X-Omat film for 3 weeks.



BIBLIOGRAPHY

Allen, R. E. 1986. Muscle cell growth and development. Designing Foods. Animal Product Options in the Marketplace. National Research Council, National Acad. Press. Wash. D. C. pp 142-162.

Allen, R. E., M. H. Stromer, D. E. Goll and R. M. Robson. 1979. Accumulation of myosin, actin, tropomyosin, and α-actinin in cultured muscle cells. Devel. Biol. 69:655-660.

Aoki, K., S. Imajoh, S. Ohno, Y. Emori, Y. Koike, G. Kosaki and K. Suzuki. 1986. Complete amino acid sequence of the large subunit of the low-Ca^{++}-requiring form of human Ca^{++}-activated neutral protease (μCANP) deduced from its cDNA sequence. FEBS Lett. 205:313-317.

Benyon, R. J. and J. S. Bond. 1986. Catabolism of intracellular protein: molecular aspects. Am. J. Physiol. 251:C141-C152.

Blau, H. M., G. K. Pavlath, E. C. Hardeman, C. P. Chiu, L. Silberstein, S. G. Webster, S. C. Miller and C. Webster. 1985. Plasticity of the differentiated states. Science 230:758-766.

Boiziau, C. and J. J. Toulme. 1991. Modified oligonucleotides in rabbit reticulocytes: uptake, stability and antisense properties. Biochimie 73:1403-1408.

Busch, W. A., M. H. Stromer, D. E. Goll and A. Suzuki. 1972. Ca^{2+}-specific removal of Z-lines from rabbit skeletal muscle. J. Cell Biol. 52:367-381.

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156-159.

Cohen, J. S. 1991. Oilgonucleotides as therapeutic agents. Pharmac. Ther. 52:211-225.

Cook, P. D. 1991. Medicinal chemistry of antisense oligonucleotides - future opportunities. Anti-cancer Drug Design 6:585-607.

Dahlman, B., L. Kuehn, M. Rutschmann and H. Reinauer. 1985. Purification and characterization of a multicatalytic high molecular mass proteinase from rat skeletal muscle. Biochem. J. 228:161-170.

Dayton, W. R., D. E. Goll, M. G. Zeece, R,. M. Robson and W. J. Reville. 1976a. A Ca^{2+}-activated protease possibly involved in myofibrillar protein turnover. Purification from porcine muscle. Biochemistry 15:2150-2158.

Dayton, W. R., W. J. Reville, D. E. Goll and M. H. Stromer. 1976b. A Ca^{2+}-activated protease possibly involved in myofibrillar protein turnover. Partial characterization of the purified enzyme. Biochemistry 15:2159-2167.

Dayton W. R. , J. V. Schollmeyer, R. A. Lepley and L. R. Cortes. 1981. A calcium-activated protease possibly involved in myofibrillar protein turnover. Isolation of a low calcium requiring form of the protease. Biochim. Biophys. Acta 659:48-61.

De Duve, C., T. De Barsy, B. Poole, A. Trouet, P. Tulkens and F. Van Hoof. 1974. Lysosomotropic agents. Biochem. Pharmacol. 23:2495-2531.

Deluca, C. L., P. L. Davies, J. A. Samis and J. S. Elce. 1993. Molecular cloning and bacterial expression of cDNA for rat calpain II 80 kDa subnunit. Biochim. Biophys. Acta. 1216:81-93.

DeMartino, G. N. and D. K. Blumenthal. 1982. Identification and partial purification of a factor that stimulates calcium-dependent proteases. Biochemistry 21:4297-4303.

Driscoll, J. and A. L. Goldberg. 1989. Skeletal muscle proteasome can degrade proteins in an ATP-dependent process that does not require ubiquitin. Proc. Natl. Acad. Sci. USA 86:787-791.

