AN ABSTRACT OF THE THESIS OF

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Title: Calcium Dependent Proteinase (Calpain) And Muscle Protein Degradation: Molecular Approach

Abstract approved: Redacted for Privacy

Dr. Neil Forsberg

In search of the relation of calpains to myofibrillar protein degradation in skeletal muscle and the regulation of their activity in vivo, I studied the effect of fasting on gene expression of calpains and calpastatin in skeletal muscle of rabbits. In response to fasting, myofibrillar protein degradation rate increased 2-fold, and mRNA levels of calpain I, calpain II and calpastatin around 6-fold, but calpains and calpastatin activities remained unchanged. To investigate this discrepancy, I analyzed polysomal calpains' mRNA. Results indicated that fasting caused 2-fold increase in the loading of calpain I and II mRNAs on the ribosome. Thus, the up-regulation of gene expression for calpain I and calpain II during fasting may be an important mechanism in modulating rate of protein synthesis. The effect of fasting on calpains and calpastatin mRNA expression is shared by cathepsin D and proteasome C2 but not by β-actin, implying that fasting invokes control of several proteolytic systems in skeletal muscle and underscores the possibility that each proteolytic system plays a role(s) in the adaptation of skeletal muscle to the fasted state.

Furthermore, co-regulation of activity of the calpain system at the transcriptional level in skeletal muscle has not previously been studied. Accordingly, I investigated effects of fasting, which increases myofibrillar protein degradation, on steady-state mRNA levels encoding calpain I and II large subunit, calpain small subunit and calpastatin using Northern blot analyses. Parallel to this, I determined changes in mRNA levels of cathepsin D, proteasome and β-actin for comparative purposes. Results indicated that fasting induced expression of calpain large subunits and small subunit, calpastatin, cathepsin D and proteasome mRNAs and reduced expression of β-actin mRNA in skeletal muscle. To determine whether the regulation observed in skeletal muscle was also present in other tissues, I examined effects of fasting on proteinase mRNA concentrations in liver, lung and kidney. Results revealed that fasting either caused no effect or reduced mRNA levels for the proteinases investigated. Based on these results and those of others, a model for calpain activation, the "Triarchy Model", was proposed.

Finally, I investigated the effect of growth hormone (GH) on expression of calpains, calpastatin and cathepsin D in skeletal muscle, as major proteases involved in myofibrillar protein degradation. GH reduced total RNA levelsin skeletal muscle ($P<0.05$) but had no effect on cytoplasmic protein level. Furthermore, GH caused a 40% reduction in mRNA levels encoding cathepsin D in skeletal muscle. However, GH did not affect cathepsin D activity. Calpain I and calpain II and their specific inhibitor calpastatin activities as well as calpain I mRNA levels in skeletal muscle were unaffected by GH treatment. In conclusion, GH may be exerting its anabolic activity in part by down-regulating protease activities. Further work needs to be done to investigate this effect.

Calcium Dependent Proteinase (Calpain) and Muscle Protein Degradation: Molecular Approach

by

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DEDECATED TO MY TEACHERS...

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CHAPTER I

HISTORICAL BACKGROUND

Proteins present in skeletal muscle, as in other tissues, are subject to continuous turnover. The overall rate of protein degradation in this tissue, like the rate of synthesis, is precisely controlled. This fundamental concept, that muscle protein is in a constant state of flux, was recognized 50 years ago by Schoenheimer and Rittenberg (1940). As increased efficiency of muscle growth and modification of the lean and fat remain outstanding goals of the livestock industry, it is generally held that the balance between protein synthesis and breakdown in muscle must be altered for these goals to be fulfilled. During normal growth, synthesis and degradation tend to move in parallel, with the synthesis rate exceeding the degradation rate (Rennie, 1985). Because these processes seem to be coupled, most studies during the period from 1940 to 1980 focused on increasing the rate of muscle growth by increasing the rate of protein synthesis (Beerman, 1989). Consequently, knowledge about the mechanism of muscle protein synthesis and how it is regulated has advanced dramatically during this period.

Strategies for manipulating degradation rate in muscle started to receive attention after it was learned in 1969 that the rate of muscle protein degradation can vary over a wide range in response to physiological demand (Goldberg, 1969a,b). If degradation rate could be decreased, net rate of protein accretion would accelerate and less energy would be expended on resynthesizing degraded protein.

Muscle protein degradation is achieved by several different pathways. It is now well-established that skeletal muscle contains multiple proteolytic pathways (Goll et al., 1989). These include lysosomal cathepsins, an ATP-dependent proteolytic system and calpains. Although various proteolytic systems mentioned above must be involved in muscle protein degradation, the precise role and substrates of these enzymes remain unclear. Since this report deals with calpains and muscle protein degradation, we will restrict our discussion in this chapter to the historical background implicating calpains in the initiation of myofibrillar protein degradation.

The history of calpains as factors initiating myofibrillar protein degradation began in 1969-1972 when Wayne Busch reported that Ca^{2+} induced specific degradation of Z-lines from rabbit skeletal muscle (Busch et al., 1972). Progress toward elucidation of the nature of the Ca^{2+}-activated sarcoplasmic fraction (CASF) responsible for the dissolution of Z-disk proteins in muscle gained considerable impetus after E. Krebs suggested to Darrel Goll that his lab had earlier worked with a Ca^{2+}-activated protease called kinase-activating factor (KAF) that activated phosphorylase kinase (Huston and Krebs, 1968). Preliminary work suggested that KAF was similar to CASF; however, complete purification of CASF at that time was not achieved due to technical difficulties.

In the period from 1972 to 1976, Bill Dayton succeeded in purifying CASF to homogeneity and elucidated its biochemical characteristics (Dayton et al., 1976a,b).

Despite the evidence presented by Dayton's work for the role of CASF, which is known now as calpain II, in myofibrillar protein turnover, the biological significance of calpain II was questioned since intracellular concentrations of calcium never reached the mM concentrations required for CASF activation. In 1980 a new dimension was added to calpain when Ron Mellgren published his paper describing the presence of a second Ca^{2+}-dependent protease now called calpain I that required μM Ca^{2+} levels for activation (Mellgren, 1980). The list of calpains did not end here, as in 1988 a third form of the calpain family, which requires high Ca^{2+} for its activity, was purified from chicken muscle (Wolfe et al., 1988). In the year 1990 a new member of the calpain enzymes (novel calpain) was characterized in rat brain (Yoshihiro et al. 1990). This proteinase is believed to be involved in production of the neuropeptide kyotrophin from calpastatin fragments.

Shortly after calpain II was purified from porcine skeletal muscle, it was realized that skeletal muscle also contained a protein inhibitor of the calpains (Okitani et al., 1976). The clear demonstration of the occurrence of a specific endogenous inhibitor for calpain, correctly named calpastatin, appeared almost concurrently from two independent laboratories (Waxman and Krebs, 1978; Nishiura et al., 1978). As with calpains, considerable controversy surrounded calpastatin with regard to its size. Human erythrocyte calpastatin migrated with a relative molecular mass of 70 kDa on SDS-polyacrylamide gels, whereas the calpastatins from cardiac and skeletal muscle and liver migrated with a relative molecular mass of 110 kDa on SDS-PAGE. These differences were not resolved until the cDNAs for calpastatin from pig (Takano et al., 1986) and rabbit (Emori et al., 1987) were cloned and sequenced. These results

showed that the primary translation product of calpastatin mRNA is a 718 amino acid precursor form; however, it is proteolytically processed to either a 110-kDa protein (liver type) or a 70-kDa protein (erythrocyte type).

To add to the complexity of the calpain system, a protein factor that stimulates proteolytic activity of calpains in the presence of Ca^{2+} has been identified (De Martino and Blumenthal, 1982; Takeyama et al., 1986). The endogenous stimulatory factor of calpain was first described by De Martino and Blumenthal in 1982 in experiments designed to test the possible effect of calmodulin on the proteinases. Although calmodulin had no effect on proteinase activity, some calmodulin preparations contained a contaminant that greatly increased proteinase activity. This factor has been isolated from bovine brain and partially purified. Many of its physical properties are similar to those of calmodulin, which explains why calmodulin prepared by conventional protein purification techniques contained this protein. The factor increases the proteolytic activity of each calpain 5- to 25-fold, but has no effect on any other protease that has been tested (trypsin, chymotrypsin, papain and macropain).

The most recent achievement is the characterization of a skeletal muscle specific calpain isozyme, designated p94 (Sorimachi et al., 1990). The mRNA for p94 is expressed only in skeletal muscle, which makes this protein particularly interesting with regard to control of muscle growth and development. Progress towards the characterization of p94 by purifying the protein and studying its biochemical properties coupled with characterization of p94 gene structure is underway in K.Suzuki's lab.

This knowledge, once available, will greatly facilitate understanding of the physiological function of p94 in skeletal muscle. Thus, the calpain system has in 15 years evolved from a single proteolytic enzyme to a seven-protein system. Considerable knowledge available on the amino acid sequences and many of the biochemical properties of the seven protein are known.

During the last 11 years, it has become obvious that the calpain system is not muscle-specific enzyme system but rather is ubiquitously distributed and exists in most if not all vertebrate animals (Murachi, 1984).

Despite the remarkable achievements in characterization of calpain structures and biochemical properties, the nature of their function and how activity of the calpain system is regulated in vivo, however, remain an enigma. Based on in vitro studies, Murachi (1989) has grouped the possible substrates of the calpains into three categories: (1) cytoskeletal proteins, (2) hormone receptors and (3) enzymes. At the present time, it is not easy to envision a common function involving all three of these categories of substrates. Because a protein is rapidly degraded in vitro does not necessarily indicate that the protein is a physiological substrate of the calpains in vivo. Consequently, the physiological substrates and function of the calpain system are very unclear at the present time.

Ubiquitous distribution of the calpain system in animal tissues suggests a constitutive "housekeeping" function of calpain in different cells, such as signal transduction. On the other hand, distinct variability in the calpains' absolute as well

as relative abundance from one tissue to the other and the expression of tissue-specific forms (i.e. p94 in skeletal muscle) is a clear indication of the individual, highly diversified significance of the calpain system in different tissues, such as initiation of myfibrillar protein degradation in skeletal muscle.

It seems likely that the extraordinary progress made during the past 18 years towards elucidating calpain structure and biochemical properties will be paralleled by an equally intensive effort during the next two decades to learn the physiological function of the calpain system and how its activity is regulated in living cells. The broad objective of conducting the research reported herein is to gain an understanding of the molecular control of activity regulation of calpains and the relation between calpains and myofibrillar protein degradation. An understanding of the process should facilitate our ability to manipulate protein degradation in muscle, which could be beneficial in ameliorating muscle disease and improving animal productivity.