Emori, Y., H. Kawasaki, S. Imajoh, S. Kawashima and K. Suzuki. 1986b. Isolation and sequence analysis of cDNA clones for the small subunit of rabbit calcium-dependent proteinase. J. Biol. Chem. 261:9472-9476.

Emori, Y., H. Kawasaki, S. Imajoh, K. Imahori and K. Suzuki. 1987. Endogenous inhibitor for calcium-dependent cysteine protease contains four internal repeats that could be responsible for its multiple reactive sites. Proc. Natl. Acad. Sci. USA 84:3590-3594.

Emori, Y., H. Kawasaki, S. Imajoh-ohmi, Y. Minami and K. Suzuki. 1988. All four repeating domains of the endogenous inhibitor for calcium-dependent protease independently retain inhibitory activity. J. Biol. Chem. 263:2364-2370.

Emori, Y., H. Kawasaki, H. Sugihara, S. Imajoh, S. Kawashima and K. Suzuki. 1986a. Isolation and sequence analysis of cDNA clones for the large subunits of two isozymes of rabbit calcium-dependent proteinase. J. Biol. Chem. 261:9465-9471.

Edmondson, D. G. and E. N. Olson. 1993. Helix-loop-helix proteins as regulators of muscle-specific transcription. J. Biol. Chem. 268:755-758.

Etlinger, J. D. and A. L. Goldberg. 1977. A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal protein in reticulocytes. Proc. Natl. Acad. Sci. USA 74:54-58.

Fagan, J. M., L. Waxman and A. L. Goldberg. 1987. Skeletal muscle and liver contain a soluble ATP + ubiquitin-dependent proteolytic system. Biochem. J. 243:335-343.

Florini, J. R. and D. Z. Ewton. 1990. Highly specific inhibition of IGF-1-stimulated differentiation by an antisense oligonucleotide to myogenin mRNA. J. Biol. Chem. 265:13435-13437.

Forsberg, N. E. and N. B. Wehr. 1990. Effects of cimaterol on muscle protein metabolism and its actions in hypothyroid and hyperthyroid rats. Domestic Anim. Endocrinol. 7:149-164.

Forsberg, N. E., M. A. Ilian, A. Ali-Bar, P. R. Cheeke and N. B. Wehr. 1989. Effects of cimaterol on rabbit growth and myofibrillar protein degradation and on calcium-dependent proteinase and calpastatin activities in skeletal muscle. J. Anim. Sci. 67:3313-3321.

Fujiwara, T., K. Tanaka, A. Kumatori, S. Shin, T. Yoshimura, A. Ichihara, F. Tokunaga, R. Aruga, S. Iwanaga, A. Kakizuka and S. Nakanishi. 1989. Molecular cloning of cDNA for proteasomes (multicatalytic proteinase complexes) from rat liver: primary structure of the largest component (C2). Biochemistry 28:7332-7340.

Fuller, J. C., G. A. Link., T. W. Huiatt, J. L. Sell and S. Nissen. 1992. Disparate responses of cultured skeletal muscle cells and growing chicks to tripeptide aldehyde protease inhibitors and an in vivo interaction with ethanol. J. Nutr. Biochem. 3:291-297.

Furuno, K. and A. L. Goldberg. 1986. The activation of protein degradation in muscle by Ca^{2+} or muscle injury does not involve a lysosomal mechanism. Biochem. J. 237:859-864.

Furuno, K., M. N. Goodman, and A. L. Goldberg. 1990. Role of different proteolytic systems in the degradation of muscle proteins during denervation atrophy. J. Biol. Chem. 265:8550-8557.

Gerard, K. W. and D. L. Schneider. 1979. Evidence for degradation of myofibrillar proteins in lysosomes. Myofibrillar proteins derivatized by intramuscular injection of N-ethymaleimide are sequestered in lysosomes. J. Biol. Chem. 254:11798-11805.

Goll, D. E. 1991. Role of proteinases and protein turnover in muscle growth and meat quality. Reciprocal Meat Conference Proceeding, 4:25-36.

Goll, D. E., W. R. Dayton, I. Singh, and R. M. Robson. 1991. Studies of the actinin/actin interation in the Z-disk by using calpain. J. Biol. Chem. 266:8501-8510.

Goll, D. E., W. C. Kleese and A. Szpacenko. 1989. Skeletal muscle proteases and protein turnover. Animal Growth Regulation. Plenum Publishing Corporation. pp 141-182.

Goll, D. E., J. D. Shannon, T. Edmunds, S. K. Sathe, W. C. Kleese and P. A. Nagainis. 1983. Properties and regulation of the Ca^{2+}-dependent proteinase. Calcium-Binding Proteins. Elsevier, Amsterdam. pp 19-35.

Goll, D. E., V. F. Thompson and R. G. Taylor. 1992. Role of the calpain system in muscle growth. Biochimie 74:225-237.

Goll, D. E., R. B. Young and M. H. Stromer. 1974. Separation of subcellular organelles by differential and density gradient centrifugation. Reciprocal Meat Conference (27) pp 250.

Goodman, M. N. 1987. Acute alterations in sodium flux in vitro leads to decreased myofibrillar protein breakdown in rat skeletal muscle. Biochem. J. 247:151-156.

Gopalakrishna, R. and S. H. Barsky. 1985. Quantitation of tissue calapin activity after isolation by hydrophobic chromatography. Anal. Biochem. 148:413-423.

Gottesman, S. and M.R. Maurizi. 1992. Regulation by proteolysis: enzyme-dependent protease and their targets. Microbiol. Rev. 56:592-621.

Gulve, E. A. and J. F. Dice. 1989. Regulation of protein synthesis and degradation in L8 myotubes. Biochem. J. 260: 377-387.

Gulve, E. A., K. Mabuchi and J. F. Dice. 1991. Regulation of myosin and overall protein degradation in mouse C2 skeletal myotubes. J. Cell. Physiol. 147:37-45.

Haeuptle, M., R. Frank and B. Dobberstein. 1986. Translation arrest by oligonucleotides complementary to mRNA coding sequences yields polypeptides of predetermined length. Nucl. Acids Res. 14:1427-1448.

Harper, J. M. M., J. B. Soar and P. J. Buttery. 1987. Changes in protein metabolism of ovine primary muscle cultures on treatment with growth hormone, insulin, insulin-like growth factor I or epidermal growth factor. J. Endocrinol. 112:87-96.

Hata, A., S. Ohno, Y. Akita and K. Suzuki. 1989. Tandemly-reiterated negative enhancer-like elements regulate transcription of a human gene for the large subunit of calcium-dependent protease. J. Biol. Chem. 264:6404-6411.

Hershko, A. and A. Ciechanover. 1982. Mechanisms of intracellular protein breakdown. Ann. Rev. Biochem. 51:335-364.

Hirai, S. I., H. Kawasaki, M. Yaniv and K. Suzuki. 1991. Degradation of transcription factors, c-Jun and c-Fos, by calpain. FEBS Lett. 287:57-61.

Holt, J. F., R. L. Redner and A. W. Nienhuis. 1988. An oligomer complementary to c-myc mRNA inhibits proliferation of HL60 promyelocytic cells and induces differentiation. Mol. Cell. Biol. 8:963-973.

Homewood C. A., D. C. Warhurst, W. Peters and V. C. Baggaley. 1972. Lysosomes, pH and the anti-malarial action of chloroquine. Nature. 235:50-52.

Hong, D. Y. 1993. Endocrine control of proteolysis in cultured muscle cells. Ph.D. Thesis, Oregon State University.

Hong, D. H. and N. E. Forsberg. 1994. Effects of serum and insulin-like growth factor-1 on protein degradation and protease gene expression in rat L8 myotubes. J. Anim. Sci. in press.