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CHAPTER II

INTRODUCTION

Proteins of skeletal muscle, like other cellular proteins, are subject to continuous turnover (Rennie, 1985). The overall rate of protein breakdown in muscle, like the rate of synthesis, is precisely controlled. The regulation of proteolysis in muscle is important in overall energy homeostasis (Kettelhut et al., 1988), in the control of muscle mass and body growth (Allen, 1988) and also in adaptations of the organism to a variety of stressful conditions (Rennie, 1985).

Classification of Muscle Proteins

Muscle proteins can be classified, in a broad sense, into myofibrillar and non-myofibrillar or sarcoplasmic proteins. Myofibrillar proteins constitute approximately 60-70% of the proteins in muscle and, in the basal state, their degradation is several times slower than that of non-myofibrillar proteins (Kadowaki et al., 1989).

However, under conditions leading to muscle hypotrophy, such as food deprivation (Kettelhut et al., 1988) or hypertrophy (Forsberg et al., 1989), myofibrillar protein breakdown can change disproportionately to that of the non-myofibrillar pool. Obviously, the rate of protein breakdown must ultimately be dependent on the activities of muscle proteolytic enzymes. The general goal of this research is aimed at investigating the role of calpains in myofibrillar protein degradation and mechanisms regulating their activity in vivo.

Pathways of Protein Breakdown in Muscle

Knowledge about the mechanisms of intracellular proteolysis has advanced considerably in recent years (Pontremoli and Melloni, 1986; Bond and Butler, 1987). It is now well established that mammalian cells contain multiple proteolytic pathways, presumably to serve distinct physiological functions (Dice, 1987).

In muscle, a variety of proteolytic systems have been characterized. These include lysosomal proteases (Gerard et al., 1988) collectively known as cathepsins, neutral serine protease (Rechsteiner et al., 1987), ATP-dependent proteases which are both ubiquitin-dependent (Hershko, 1988) and independent (Driscoll and Goldberg, 1989) and calcium-dependent proteinase (Goll et al., 1985). Despite the voluminous literature on the biochemistry of these proteolytic enzymes, the roles of these enzymes in myofibrillar and non-myofibrillar protein degradation may be best described as controversial (Gerard et al., 1988, Lowell et al., 1986, Arakawa et al., 1983, Goodman, 1987). Hence, identification of the proteolytic systems involved in myofibrillar and non-myofibrillar proteins of skeletal muscle is an important task for the future. The research pursued in this thesis was aimed at determining the role of calpains in muscle protein turnover by utilizing models of accelerated and attenuated muscle protein degradation rate. Fasting and growth hormone treatments as models for accelerated and attenuated myofibrillar protein turnover will be discussed in detail in Chapters III and V, respectively.

Overview on Calpains

The first evidence for a calpain was provided by Huston and Krebs (1968) who reported a calcium-activated protease that phosphorylase kinase. In 1976, Dayton et al. purified from porcine skeletal muscle a calpain which was found to have restricted proteolytic activity on troponin I and T and to release α-actinin (intact) from the Z-lines of muscle (Dayton et al., 1976a,b). They suggested that it played a role in myofibrillar protein degradation. Similar enzymes have now been isolated from virtually every cell type known (e.g. brain, liver, heart, kidney, skeletal, smooth and cardiac muscles, blood cells, oviduct, nerve tissue, spinal cord and even tumor cells) (Pontremoli and Melloni, 1986).

In 1980, a second form of the enzyme was detected (Mellgren, 1980) and this has also now been purified from a variety of tissues including cardiac (Szpacenko et al., 1981) and skeletal (Dayton et al., 1981) muscles. The major operational distinction between the two forms of the enzyme is that, while the original form required high (millimolar) concentrations of Ca^{+2} for maximal activity, the new form is activated by much lower (i.e. micromolar) concentrations of Ca^{+2}. In this text, the original form will be designated calpain II and the new form calpain I. Since their discovery, a wide and intriguing range of functions have been ascribed to the calpains. These include roles in membrane protein turnover (Mellgren, 1987), enzyme activation (Suzuki et al., 1984), processing of receptors for hormones or growth factors (Ek and Heldin, 1986) and myofibrillar protein degradation (Goll et al., 1989).

Cellular Functions of Calpain

In contrast to the outstanding advances in the chemistry of calpain and calpastatin, the true function of the calpain-calpastatin system still remains largely obscure. Most hypotheses concerning the physiological role of calpain have been deduced from the susceptibility of several proteins to in vitro proteolytic modification by calpains (Pontremoli and Melloni, 1986; Suzuki et al., 1987a; Mellgren, 1987; Murachi, 1989). The results obtained indicate that calpain is involved in the processing of constituent proteins of cell membranes, cytoskeleton, neurofilaments, and of intermediate filaments and microtubules. These observations have been extrapolated to a participation of calpain in the turnover of myofibrillar proteins, in the rearrangement of submembrane-cytoskeleton-connecting proteins, in microtubule assembly and, more generally, in the dynamic reorganization of the intracellular network that occurs during specialized cell function, including movement of secretory granules (Perrin et al., 1987) and mitosis (Schollmeyer, 1988). Calpain has also been implicated in the modulation, through selective limited proteolysis, of membrane receptors, Ca^{2+} channels (Chad and Eckert, 1986; Bellers et al., 1988) and a number of enzymes, in particular protein kinases (Murachi, 1989). In this respect, calpain appears to be involved in those biochemical pathaways that are activated in response to stimulation of the cell with an external stimulus. Calpain can also act on a soluble intracellular proteinase and thereby may be involved in the proteolytic degradation of non-functional or denatured proteins (Pontremoli and Melloni, 1986). Possible biological functions of calpain will not be discussed in more detail in the following section, except for the role of calpains in myofibrillar protein degradation, which constitutes the aim of this thesis.

Calpain and Myofibrillar Protein Degradation

Ever since the discovery that calpain is a major intracellular cytosolic proteinase in muscle (Busch et al., 1972; Dayton et al., 1976a,b), a major question has been, and still is, do calpains initiate myofibrillar protein degradation? Evidence supporting a role of the calpains in initiating myofibrillar protein turnover is extensive. Ca^{2+}-dependent proteolytic activity was isolated from myofibrils and degraded myofibrillar proteins at the pH and ionic strength of the cell (Busch et al., 1972). Release of myofilaments is Ca^{2+}-dependent and is reduced by inhibitors of calpain (van der Westhuyzen et al., 1981). Goll et al. (1989) reported that the calpain/calpastatin system is located throughout the sarcoplasm but is present in highest concentration at the Z-disk, a site where initiation of myofibrillar disassembly occurs. Calpains are unique in their ability to rapidly remove Z-disks without causing other large structural changes in the myofibril (Bullard et al., 1990). Alterations in Z-disks, including complete loss of Z-disks, are the most consistent features seen in rapidly atrophying muscle (Cullen and Fulthorpe, 1982). Furthermore, a positive correlation between calpain activities and rate of myofibrillar protein degradation exists in muscle diseases.

Duchenne dystrophy, which is caused by lack of dystrophin protein (Hoffman et al., 1987) is characterized by enhanced permeability of muscle membranes to Ca^{2+} (Turner et al., 1988), which may result in uncontrolled activation of calpains. Conditions in which myofibrillar protein degradation is enhanced and is associated with enhanced calpain activities include vitamin E deficiency (Dayton et al., 1979), starvation and diabetes (Nakamura et al., 1988; Arakawa, 1983). Since calpains

degrade Z-disks on the periphery of myofibrils to release filaments from the myofibrillar surface (Dayton et al., 1976a; Zeman et al., 1987), such a mechanism for metabolic turnover of myofibrils is consistent with the findings that myofibrils in rapidly atrophying muscle typically have diminished diameters rather than being fragmented or having missing sections (Pellegrino and Franzini, 1963). Further evidence for the role of calpains in myofibrillar protein degradation is that calpains degrade a variety of myofibrillar proteins including desmin, filamin, titin, troponin-I, troponin-T, c-protein, tropomyosin and nebulin (Ishiura et al., 1979; Goll et al., 1983) in vitro and calpains degrade a mixture of myofibrillar proteins at 20-fold the rate of a mixture of sarcoplasmic proteins (Tan et al., 1988).

Despite evidence that calpain may initiate myofibrillar protein degradation, contrary evidence exists which suggests that calpain may not be involved in myofibrillar protein degradation. For example, Goodman (1987) reported that acute treatment of isolated rat muscle with agents that increase cytosolic Ca^{2+} concentrations (i.e., the ionophore A23187) increased tyrosine release (an index of total proteolysis) but not 3-methylhistidine (an index of myofibrillar proteolysis), indicating lack of involvement of calpain in myofibrillar protein turnover. With this background, it is obvious that calpain participation in muscle protein degradation is not definite and further research is definitely required.

Structure of Calpain, Calpastatin and Protein Activator

Most mammalian cells have two calpain isozymes, calpain I and II (Suzuki et al., 1987). Both calpain I and II contain an 80-kDa large subunit (catalytic) and a smaller 30-kDa (regulatory subunit). The smaller or regulatory subunit is identical for both enzymes, whereas the 80 kDa subunits of calpain I and II represent separate gene products with 51% sequence homology (Suzuki et al., 1987a).

The primary structure of the 80 kDa subunit was first elucidated in 1984 for chicken calpain from its cDNA sequence (Ohno et al., 1984). Later, the 80 kDa cDNA of calpain I and II of rabbit (Emori et al., 1986a), human calpain I (Aoki et al., 1986) and human calpain II (Imajoh et al., 1988) were isolated and sequenced. These cDNA sequences are shown in Figure 1. The predicted sizes of chicken calpain and human calpain I and II are 705, 714 and 700 amino acid residues, respectively. Analysis of the end termini reveals that the initiation methionine is removed during posttranslational modification but the C terminus remains intact. The structures of the 80-kDa subunits are highly homologous. Sequence comparison between human and rabbit calpain I and II large subunits indicated that the same isozymes from different species are about 95% homologous. Structural homology is much less between different isozymes of the same species and lies in the 60% range.