Ilian, M. A. and N. E. Forsberg. 1992. Gene expression of calpains and their specific endogenous inhibitor, calpastatin, in skeletal muscle of fed and fasted rabbits. Biochem. J. 287:163-171.

Imajoh, S.,H. Kawasaki, Y. Emori, S. Ishiura, Y. Minami, H. Sugita, K Imahori and K. Suzuki. 1987. A fragment of an endogenous inhihitor produced in Escherichia coli for calcium-activated neutral protease retains an inhibitory activity. FEBS Lett. 215:274-278.

Ishida, S., Y. Emori and K. Suzuki. 1991. Rat calpastatin has diverged primary sequence from other mammalian calpastatin but retains functionally important sequences. Biochim. Biophys. Acta. 1088:436-438.

Ishiura, S., S. Tsuji, H. Murofushi and K. Suzuki. 1982. Purification of an endogenous 68000-dalton inhibitor of Ca^{++}-activated neutral protease from chicken skeletal muscle. Biochim. Biophys. Acta. 701:216.

Johnson, J. D. and D. L. Wetmore. 1984. Protein turnover in heart and diaphragm of rat fetus. Am. J. Physiol. 247:E781-E785.

Kadowaki, M., N. Harada, S. Takahashi, T. Noguchi and H. Naito. 1989. Differential regulation of myofibrillar and total protein in skeletal muscle of rats: effects of streptozotocin-induces diabetes, dietary protein and starvation. J. Nutr. 119:471-477.

Kapprell, H. P. and D. E. Goll. 1989. Effect of Ca^{++} on binding of the calpains to calpastatin. J. Biol. Chem. 264:17888-17896.

Kar, N. C. and C. M. Pearson. 1980. Elevated activity of neutral proteinase in human muscular dystrophy. Biochem. Med. 26:238-243.

Kawasaki, H., Y. Emori, S. Imajoh, Y. Minami and K. Suzuki. 1989. Identification and characterization of inhibitory sequences in four repeating domains of the endogenous inhibitor for calcium-dependent protease. J. Biochem. 106:274-281.

Kawasaki, H., Y. Emori and K. Suzuki. 1993. Calpastatin has two distinct sites for interation with calpain: effect of calpastatin fragments on the binding of calpain to membranes. Arch. Biochem. Biophys. 305:467-472.

Kay, J., L. M. Siemankowski, R. F. Siemankowski, J. A. Greweling and D. E. Goll. 1982. Degradation of myofibrillar protein by trypsin-like serine protease. Biochem. J. 201:279-285.

Kayali, A., V. R. Young, and M. N. Goodman. 1987. Sensitivity of myofibrillar proteins to glucocorticoid-induced muscle proteolysis. Am. J. Physiol. 252:E621-E626.

Kleese, W. C., D. E. Goll, T. Edmunds and J. D. Shannon. 1987. Immunofluorescent localization of the Ca^{2+}-dependent proteinase and its inhibitor in tissue of Crotalus atrox. J. Exp. Zool. 241:277-289.

Konigsberg, I. R. 1979. Skeletal myoblasts in culture. Methods Enzymol. 58:511-522.

Koohmaraie, M. 1992. Ovine skeletal muscle multicatalytic proteinase complex (proteasome): purification, characterization, and comparison of its effects on myofibrils with μ-calpains. J. Anim. Sci. 70:3697-3708.

Kumamoto, T., S. Araki, I. Toyoshima and N. Fukuara. 1984. Experimental chloroquine myopathy-morphological and biochemical studies. Clin. Neutrol. 24:505-512.

Laemmli U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lee, F., R. Mulligan, P. Berg and G. Ringold. 1981. Glucocorticoids regulate expression of dihydrofolate reductase cDNA in mouse mammary tumour virus chimaeric plasmids. Nature 294:228-234.