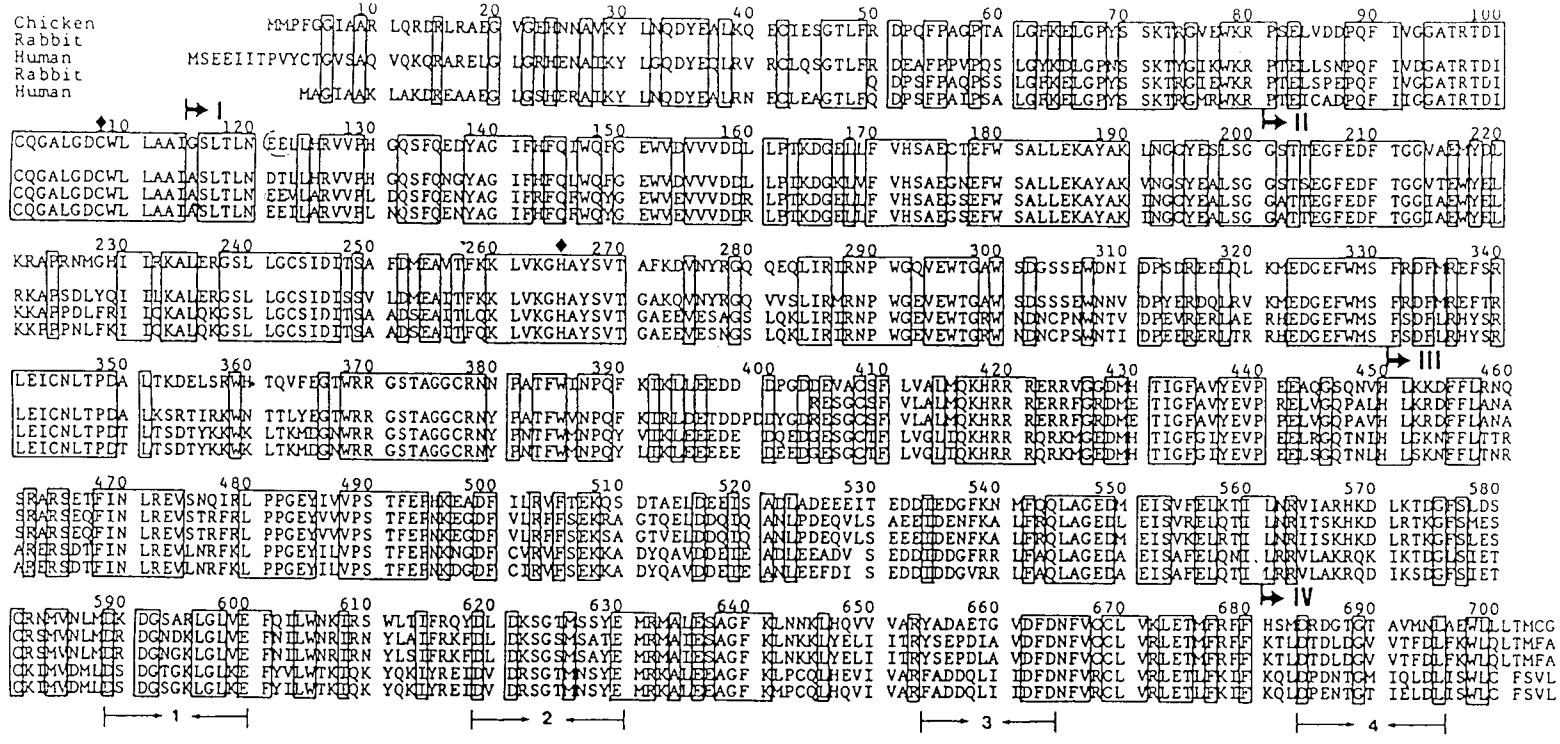

Figure II.1. Amino acid sequences of the 80 kDa subunits of chicken, rabbit calpain I and II and human calpain I and II. Starting points of domains I-IV are shown by side arrows. Amino acid residues Cys and His of the active site are indicated by rectangles (Suzuki, 1990).

The most recent achievement in structural analysis of calpains is the characterization of a novel calpain (n-calpain) by Sorimachi and his associates(1989). The encoded protein consists of 821 amino acid residues with a molecular weight of 94 kDa (hence designated p94) and shows significant sequence homology with both calpain I and II large subunits of 54% and 51%, respectively. A unique feature of this n-calpain is that, in contrast to the ubiquitous expression of calpain I and II, n-calpain is expressed only in skeletal muscle. This makes this protein of particular interest for muscle biologists, as the specific expression of n-calpain suggests some special function in skeletal muscle not shared with other calpains. The structural features of the 80 kDa subunit can be divided into four domains as illustrated in Figure 2, on the basis of sequence homology of other known proteins (Suzuki et al., 1987b; Ohno et al., 1984).The second domain (res. 80-330, chicken calpain) is homologous to typical cysteine proteinases such as papain and cathepsin B, L and H. Sequence homologies are most centered around Cys 108 and His-265 of the enzyme active site. The amino acid residue Cys 108 of chicken 80 kDa subunit has been characterized as its active site by selective modification with cysteine proteinase inhibitors such as iodoacetic acid, E64 and Leupeptin (Suzuki et al., 1983). The C-terminal domain or domain IV (res. 561-705) is a calmodulin-like Ca^{2+}-binding domain with four consecutive EF hand structures typical Ca^{2+}-binding structure comprising helix-loop-helix structures of about 30 residues with Ca^{2+} binding to the loop region (Kretsinger, 1976). This structural motif is a typical characteristic of Ca^{2+}-binding proteins of the calmodulin family such as calmodulin, troponin C and myosin light chain. The other two domains I and II have no clear sequence homology with known proteins.

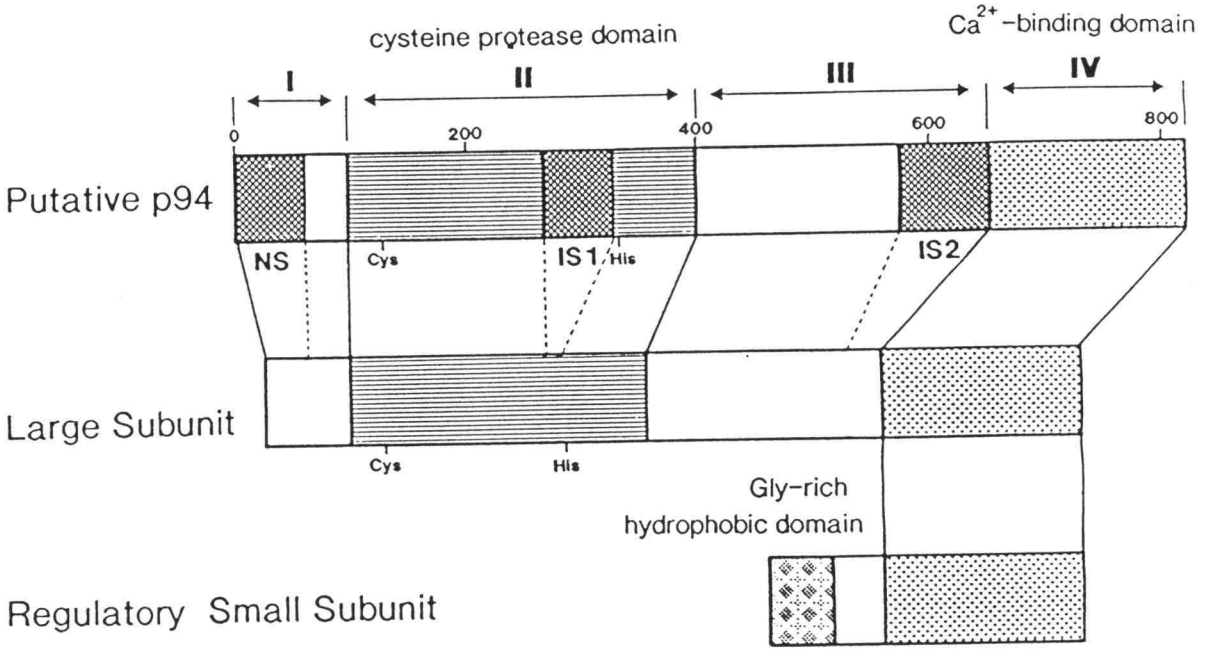

Figure II.2. Domain structure of calpain (Sorimachi et al., 1990).

The structures of the small subunit have been determined in humans (Ohno et al., 1986), rabbits (Emori et al., 1986b) and pigs (Sakihama, 1985). Analysis of cDNAs of human, rabbit and pig small subunit indicates that they are composed of 266, 268

and 266 amino acid residues, respectively, with a calculated molecular weight of 28 kDa. Comparison of the amino acid sequence of the three small subunits shows about 97% sequence homology (Figure 3), and their mature proteins start with the N-acetylated initiation methionine (Sakihama et al., 1985). The structure of the 30 kDa subunit shows some interesting features (Figure 2). First, the N-terminal 60-70 residues are mostly glycine residues. Blocks of 10, 19 and 20 glycine sketches are found. Because residues other than glycine are mostly hydrophobic, this region (domain V) is termed a glycine-rich hydrophobic domain (Suzuki et al., 1987b). Another interesting structural feature of the 30 kDa subunit is the existence of a calmodulin like calcium binding domain (domain IV') in the C-terminal region. This domain shares significant sequence homology (50%) with domain IV in the 80 kDa subunit of calpains I and II (Suzuki, 1990). Furthermore, the four EF hand structures in these sequences can be aligned exactly at the same positions.

The primary structure of calpastatin is also known for humans (Asada et al., 1989), rabbits (Emori, 1987) and pigs (Takano et al., 1986). Furthermore, in 1991, rat calpastatin was cloned and sequenced (Ishida et al., 1991). Figure 4 shows the deduced amino acid sequences of human, pig and rabbit calpastatins. They comprise 673, 715 and 718 amino acid residues, respectively. They have four repeats of mutually homologous sequences at the interval of about 140 amino acid residues. Comparison of amino acid sequence of the three calpastatins indicates that this protein seems to be a more diverged molecule as compared to 30 kDa subunit, only 70-80%. Sequence homology is observed between any pair of the three species. This is further

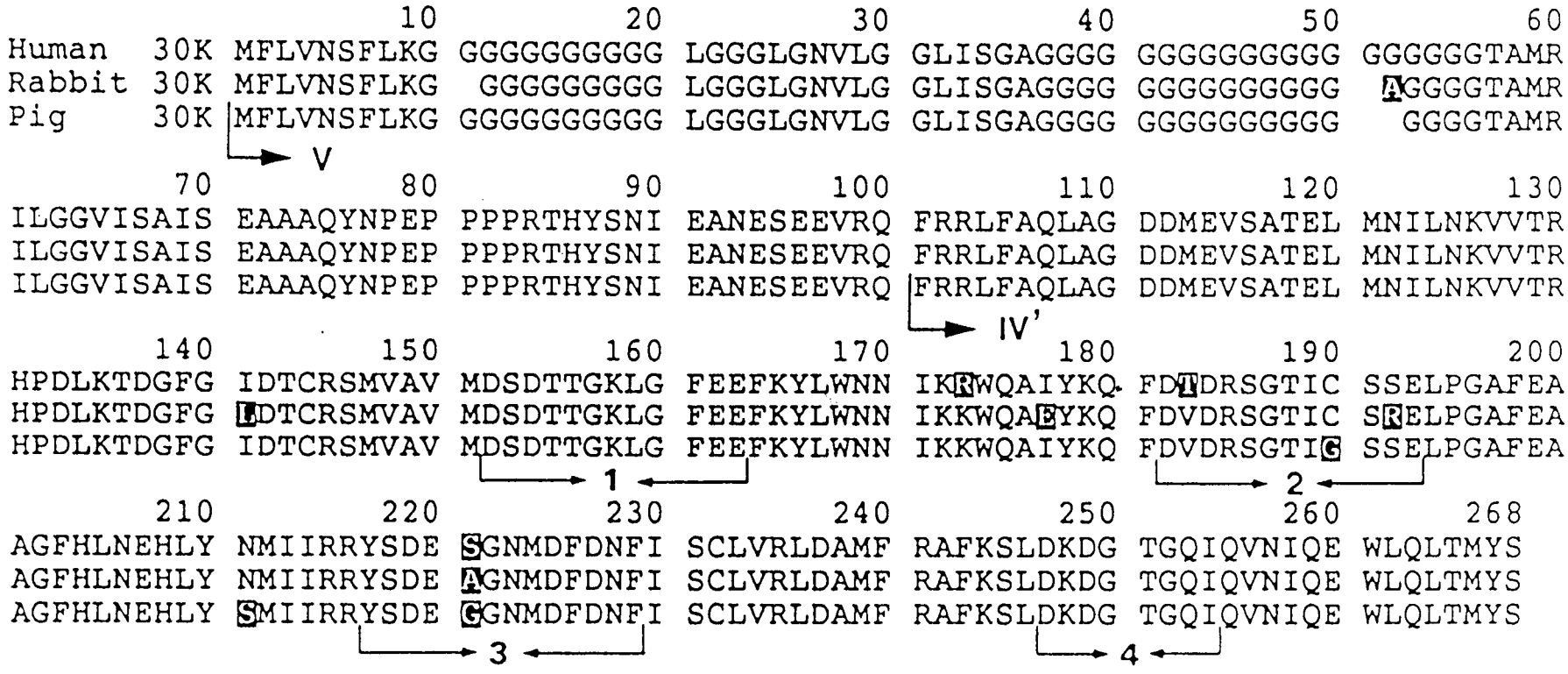

Figure II.3. Amino acid sequences of the 30 KDa subunits of human, rabbit and porcine calpains. Residues different among the three sequences are marked by shading. Loop regions of EF hand structures (1 to 4) and starting points of domain V and IV are also shown (Suzuki, 1990).

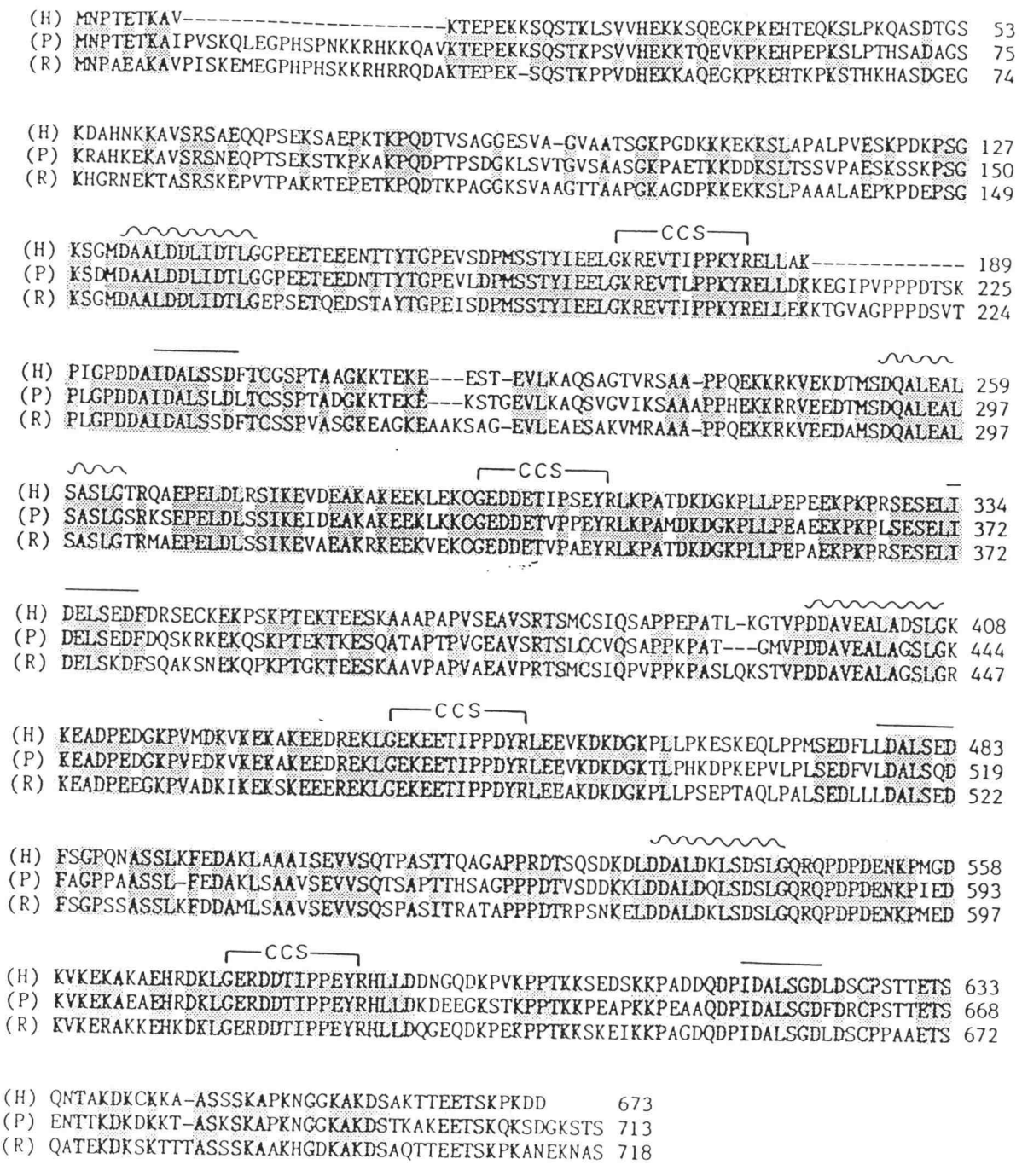

Figure II.4. Comparison of amino acid sequence of human (H), pig (P) and rabbit (R) calpastatins. Residues identical in the three calpastatins are shaded. The regions indicated with CCS are functionally important and have highly conserved sequences which are referred to as "central consensus sequence". Conserved regions in amino-terminal and carboxyl-terminal sides in each domain are marked with waved and thin lines above the human calpastatin sequences, respectively (Masatoshi et al., 1990).

illustrated from the results of determining the structure of rat calpastatin (Ishida et al., 1991). Rat calpastatin is considerably shorter than calpastatin from humans, rabbits and pigs (603 amino acid residues only). However, important sequence regions for its inhibitory activity against calpain are conserved and contain four repeats as in other calpastatins. No significant sequence homology has been found between calpastatins and any other known proteins. Hence, it is concluded that calpastatins contribute a unique family of cysteine proteinase inhibitors quite independent from the cystatin superfamily (Masatoshi et al., 1990).

The amino terminus of calpastatin isolated from pig was blocked and could not be determined by Edman degradation (Takano et al., 1988). However, the amino terminus of calpastatin from rabbits was not blocked and was determined as glutamic acid, which corresponds to the 8th residue from initiation methionine (Emori et al., 1987).

Among the proteins of the calpain system, the endogenous stimulatory protein is the least well characterized component. Knowledge about its structure is not available. Factors limiting research on endogenous stimulatory protein may be related to technical problems associated with its purificaiton to homogeneity in suitable quantites for strutural analysis. Preliminary structural analysis data indicate that this factor is a heat stable protein having an apparent molecular mass of about 40 kDa (Pontremoli et al., 1988). It is hoped that the primary structure of this activator will be determined and that this knowledge will shed light on the nature and function of

this activator. Furthermore (on the basis of structural analysis), the interaction of this activator with calpain and possibly calpastatin may be understood.

The Calpain Genes

Knowledge about calpain gene structures is growing very rapidly. This remarkable achievement is mainly credited to the laboratory of Koichi Suzuki in Japan. The gene structures of the chicken 80 kDa (Emori et al., 1986b) and rabbit 30 kDa (Miyake et al., 1986) have been analyzed. The gene for the chicken 80 kDa subunit is about 10 kb and is composed of 21 exons of various sizes. Intron-exon junctions correspond very closely to the domain functions as defined from sequence homology. In the protease domain (domain II), the regions around the active site Cys 108 and His 265 are split by intron. In the calmodulin domain (domain IV), each of the four EF hand structures is encoded by one exon.

The gene for the rabbit 30 kDa subunit is about 11 kb long with 11 exons. Again, correspondence of the domain junctions to the intron-break points is observed (Miyake et al., 1986). The second exon codes for the glycine-rich hydrophobic domain (domain V). Each of the four consecutive EF hand structures in the calmodulin domain (domain IV) is encoded by one exon.

Careful analysis of the 5'-upstream region revealed multiple initiation sites both for the 30 kDa and 80 kDa subunits (Miyake et al., 1986; Hata et al., 1989). These upstream regions lack the typical promoter elements CAAT and TATA boxes and show

very high GC content. Such structural features are common to house keeping genes. The promoter region of the human 80 kDa subunit which exists between -220 and -80 has been characterized into upstream (-220 to -130) and downstream (-130 to -80) promoter elements (Hata et al., 1989). The downstream region comprises potential interacting sites for Sp1 and Ap-1 (Suzuki and Ohno, 1990). Upstream of the promoter region exist four tandemly reiterated regulatory regions which negatively regulate the promoter activity. The genes for the human 30 kDa and 80 kDa subunits share 50% sequence homology in the upstream region (-360 to -60) including Sp1 and Ap-1 recognition sites, suggesting that expression of these two genes is co-regulated at the transcriptional level by a similar mechanism (Suzuki and Ohno, 1990). The most recent contribution to calpain genes is the mapping of the genes for the large subunits of calpain I, II and n and the small subunit in the human genome (Ohno et al., 1990). Results indicate that each gene of the calpain family is located on a distinct chromosome. Genes for the large subunit of calpain I, II and n were respectively assigned to human chromosomes 11, 1 and 15. The gene for the small subunit was located on chromosome 19 in the human genome. From the point of view of muscle biology, the presence of the small subunit gene on human chromosome 19 is interesting as myotonic dystrophy is associated with this chromosome (Eiberg et al., 1981).