Lewis, S. E. M., P. Anderson and D. F. Goldspink. 1982. The effects of calcium on protein turnover in skeletal muscle of the rats. Biochem. J. 204:257-264.

Libelius, R., J. O. Josefsson and I. Lundquist. 1979. Endocytosis in chronically denervated mouse skeletal muscle. A biochemical and ultrastructural study with horseradish peroxidase. Neuroscience 4:283-290.

Liebhaber, S. A., J. E. Russell, F. Cash and S. S. Eshelman. 1991. Intramolecular duplexes in eukaryotic mRNA supress translation in a position-dependent manner. J. Cell. Biochem. 15D:CD 007.

Loke, S. L., C. A. Stein, X. H. Zhang, M. Nakanishi, C. Subasinghe, J. S. Cohen and L. M. Neckers. 1989. Characterization of oligonucleotide transport into living cells. Proc. Natl. Acad. Sci. USA 86:3473-3478.

Maher, L. J. and B. J. Dolnick. 1987. Specific hybridization arrest of dihydrofolate reductase mRNA in vitro using anti-sense RNA or anti-sense oligonucleotides. Arch. Biochem. Biophys. 253:214.

Malik, M. N., M. D. Fenko, K. Iqbal and H. M. Wisniewski. 1983. Purification and characterization of two forms of Ca^{2+}-activated neutral protease from calf brain. J. Biol. Chem. 258:8955-8962.

Maniatis, T. E., F. Frisch and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor.

Matsukura, U., A. Okitani, T. Nishimuro and H. Kato. 1981. Mode of degradation of myofibrillar proteins by an endogenous protease, cathepsin L. Biochim. Biophys. Acta 662:41-47.

Miner, J. H. and B. J. Wold. 1991. c-myc inhibition of MyoD and myogenin-initiated myogenic differentiation. Mol. Cell. Biol. 11:2842-2851.

Miyake, S., Y. Emori and K. Suzuki. 1986. Gene organization of the small of human calcium-activated neutral protease. Nucleic acids Res. 14:8805-8817.

Murachi, T. 1983. Calpain and calpastatin. Trends Biochem. Sci. 8:167-169.

Obinata, T., K. Maruyama, H. Sugita, K. Kohama and S. Ebashi. 1981. Dynamic aspects of structural proteins in vertebrate skeletal muscle. Muscle Nerve 4: 456-462.

Murakami Y., S. Matsufuji, T. Kameji, S. Hayashi, K Igarashi, T. Tamura, K. Tanaka and A. Ichihara. 1992. Orthnithine decarboxylase is degraded by the 26S proteasome without ubiquitination. Nature 360:597-599.

Nadal-Ginard, B. 1978. Commitment, fusion and biochemical differentiation of a myogenic cell line in the absence of DNA synthesis. Cell 15:855-864.

O'Farrell P. H. 1975. High resolution two-dimentional electrophoresis of proteins. J. Biol. Chem. 250:4007-4021.

Ohno, S., Y. Emori, S. Imajoh, H. Kawasaki, M. Kisaragi and K. Suzuki. 1984. Evolutionary origin of a calcium-dependent proteinase by fusion of gene for a thiol-protease and calcium-binding protein. Nature 312:566-570.

Ohno, S., Y. Emori and K. Suzuki. 1986. Nucleotide sequence of a cDNA coding for the small subunit of human. Nucleic Acids Res. 14:5559.

Orlowski, M. 1990. The multicatalytic proteinase complex, a major extralysosomal proteolytic system. Biochemistry. 29:10289-10297.

Otsuka, Y. and D. E. Goll. 1987. Purification of the Ca^{++}-dependent proteinase inhibitor from bovine cardiac muscle and its interaction with the millimolar Ca^{++}-dependent proteinase. J. Biol. Chem. 262:5839-2851.

Ou, B. R. and N. E. Forsberg. 1991. Determination of skeletal muscle calpain and calpastatin activities during maturation. Am. J. Physiol. 261:E677-E683.