Substrate Specificity of Calpain

Calpain hydrolyzes its substrate protein in its native state to large fragments only and not to small peptides or amino acids. This observation is substantiated by the

increasing numbers of reports concerning the proteolysis by calpain of various endogenous proteins (Pontremoli and Melloni, 1986; Murachi et al., 1987). Calpain substrates include myofibrillar proteins (Ishiura et al., 1979), cytoskeletal proteins (Ishizaki et al., 1985), hormone receptors (Lynch et al., 1986), protein kinases (Inoue et al., 1977), and several others. Limited proteolysis of protein substrates is the most significant feature of the action of calpain. Studies on cleavage site specificity clearly indicate that the substrate specificity of calpain seems to be determined by conformational factors of substrate protein rather than by some specific amino acid residues or sequence (Siezen and Hoenders, 1979). Studies on substrate specificity clearly indicate that calpains are unique and different from those of other cysteine proteases, such as papain and cathepsins B and L (Kargel et al., 1980). Furthermore, each calpain isozyme appears to have some preference for specific types of proteins and this specificity appears not to be always identical among the same calpain isozyme isolated from various sources (Takahashi, 1990). This observation explains why tissues have more than one form of calpain and also the ubiquitous distribution of calpains in various tissues. It is hoped that studies on calpain substrate specificity in the future will help to fully define the native physiological substrates of these proteinases, an issue badly needed.

Regulation of Calpain Activity

Regulation of enzyme activity may be manifest at many metabolic levels, including transcriptional, translational or post-translational levels. Figure 5 depicts such levels for calpain activity regulation.

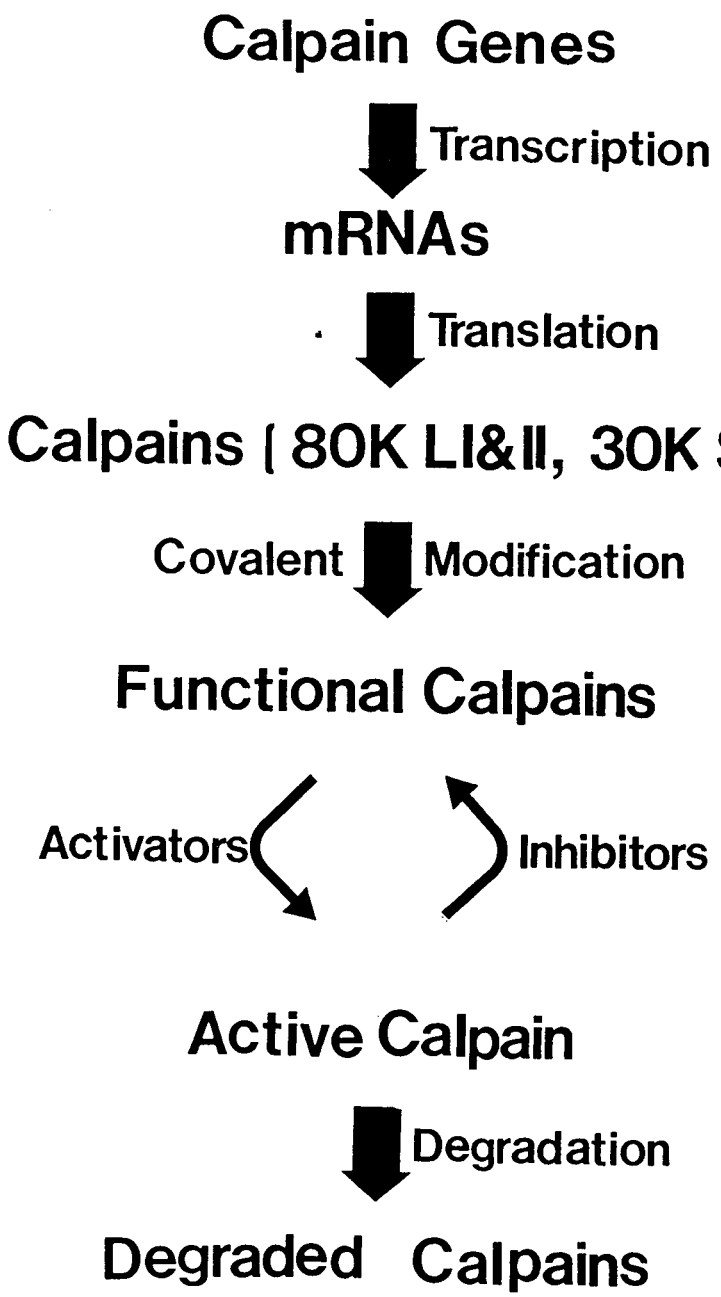

Figure II.5. Metabolic levels at which calpain activity can be regulated.

The regulation of calpain activity at the transcriptional and translational levels have not been identified.

Knowledge of the mechanism underlying the regulation of genes for house keeping proteins, such as calpain, is quite limited compared with that of genes of tissue specific proteins. Furthermore, since calpain is a cytosolic proteinase, both its activity and gene expression are under very tight control. A major reason, therefore, blocking progress towards elucidation of the molecular mechanisms of calpain genes regulation is the lack of find suitable experimental cellular systems in which significant changes in the levels of calpain mRNAs occur upon certain treatments.

Evidence suggesting mRNA concentration as a determinant of activity, however, is limited to the observations of Emori et al. (1986) that mRNAs encoding the calpains are found at variable concentrations in tissues. Recently, analysis of the promoter region of 30 kDa and 80 kDa subunit genes (Miyake et al., 1986; Hata et al., 1989) revealed four tandemly reiterated regulatory regions which negatively regulate the promoter activity. The presence of cellular trans-acting factors modulating the function of these negative regulatory elements was suggested (Hata et al., 1989).

Since calpains are present essentially in all tissues of animal species studied but their absolute as well as relative abundance from one tissue to the other are variable (Murachi, 1989), this observation may be explained by the presence of rather general promoter elements responsible for the constitutive expression of calpains in all tissues and is controlled by common trans-acting factors such as Sp (Hata et al., 1989).

However, negative regulatory elements, whose activity may be mediated by a ubiquitous trans-acting factor whose concentration is cell type-specific, may be responsible for the variability in the relative abundance of calpain in various tissues. The research presented in this thesis furnishes strong experimental evidence for the transcription regulation of genes of the calpain family including calpain I and II large subunits (LI, LII), calpain small subunit (S) and calpastatin. Furthermore, my findings demonstrate that the transcription of calpain genes (L1, L2, S and calpastatin) are co-regulated in muscle, liver, kidney and lung under normal and stress conditions. Thus fasting may provide a suitable model in which important regulatory elements underlying expression of genes of the calpain system may be elucidated.

At the post-translational level, there exist considerable opportunities for calpain activity regulation. These include Ca^{2+} concentration, phospholipids, calpastatin, protein and metabolite activators and compartmentation. As we shall see, regulation of the calpain activity in vivo continues to remain a mystery. However, in vitro studies indicate that the two distinct calpain I and II isozymes appear to be regulated similarly by the abovementioned factors. The Ca^{2+} concentration requirement for enzyme activation and subsequent proteolytic activity remain the sole major distinguishing feature between calpain I and calpain II. On the basis of their Ca^{2+} requirements, calpains are present in all tissues or cells in an inactive state. Accordingly, activation of the proteinase must induce an increase in its Ca^{2+} sensitivity, in order to reconcile the physiological variations in the concentration of Ca^{2+} with the expression of catalytic activity.

Two basic mechanisms have been proposed to account for an increase in the Ca^{2+} sensitivity of calpain. The first involves an autoproteolytic conversion (Coolican and Hatahaway, 1986; Imajoh et al., 1986; Inomata et al., 1985; Mellgren et al., 1982). The second mechanism is accomplished through interaction with natural positive effectors (Pontremoli et al., 1987).

Several groups have isolated a protein factor that stimulates the activity of calpains. This factor was originally shown in brain (De Martino and Blumenthal, 1982; Takeyama et al., 1986) and has recently been isolated and purified from human neutrophils (Pontremoli et al., 1988). The mechanism of action of this stimulator is not clear. Kinetic data indicate that it does not influence the K_m of the calpains for substrates but does increase V_{max} of proteolysis.

In the presence of the activator, the affinity of calpains for Ca^{2+} increases approximately 100-fold. A recent observation (Pontremoli et al., 1987), though not confirmed by other, concerns the activation of human neutrophil calpain (calpain II) by isovaleryl carnitine, a product of the catabolism of leucine, which is synthesized from isovaleryl CoA and carnitine. Activation involves a significant increase in Ca^{2+} sensitivity and desensitization to inhibition by calpastatin. Research on stimulatory factors of calpains and their role in activity regulation has not been progressing along with other areas of calpain research. Consequently, further research on the interaction of stimulatory factor(s) and calpain activation will significantly augment our ability to define physiological targets and functions of the calpain system.

The second mechanism proposed to explain the increase of Ca^{2+} sensitivity of calpain is autoproteolysis. Very rapid autolysis of calpain II occurs upon incubation in mM Ca^{2+} (Suzuki et al., 1984). The Ca^{2+} sensitivity of calpain increases significantly during autolysis (Imajoh et al., 1986); Ca^{2+} concentration for 50% activity for calpain I and calpain II changes from 30 μM to 7 μM and from 0.5 mM to 70 μM, respectively (Suzuki et al., 1988). Although autolysis lowers the Ca^{2+} requirement for both calpain I and II, the Ca^{2+} concentration required for autolysis is much higher than that in vivo (Cong et al., 1989). A possible solution for this situation was furnished by the discovery that phosphatidylinositol lowers the Ca^{2+} concentration required for autolysis (Coolican and Hathaway 1986). Rozanov and Mellgren (1988) have recently shown that a number of phospholipids, incuding phosphatidyl serine, phosphatidylethanolamine and phosphatidylcholine, all lower the concentration required for autolysis of calpain II. Hence, several membrane-binding sites could be available for the activation of calpains at the membrane. On the basis of this finding, an interesting theory was forwarded. This theory, which is called "membrane activation theory", proposes that the unautolyzed calpains as they exist in the cytoplasm with 80 kDa and 30 kDa subunits are inactive "proenzymes" which become active proteolytically only after they have been autolyzed to 78 kDa and 18 kDa subunits at the membrane surface (Figure 6). In brief, calpain exists mainly in cytosol, but when the cytosolic Ca^{2+} concentration is increased, calpain binds to the inner side of cell membrane through the N-terminal region of the 30 kDa subunit. Autolysis of calpain occurs at the cell membrane with the aid of Ca^{2+} and phospholipid and the N-terminal domain of the 80 kDa subunit is hydrolyzed. Some of the activated, autolyzed calpain remains at the cell membrane for processing of various substrates such as membrane

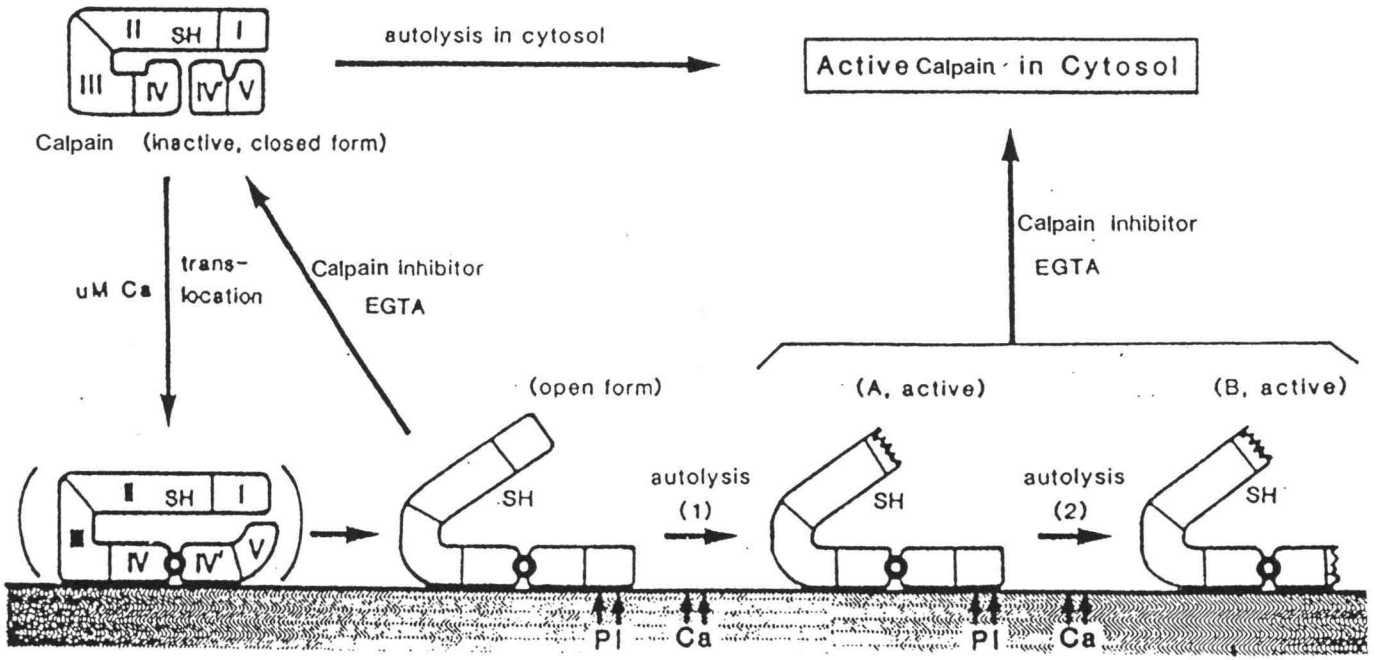

Figure II.6. Mechanism for activation of calpain at the cell membrane. The effect of calpastatin on the regulation of calpain activity is also shown. I-V, IV are domains of calpain shown in Figure 2. SH refers to the sulfhydroxyl group of active site cysteine. PI reads phosphatidylinositol, o sign indicates bound Ca^{2+}.

and cytoskeletal proteins. Other autolyzed calpain may be released in the cytosol for cytosolic functions. Further autolysis of the glyicne-rich hydrophobic domain (domain V) of the 30 kDa subunit detaches calpain from membranes into cytosol (Suzuki et al., 1988).

Although generally accepted, the membrane activation theory via autolysis is not without limitation. A fundamental problem remains, however, because, even in the presence of phospholipids, the Ca^{2+} concentration required for autolysis is at least five to ten times higher than the free Ca^{2+} concentrations that exist in the cell (Cong et al., 1989). Furthermore, Samis and Elce (1989) demonstrated that Ca^{2+} did not induce translocation of calpain II to intact erythrocyte membranes and concluded that if translocation had occurred it was transient.

Because of these differences, further studies were initiated to investigate the role of autolysis in activity of calpain I and II (Cong et al., 1989). Results of these studies showed that calpain I required 60 μM Ca^{2+} for half maximal activation, but required 200 μM Ca^{2+} for half maximal rate of autolysis. Phosphatidylinositol lowered the Ca^{2+} concentration required for a half maximal autolysis of calpain I to 140 μM. From this result, the authors concluded that native calpain I is an active proteinase and not a proenzyme. With regard to calpain II, it was found that this enzyme required 700 mM Ca^{2+} for a half maximal rate of proteolytic activity, 720 mM Ca^{2+} for a half maximal rate of autolysis and 380 mM Ca^{2+} for a half maximal rate of autolysis in the presence of phospholipids. On the basis of these results, the authors concluded that both

calpain I and II are active enzymes as is and suggested that autolysis could be the first step in calpain degradation and turnover.

One regulation of the calpains that might be affected by autolysis is its interaction with calpastatin. It has been shown that less Ca^{2+} is required for calpastatin to bind to calpain II than is required for half maximal activity (Otsuka and Goll, 1987). Consequently, calpain II could never be active if it existed in the same cellular compartment as calpastatin. To address this point, Kapprell and Goll (1989) studied the effects of Ca^{2+} on binding of autolyzed and native forms of calpain I and II to calpastatin. The results of this study showed that autolysis does not alter the Ca^{2+} induced control of calpain activity by calpastatin. Indeed, of the four forms of calpain, only unautolyzed calpain I could ever be active in living cells unless the calpain can be released from the calpastatin inhibition either by compartmentation or activators. To test the possibility of regulation of calpain activity by compartmentation, several studies have described immunolocalization of calpain I, calpain II and calpastatin in tissues (Kitahara et al., 1986, and Kleese et al., 1987). In general, the immunolocalization studies have found that calpain I, calpain II and calpastatin are located in all regions of the cell. Hence, calpain is always in contact with cytosolic protein substrates. If calpains were present in a different cellular compartment away from their inhibitor calpastatin, their protein substrates would be constantly hydrolyzed, resulting in serious cellular damage. Thus, it is reasonable to expect that the calpain and calpastatin are co-localized in various cellular compartments. This hypothesis is supported by immunohistochemical studies both at the electron microscope level and at the light microscope level under different physiological

conditions (Goll et al., 1989). Results of these studies indicated that both calpains and calpastatin are widely distributed throughout the interior of muscle cells from starved or denervated animals. Hence, the stress caused by starvation or denervation does not alter the intracellular distribution of the calpains or calpastatin and calpain-calpastatin remain co-localized. Co-localization of calpain and calpastatin necessitate the availability of effector to liberate calpain from the inhibition of calpastatin to perform its catalytic activity on its substrates.

As stated previously, an endogenous stimulatory protein has been identified but, until now, it has remained the least well characterized component of the calpain system. The possible localization of this stimulatory protein in a putative cytoskeletal fraction (Ishizaki et al., 1985) has important functional implications as the effector could play a major role in modulating the proteolytic activity of the calpain system and the membrane and/or cytoskeletal fractions. Consequently, further research on this stimulatory protein is needed,such as the complete purification and characterization of its chemical structure in order to determine its role in the ineraction of the calpain/calpastatin unit with its protein substrate.

Another important observation pertaining to calpain activators is the observation that isovaleryl carnitine (IVC) is a specific activator of calpain II of human neutrophils (Pontremoli et al., 1987). Isovaleryl carnitine is a product of the catabolism of leucine and is synthesized from isovaleryl CoA and carnitine. Results of the above study indicated that IVC increased the Ca^{2+} sensitivity of calpain II to low μM range and

released inhibition of calpain II by calpastatin in a reversible manner. Furthermore, the activation of neutrophil calpain by IVC was fully reversible.

Clearly, many important questions about the regulation of the calpain system remain unanswered. Ca^{2+} plays a central role in activity of the calpain system. Slightly different Ca^{2+} concentrations are required for proteolytic activity of the calpains, for autolysis of the calpains, and for the calpain/calpastatin interaction. Thus the interactions of the calpains, calpastatin, and calcium ion in the intracellular environment are not yet elucidated. It is hoped that in the near future the regulation of calpain system activity will be clarified. The major goal of this research is to characterize the role of calpain I, calpain II and calpastatin in myofibrillar protein degradation and to elucidate the transcriptional regulationof their activity in vivo.

The major goal of this research is to characterize the role of calpain I, calpain II and calpastatin in myofibrillar protein degradation and to elucidate the transcriptional regulation of their activity <u>in vivo</u>.

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CHAPTER III

GENE EXPRESSION OF CALPAINS AND THEIR SPECIFIC ENDOGENOUS INHIBITOR, CALPASTATIN, IN SKELETAL MUSCLE OF FED AND FASTED RABBITS

by

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CONTRIBUTIONS

Mohammad A. Alyan participated in developing the experimental design, development and streamlining of analytical techniques utilized to perform this study, performing the animal trial, sampling tissues, analyzing the data and calculating the results, performing the statistical analysis and writing the manuscript. Dr. Neil E. Forsberg participated in the experimental design, supervising progress of study and editing the manuscript.

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ABSTRACT

To examine the relation of calpains to myofibrillar protein degradation in skeletal muscle and the regulation of their activity in vivo, we studied the effect of fasting on gene expression of calpains and calpastatin in skeletal muscle of rabbits. In response to fasting, myofibrillar protein degradation rate increased 2 fold, and mRNA levels of calpain I, calpain II and calpastatin around 6 fold, but calpains and calpastatin activities remained unchanged. To investigate this discrepancy, we analyzed polysomal calpains mRNA. Results indicated that fasting caused a 2-fold increase in the loading of calpain I and II mRNAs on the ribosome. Thus the up-regulation of gene expression for calpain I and calpain II during fasting may be an important mechanism in modulating rate of protein synthesis. The effect of fasting on calpains and calpastatin mRNA expression is shared by cathepsin D and proteasome C2 but not by β-actin, implying that fasting invokes control of several proteolytic systems in skeletal muscle and underscores the possibility that each proteolytic system plays a role(s) in the adaptation of skeletal muscle to the fasted state.

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INTRODUCTION

Calpain refers to Ca^{2+}-dependent proteinase (EC 3.4.22.17) and calpastatin to its specific endogenous inhibitor protein. Calpain and calpastatin are ubiquitously distributed intracellular proteins in animal tissues including skeletal muscle (Murachi, 1985). The proteinase exists at least in two forms: calpain I is active at μM levels of Ca^{2+} while calpain II requires mM concentrations of calcium (Dayton et al., 1976a,b, Mellgren, 1980). These two isozymes are composed of different large subunits (80 kDa subunit) and identical 30 kDa small subunits (Emori et al., 1986a,b; Kawasaki et al., 1986). The large subunit is composed of four distinct domains including a papain-like cysteine proteinase as well as a calmodulin-like Ca^{2+} binding domain (Suzuki et al., 1987). The small subunit is composed of a glycine-rich hydrophobic domain at the NH_2-terminus and a calmodulin-like domain at the COOH-terminus (Suzuki et al., 1987).