Ou, B. R., H. H. Meyer and N. E. Forsberg. 1991. Effects of age and castration on activities of calpains and calpastatin in sheep skeletal muscle. J. Anim. Sci. 69:1919-1924.

Pontremoli, S. and E. Melloni. 1986. Extralysosomal protein degradation. Ann. Rev. Biochem. 55:455-481.

Pontremoli, S., E. Melloni, M. Michetti, B. Sparatore, F. Salamino, N. Siliprandi and B. L. Horecker. 1987. Isovalerylcarnitine is a specific activator of calpain of human neutrophils. Biochem. Biophys. Res. Comm. 148:1189-1195.

Pontremoli, S., E. Melloni, M. Michetti, F. Salamino, B. Sparatore and B. L. Horecker. 1988. An endogenous activator of Ca^{2+}-dependent proteinase of human neutrophils that increases its affinity for Ca^{2+}. Proc. Natl. Acad. Sci. USA 85:1740-1743.

Publicover, S.J., C. J. Duncan and J. L. Smith. 1978. The use of A23187 to demonstrate the role of intracellular calcium in causing ultrastructural damage in mammalian muscle. J. Neuropathol. Exp. Neurol. 37:544-550.

Reeds. P. J., S. M. Hay, P. M. Dorwood and R. M. Palmer. 1986. Stimulation of muscle growth by clenbuterol: lack of effect on muscle protein biosynthesis. Br. J. Nutr. 56:249-258.

Reed, J. C., C. A. Stein, C. Subasinghe, S. Haldar, C. M. Croce, S. Yum and J. S. Cohen. 1990. Antisense-mediated inhibition of BCL2 proto-oncogene expression and leukemic cell growth; comparisons of phosphorothioate oligonucleotides. Cancer Res. 50:6565-6570.

Richler, C. and D. Yaffe. 1970. The in vitro cultivation and differentiation capacities of myogenic cell lines. Devel. Biol. 23:1-22.

Roeder, R. A., K. L. Hossner, R. G. Sasser and J. M. Gunn. 1988. Regulation of protein turnover by recombinant human insulin-like grwoth factor-I in L6 myotube cultures. Horm. Metabol. Res. 20:698-700.

Roeder, R. A., S. D. Thorpe, F. M. Byers, G. T. Schelling and J. M. Gunn. 1986. Influence of anabolic agents on protein synthesis and degradation in muscle cells grown in culture. Growth 50:485-495.

Sakihama, T., H. Kakidani, K. Zenita, N. Yumoto, T. Kukichi, T. Sasaki, R. Kannagi, S. Nakanishi, M. Ohmori, K. Takio, K. Titani and T. Murachi. 1985. A putative Ca^{2+}-binding protein:structure of the light subunit of porcine calcium elucidated by molecular cloning and protein sequence analysis. Proc. Natl. Acad. Sci. USA 82:6075-6079.

Salamino, F., R. De Tullio, P. Mengotti, P. L. Viotti, E. Melloni and S. Pontremoli. 1993. Site-directed activation of calpain in promoted by membrane-associated natural activator protein. Biochem. J. 290:191-197.

Sartorius, C. and R. M. Franklin. 1991. The use of antisense oligonucleotides as chemotherapeutic agents for parasites. Paras. Today 7:90-93.

Schwartz, W. N. and J. W. C. Bird. 1977. Degradation of myofibrillar proteins by cathepsins B and D. Biochem. J. 167:811-820.

Silver, G., and J. D. Etlinger. 1985. Regulation of myofibrillar accumulation in chick muscle cultures: evidence for the involvement of calcium and lysosomes in non-uniform turnover of contractile proteins. J. Cell Biol. 101:2383-2391.

Smidt, P., T. L. Doan, S. Falco and T. Berkel. 1991. Association of antisense oligonucleotides with lipoproteins prolong the plasma half-life and modifies the tissue distribution. Nucl. Acids Res. 19:4695-4700.

Sorimachi, H., S. Ishiura and K. Suzuki. 1993a. A novel tissue-specific calpain species expressed predominantly in the stomach comprises two alternative splicing products with and without Ca^{2+}-binding domain. J. Biol. Chem. 268:19476-19482.

Sorimachi, H., S. I. Ohmi, Y. Emori, H. Kawasaki, S. Ohno, Y. Minami and K. Suzuki. 1989. Molecular cloning of a novel mammalian calcium-dependent protease distinct from both m- and μ-type. J. Biol. Chem. 264:20106-20111.

Sorimachi, H. and K. Suzuki. 1992. Sequence comparison among muscle-specific calpain, p94, and calpain subunits. Biochim. Biophys. Acta. 1160:55-62.

Sorimachi, H., N. Toyama-Sorimachi, T. C. Saido, H. Kawasaki, H. Sugita, M. Miyasaka, K. Arahata, S. Ishiura and K. Suzuki. 1993b. Muscle-specific calpain, p94, is degraded by autolysis immediately after translation, resulting in dispearance from muscle. J. Biol. Chem. 268:10593-10605.

Steel D. M. and H. Harris. 1989. The effect of antisense RNA to fibronectin on the malignancy of hybrids between melanoma cells and normal fibroblasts. J Cell Sci. 93:515-524.

Steel, R. G. D. and L. H. Torrie. 1980. Principles and Procedures of Statistics. Mcgraw-Hill Book Co., Inc., New York.

Sugita, H., S. Ishiura, K. Suzuki and K. Imahori. 1980. Ca^{2+}-activated neutral protease and its inhibitors: in vitro effect on intact myofibrils. Muscle Nerve 3:335-345.

Suzuki, K. 1987. Calcium-activated neutral protease: domain structure and activity regulation. Trends Biochem. Sci. 12:103-105.

Suzuki, K., S. Imajoh, Y. Kawasaki, Y. Minami and S. Ohno. 1988. Regulation of activity of calcium-activated neutral protease. Adv. Enzyme Reg. 27:153-169.

Suzuki, K. and S. Ohno. 1990. Calcium activated neutral protease--structure-function relationship and functional implications--. Cell Structure and Function 15:1-6.

Takio, K., T. Towatori, N. Katunuma, D. C. Teller and K. Tirani. 1983. Homology of amino acid sequences of rat liver cathepsin B and H with that of papain. Proc. Natl. Acad. Sci. U.S.A. 80:3666-3670.

Tanaka, K., T. Tamura, T. Yoshimura and A. Ichihara. 1992. Proteasomes: protein and gene structures. New Biologist. 4:173-187.

Tanaka, K., T. Yoshimura, A. Ichihara, A. Ikai, M. Nishigai, M. Morimoto, M. Sato, N. Tanaka, Y. Katsube, K. Kameyama and T. Takagi. 1988. Molecular organization of a high molecular weight multi-protease complex from rat liver. J. Mol. Biol. 203:985-996.

Teichman-Weinberg, A., U. Z. Littauer and I. Ginzburg. 1988. The inhibition of neurite outgrowth in PC12 cells by tubulin antisense oligodeoxyribonucleotides. Gene 72:297-307.

Thinakaran, G. and J. Bag. 1991. Alterations in the expression of muscle-specific genes mediated by troponin C antisense oligonucleotide. Exp. Cell Res. 192:227-235.

Tomas, F. M., S. E. Knowles, P. C. Owens, L. C. Read, C. S. Chandler, S. E. Gargosky and F. J. Ballard. 1991. Increased weight gain, nitrogen retention and muscle protein synthesis following treatment of diabetic rats with insulin-like growth factor (IGF)-1 and des(1-3)IGF-1. Biochem. J. 276:547-554.

Towbin, H., T. Staehelin and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamine gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350-4354.