Calpains participate in numerous cellular functions through limited hydrolysis of various substrate proteins. These observations have been related to participation of calpains in the rearrangement of submembrane-cytoskeletal connecting protein (Thomas et al., 1983), brain functioning (Zimmerman and Schlaepfer, 1982; Schlaepfer et al., 1985), movement of secretory granules (Perrin et al., 1987), selective limited proteolysis of receptors (Chad and Eckert, 1986) and a number of enzymes such as protein kinase C (Kishimoto et al., 1983) and initiation of

myofibrillar protein degradation (Goll et al., 1985). This paper focuses on the relation of the calpain system to initiation of myofibrillar protein degradation and the molecular mechanisms regulating their activities in association with this function.

Evidence supporting a role for calpains in initiating myofibrillar protein degradation is extensive (Goll et al., 1989). Despite this circumstantial evidence, however, no unequivocal data exclude other proteases from some role in myofibrillar protein degradation. In fact, growing evidence suggests that although calpains could initiate metabolic turnover of the myofibrillar protein, other proteases must also be involved in this turnover (Furano et al., 1990). Clearly, further research is needed to elucidate the role of calpains and other proteinases in myofibrillar protein degradation.

Intracellular proteinases, especially the extralysosomal ones such as calpain possess elaborate mechanisms for regulation of their activity. Regulation of enzyme activity may exist at many metabolic levels including transcriptional, translational and post translational levels. In the past decade, there have been substantial advances in our understanding of the regulatory processes of calpain activity at the post translational level (Suzuki et al., 1988; Murachi, 1989; Melloni and Pontremoli, 1989; Cong et al., 1989). However, knowledge concerning the in vivo activity regulation of calpains at the transcriptional level is limited to the observation by Emori et al. (1986a) that considerable variability exists for mRNA levels of calpain I and II in various rabbit tissues and this variability correlates closely with published results of calpain I and II activities in respective rat tissues

(Murachi, 1989). A major obstacle hindering progress in research on transcriptional regulation of calpain activity is the lack of a suitable biological model in which significant changes in calpain activities and mRNA levels occur upon certain treatments (Suzuki and Ohno, 1990).

One approach to the elucidation of transcriptional regulation of activity of calpains is the determination of enzyme activity and mRNA concentrations of the isozymes during certain treatment that preferentially affect certain physiological functions of calpains in vivo. For this purpose, we analyzed expression of calpain I, calpain II and calpastatin genes in skeletal muscle of rabbits during fasting to determine the transcriptional regulation of calpains in association with enhanced myofibrillar protein degradation rate. Our findings reveal significant changes in mRNA levels of skeletal muscle calpain I and II and their specific inhibitor, calpastatin, in response to fasting. This is the first paper, according to our knowledge, that describes the transcriptional regulation of calpain I and II during some physiological process (enhanced myofibrillar protein degradation) in response to a certain treatment (fasting). The implication of this finding is that fasting may provide a suitable experimental model for the elucidation of cis and trans-regulatory elements involved in this mechanism.

MATERIALS AND METHODS

Experimental model

Fasting was used as an experimental model in this study as it is characterized by the mobilization of body protein reserves, mainly in skeletal muscle, to support the organism's energy requirements (Cahill, 1970). This effect results from a decreased protein synthesis and a marked rise in muscle protein degradation (Li and Goldberg, 1976; Fryburg et al., 1990). An important aspect of fasting is that in skeletal muscle, it causes a preferential increase in the degradation of myofibrillar proteins (Li and Wassner, 1984; Lowell et al., 1986b). In a recent study, Kadowaki et al. (1989) observed no change in total protein degradation in muscle, but myofibrillar protein degradation was increased to 203% of the control group by 3 d of fasting. They suggested that different mechanisms may be responsible for the degradation of myofibrillar and sarcoplasmic proteins during fasting. Since calpains are implicated in initiating myofibrillar protein degradation (Goll et al., 1989), a process preferentially accelerated during fasting, the model utilized in this study will allow the critical investigation of the molecular bases for the regulation of expression of calpains in association with myofibrillar protein degradation as their physiological function in skeletal muscle.

Animals

Twelve rabbits (ca. 2000 g) were obtained from the Oregon State University Rabbit Research Center and were individually housed in metabolic cages and assigned to one of two treatments: 8 days of ad libitum intake of a commercial rabbit diet (composition is listed in Appendix I) or 8 days of fasting (6 animals per treatment). Water was available ad libitum and urine was collected into 20 ml of 5 N HCl daily and frozen. At the end of the study, the rabbits were euthanized by intraperitoneal injection of T-61 Euthanasia solution, after which *biceps femoris* was dissected and frozen in liquid nitrogen. Tissues were stored in plastic bags at -90° C.

Assessment of myofibrillar protein degradation rate

To evaluate the myofibrillar protein degradation rate during fasting, we measured daily output of 3-methylhistidine (3-MH) in urine of control and fasted rabbits during the experimental period. This amino acid is formed by post-translational modification of certain histidine residues in actin and in myosin of pale muscle fibers (Young et al., 1972). Since it is neither reutilized for protein synthesis nor catabolized (Young et al., 1972) and is quantitatively released from the tissue and excreted in urine under normal and accelerated myofibrillar protein degradation rates (Emery et al., 1986), its release would directly reflect myofibrillar protein degradation. The method used to determine 3-MH in this study is according to Forsberg and Liu (1989) with some modifications.

Samples of urine (2 ml) were heated to 100° C for 2 h with an equal volume of 6 N HCl to deacetylate 3-MH. Deacetylated urine samples (0.5 ml) were freeze dried in a Virtus Uni-Top Model 600 SL freeze dryer, then resuspended and derivatized as phenylisothiocyanate (PITC) derivaties with 0.5 ml of a 7:1:1:1 mixture of ethanol:PITC:triethylamine:water. Samples were incubated at room temperature for 20 mintues and then were lyophilized and redissolved in 0.5 ml of 5 mM phosphate buffer (pH 6.5) in 5% acetonitrile. Afterward, samples were analyzed by HPLC for total PITC-3-MH. Results of 3-MH analysis was expressed as μmol/100 g body weight/day or as 3-MH/creatinine ratio. The molar ratio appears to be a valid approach since 98% of all creatinine in the body is present in skeletal muscle (Afting et al., 1981). Thus, the molar ratio of 3-MH to creatinine excretion provides an index of myofibrillar protein degradation per unit of muscle mass. Creatinine was determined colorimetrically by the picric acid method (Faulkner and King, 1976).

Preparation of ^{32}P-labeled cDNA probes.

A variety of cDNA fragments were provided to us. These included pLU1001, pLM28 and pCI21 (Emori et al., 1986a. 1987), which encode rabbit muscle calpain I and calpain II (80 kDa subunits) and calpastatin, respectively, and were provided by Drs. Y. Emori and K. Suzuki (Tokyo Metropolitan Institute of Medical Science); pC2-α, which encodes rat liver proteasome (30.8 kDa C2 subunit; Fujiwara et al., 1989) and was provided by Dr. K. Tanaka (Institute for Enzyme Research,

University of Tokushima, Japan); pHCpDEco1.1, which encodes human cathepsin D and was provided by Dr. John Chirgwin (University of Texas Health Science Center; Faust et al., 1985); and pRß-A1, which encodes rat ß-actin and was provided by Dr. Larry Kedes (University of Southern California; unpublished observations). Plasmids were prepared by transformation of E. coli HB101 and amplified and recovered by using standard techniques (Maniatis et al., 1982). Identity of plasmids used in this study was checked by establishing their physical maps with restriction enzymes and comparing these maps to those expected based on published results (details of this analysis are presented in Appendix II, III, IV, V, VI). Plasmid DNA was recovered and purified and cDNA inserts were then recovered by restriction enzyme digestion. RsaI/DdeI and RsaI fragments of pLU1001 and pLM28, respectively, which correspond to 3'-non-coding regions of calpain I and II mRNA, were used to discriminate between calpain I and II mRNAs. EcoRI fragment of about 1100bp corresponding to the coding region of calpastatin was used to assay for its mRNA levels. A 1000bp EcoRI coding-region fragment of pHCpDEco1.1 was utilized to assay cathepsin D mRNA. A 1000bp HindIII/PvuII coding-region fragment of pC2-α was prepared to assay proteasome C2 mRNA. Finally, a 1057bp coding-region HindIII/PvuII fragment of pRß-A1 was used to assay ß-actin mRNA. cDNA inserts were purified following restriction enzyme digestion via agarose gel electrophoresis which was followed by electroelution of the cDNA into a dialysis membrane. The cDNA was recovered from the dialysis and then purified by ethanol precipitation and phenol:chloroform extraction. cDNA fragments (25-50 ng) were radiolabeled to high specific activity (1.9×10^9 d.p.m./μg cDNA) with α^{32}P-dCTP (6000 Ci/mmol; New England Nuclear, Boston, MA) using a random

primer kit (5-prime 3-prime Inc., West Chester, PA) after which they were purified from unincorporated α^{32}-dCTP using P60 gel chromatography. (A typical elution chromatogram is presented in Appendix VII)

Cellular RNA preparation and Northern Analysis

Total RNA was extracted from tissue samples by using guanidinium isothiocyanate followed by purification on a cesium chloride gradient as outlined by Maniatis et al. (1982). Fifty μg of total RNA was electrophoresed on 2.2M formaldehyde-containing agarose gels. Quality of extracted RNA samples was checked by ethidium bromide staining of agarose gels to verify the 28S and 18S bands and intensity (a representative picture of electrophoresed RNA samples is given in Appendix VIII). High quality gels were transferred overnight to a BAS-85 nitrocellulose membrane (Schleicher and Schuell, Keene, NH). Fifty micrograms of total RNA yielded detectable bands which were in a linear range of image density. RNA was immobilized by baking the filter for 3 to 4 h under vacuum at 80° C after which the membrane was prehybridized overnight at 42° C in Stark's buffer (Maniatis et al., 1982) containing 100μg/ml sheared salmon sperm DNA (Sigma Chemical Co., St. Louis, MO). Following pre-hybridization, the immobilized RNA was hybridized for 48 hr at 42° C to ^{32}P-labeled cDNA probes in Stark's buffer containing dextran sulfate (10% w/v) and 5 x 10^5cpm radiolabeled cDNA per ml of hybridization buffer. Following hybridization membranes were washed. Washing conditions used for each cDNA are given in legends of figures. Membranes were wrapped in Saran wrap and exposed with two Dupont Cronex Plus intensifying

screens for .5 to 4 days to Kodak X-Omat film at -90° C. Quantitation of mRNA was completed via scanning densitometry using a Hoefer GS-350H scanning program.