Ugai, S. I., T. Tamura, N. Tanahashi, S. Takai, N. Komi, C. H. Chung, K. Tanaka and A. Ichihara. 1993. Purification and characterization of the 26S proteasome complex catalyzing ATP-dependent breakdown of ubiquitin-ligated proteins from rat liver. J. Biochem. 113:754-768.

Van der Westhuyzen, D. R., K. Matsumoto and J. D. Etlinger. 1981. Easily releasable myofibrillar assembly and turnover. J. Biol. Chem. 256:11791-11797.

Vernon, B. G. and P. J. Buttery. 1978. Protein turnover in rats treated with trenbolone acetate. Br. J. Nutr. 36:575-579.

Wakelam, M. J. O. The fusion of myoblasts. Biochem. J. 228:1-12, 1985.

Whalen, R. C., K. Schwartz, P. Bouveret, S. M. Sell and F. Gros. 1979. Contractile muscle isozymes in muscle development: identification of an embryonic form of myosin heavy chain. Proc. Natl. Acad. Sci. USA 76:5194-5201.

Wibo, M. and B. Poole. 1974. Protein degradation in cultures cells. II. The uptake of chloroquine by rat fibroblasts and inhibition of cellular protein degradation and cathepsin B. J. Cell. Biol. 63:430-440.

Wickstorm, E. L., T. A. Bacon, A. Gonzalez, D. L. Freeman, G. H. Lyman. 1988. Human promyelocytic leukemia HL-60 cell proliferation and c-myc protein expression are inhibited by an antisense pentadecadeoxynucleotide targeted against c-myc mRNA. Proc. Natl. Acad. Sci, USA 85:1028.

Widnell, C. C. and K. H. Pfenninger. 1989. Essential Cell Biology. Williams and Wilkins, Baltimore.

Wilcox, D. and R. W. Mason. 1992. Inhibition of cysteine proteinases in lysosomes and whole cells. Biochem. J. 285:4995-502.

Wolfe, F. H., S. K. Sathe, D. E. Goll, W. C. Kleese, T. Edmunds and S. M. Duperret. 1989. Chicken skeletal muscle has three Ca^{2+}-dependent proteinases. Biochem. Biophys. Acta 998:236-250.

Wolitzky, B. A., M. S. Hudecki and H. L. Segal. 1984. Turnover of myofibrillar proteins in cultured muscles from normal and dystrophic chick embryos. Biochim. Biophys. Acta 803:106-114.

Yakubov, L. A., E. A. Deeva, V. F. Zarytova, A. S. Ryte, L. V. Yurchenko and V. V. Vlassov. 1989. Mechanism of oligonucleotide uptake by cells: involvement of specific receptor? Proc. Natl. Acad. Sci. USA 86:6454-6458.

Yaffe, D. 1968. Retention of differentiation potentialities during prolonged cultivation of myogenic cells. Proc. Natl. Acad. Sci. USA 61:477-483.

Yeh, J. Y., B. R, Ou and N. E. Forsberg. 1994. Effects of dexamethasone on muscle protein homeostasis and on calpain and calpastatin activities and gene expression in rabbit. J. Endocrinol. 141:209-217.

Yoshimura, N., T. Murachi, R. Health, J. Kay, B. Jasani and G. R. Newman. 1986. Immunogold electron-microscopic localization of calpain I in skeletal muscle of rats. Cell Tissue Res. 244:265-270.

Zeman, R. J., T. Kameyama, K. Matsumoto, P. Bernstein and J. D. Etlinger. 1985. Regulation of protein degradation in muscle by calcium. Evidence for enhanced non-lysosomal proteolysis associated with elevated cystolic calcium. J. Biol. Chem. 260:13619-13624.

Zon, G. 1990. Innovations in the use of antisense oligonucleotides. AIDS: Anti-HIV Agents, Therapies, and Vaccines 616:161-172.