In preliminary studies we validated cross-species hybridization of non-rabbit cDNAs to rabbit and rat mRNA using Northern blot analysis. Furthermore, we performed calibration curves for all probes utilized in this study to establish linearity of signal response with gradation of RNA concentrations. Calibration curves were constructed using 12.5, 25 and 50 μg of total RNA from skeletal muscle of control animals.

Preparation of polysomes

The procedure of Buckingham et al., 1976, was followed to prepare polysomes from muscle tissues. This procedure is particularly suitable for muscle work as it employs high salt concentrations in homogenization buffer to prevent precipitation of contractile proteins. One gram muscle sample (one sample was analyzed per animal) was homogenized in ice cold polysomal buffer (20 mM Tris-HCl, pH 7.6; 0.25 M sucrose; 0.25 M NaCl; 5 mM magnesium acetate; heparin, 1 mg/ml; and 6 mM 2 mercaptoethanol) by using a Polytron at half maximum setting for 15 seconds for 3 times. Afterward, the homogenate was centrifuged at 10,000g for 10 min to remove debris, plasma membranes, nuclei and mitochondria. Then Triton X-100 was added to the post-mitochondrial supernatant in 1% (v/v) final concentration and put on an end-to-end shaker for 10 min while immersed in ice.

Afterward the mixture was layered over an equal volume of polysome buffer containing 1.0 M sucrose in a polyallomer tube (Beckman). The tubes were centrifuged in an ultracentrifuge at 140,000g for 3.7 h at 4° C in an SW28.1 rotor. After completion of the centrifugal run, the supernatant was aspirated very gently and discarded. Then the tube wall was rinsed with cold polysome buffer lacking sucrose. The polysome pellet was allowed to drain for a few minutes by placing it upside down over tissue paper. Finally the polysome pellet was suspended gently in cold sucrose-free polysome buffer, for RNA extraction.

Polysomal RNA extraction

The method of Chomczynski and Sacchi (1987) was used to extract RNA from polysomes with some modification. To a suspension of polysomes (0.5 ml), 10 ml guanidinium thiocyanate (GI) buffer (4 M GI; 25 mM sodium citrate, pH 7.0; 0.5% sarcosyl; 0.1 M 2-mercaptoethanol) was added and homogenized by using 15 strokes of a Teflon/glass (Dounce type) homogenizer. An 0.1 x volume of 2 M sodium acetate (pH 4) was added, and the RNA was extracted with equal volume of 1:0.2 mixture of phenol and chloroform-isoamyl alcohol (49:1). RNA was collected by two isopropanol precipitations. The RNA pellet was given a final wash in 80% ethanol and was resuspended at 65° C in 0.5% sodium dodecyl sulfate (SDS) with gentle pipetting. RNA quantity and quality were determined by optical density and 260/280 nm analysis and RNA sample were aliquotted to appropriate concentration size and stored at -86° C until use.

Dot Blot Analysis

Polysomal RNA was denatured as described for Northern blots and then diluted with an equal volume of 20 x SSC. It was diluted with 10 x SSC to give serial concentration of 2.5, 5.0, 10.0 and 20.0 μg polysomal RNA/0.1 ml and aliquots of 0.2 ml were applied to a nitrocellulose membrane in a dot-blot apparatus (Schleicher and Schuell). Each well was washed with 0.1 ml of 10 x SSC. Then the membrane was allowed to air dry, followed by baking at 80° C for 3 h under vacuum.

Analysis of calpain I and calpain II mRNA in polysomal RNA was performed essentially as described earlier for Northern analysis. Experimental conditions for prehybridization, hybridization, washing and probe preparation to examine the composition of translated calpain I and II mRNA in muscle tissue were identical to those used for Northern analysis. Finally, dot-blots were scanned by visible light densitometer (Hoefer TI-350H) and after establishing that the absorbance and RNA concentration (2.5-20 μg RNA) were linearly related, the absorbance of dots from fasted groups was expressed as a percentage of the absorbance of dots of the control group.

Isolation and quantitation of calpains and calpastatin

Phenyl-sepharose chromatography was utilized to separate calpains and calpastatin according to the procedure of Gopalakrishna and Barsky (1985).

Skeletal muscle samples (5 g) were homogenized in 5 vol of ice cold 50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 10 mM 2-mercaptoethanol, and 150 nM pepstatin A by using polytron at half maximum setting, three times for 15 s each while immersing the tube in ice. All separation and chromatography steps were performed at 4° C. The homogenate was centrifuged at 13,000 g for 30 min and supernatant was decanted through glass wool to separate lipids. The supernatant was divided into three portions: 1 ml for protein analysis, 1 ml for calpastatin determination, and 20 ml for calpain isolation. To supernate (20 ml) 40 μl of 1 mM leupeptin, 4 ml of phenyl-sepharose (Sigma Chemical Co., St. Louis, MO) beads (previously washed with buffer A (20 mM Tris-HCl, pH 7.5; 0.1 mM $CaCl_2$, 10 mM 2-mercaptoethanol, 20 μM leupeptin) containing added 0.25 M NaCl), and 1.2 ml of 5 M NaCl were added. After shaking the mixture for 5 min while immersed in ice, 0.8 ml of 0.1 M $CaCl_2$ was added and the shaking was continued for another 10 min. Then this gel suspension was packed into a 10-ml plastic column (Bio Rad). The packed column was then washed successively with 8 ml of buffer A with added 0.2 M NaCl, buffer A without NaCl, and buffer A without leupeptin. Afterwards, calpain II and calpain I were eluted successively with 16 ml of buffer B (20 mM Tris-HCl, pH 7.5; 1 mM EGTA, 10 mM 2-mercaptoethanol) with added 0.1 M NaCl and 8 ml of buffer B alone.

Calpastatin was partially purified by boiling supernatant (1 ml) for 5 min, followed by centrifugation to precipitate heat denatured proteins in a

microcentrifuge. The calpastatin containing supernatant was collected by a Pasteur pipet and stored at -20° C for activity analysis.

Calpain and calpastatin assays

Calcium-dependent proteolytic activity was determined by measuring the release from Hammersten casein of trichloroacetic acid-soluble amino acid, absorbing at 280 nm (Yoshimura et al., 1984). Proteolytic activity was measured in triplicate. Samples of eluants (3 ml) were combined with 1.5 ml of Ca^{2+} assay medium [100 mM Tris, pH 7.5; 2 mM 2-mercaptoethanol; 1mM NaN_3; 0.05 mM $CaCl_2$ (for calpain I) or 1 mM $CaCl_2$ for calpain II; 5 mg/ml casein] and incubated for 30 min at 25° C. The reaction was stopped by adding 0.25 ml of ice cold 50% trichloroacetic acid. The trichloroacetic acid-soluble digestion products were measured spectrophotometrically at 280 nm. One unit of calpain activity was defined as the amount of enzyme which produced an increase of one absorbance unit at 280nm after 30 min incubation at 25° C, corrected by subtracting the activity found in the presence of 1 mM EDTA.

Calpastatin activity was assayed on the basis of its inhibition of calpain II activity. Inhibitor activity was measured by introducing portions (150 μl) of boiled fractions to a calpain II preparation from rabbit tissues (0.2 enzyme units) and preincubating for 10 min at 25° C. After preincubation the remaining (uninhibited) protease activity was determined by the proteinase assay described above. In this assay system, one unit of calpastatin activity completely inhibits the proteolytic

activity of one unit of calpain II. Inhibitory units were calculated only from the dilution which showed 40 to 50% inhibition.

Statistical Analysis

Differences ($P<.05$) between the two experimental treatments were examined using an unpaired Student's t-test (Steel and Torrie, 1980).

RESULTS

Characterization of fasted rabbits

To provide a frame of reference for our work, body weight and weight of cranial *biceps femoris* and its RNA content in control and fasted rabbits are given in Table 1. Rabbits allowed access to food continued to grow and gained on the average 14% in body size during the 8 d experimental period. However, fasted rabbits experienced a significant loss in body size and were 38% smaller than their fed counterparts at the conclusion of the experiment. *Biceps femoris* was reduced ($P<0.05$) by fasting to a similar level as body weight. Total and polysomal RNA concentrations were reduced by 29% and 43% in *biceps femoris* in response to fasting.

The rate of protein synthesis is dependent on both the capacity and the efficiency of the translation process (Millward et al., 1975). Based on results of this study, we cannot critically discuss the efficiency of protein synthesis in skeletal muscle of control versus fasted rabbits as this parameter is related to the activity of the components involved in the synthetic process and is expressed as the quantity of protein synthesized per amount of RNA per unit time. However, we can comment on the capacity of protein synthesis of skeletal muscle in control versus fasted rabbits as this index is dependent upon the number of ribosomes and can be

Table III.1. Effect of fasting on rabbit body weight and muscle weight and RNA levels.

Parameter*	Control	Fasted	% Change
Body wt (g)			
- Initial	2003 ± 72	2008 ± 45	--
- Final	2284 ± 66[a]	1420 ± 20[b]	-38
Cranial *biceps femoris*			
- Weight at end of study (g)	15.4 ± 0.8[a]	10.0 ± 0.4[b]	-35
- Total cellular RNA (μg/g tissue)	1377 ± 94[a]	972 ± 57[b]	-29
- Polysomal RNA (μg/g tissue)	550 ± 59[a]	311 ± 79[b]	-43
- Polysomal/Total	0.40 ± 0.08[a]	0.32 ± 0.09[a]	-20

* Values are means of 6 observations ± SEM except for polysomal RNA analysis, with only 3 observations ± SEM. Values in the same row with differing superscripts differ significantly ($P<0.05$). RNA concentrations were determined spectrophotometrically at wavelengths 260/280 nm following its purificaiton as described in the Materials and Methods section (Maniatis et al., 1982).

expressed as the content of total RNA per g of tissue using the generalization that, in most tissues, the majority of RNA is ribosomal RNA (Young, 1970). Thus an 8 d fast caused a significant decrease (29%) in the capacity of rabbit skeletal muscle (*biceps femoris*) for protein synthesis. Polysomal RNA level was lowered more drastically (43%) in skeletal muscle in response to fasting. Utilizing the same principle that total RNA level in a certain pool is a good indication of the size of the ribosomal population in that pool, one can visualize the total cellular RNA as an index for the population of total ribosomes of active and inactive ones and the polysomal RNA level as an index for the population of active ribosomes. Thus the

ratio of polysomal RNA concentration to total cellular RNA concentration is indicative of the proportion of active ribosomes in the total population. Results of this mathematical exercise, as presented in Table 1, show a significant decrease in the pool of active ribosomes in skeletal muscle after fasting. These findings suggest that, in fasting, not only the pool of total ribosomes is reduced but also the rate of initiation of protein synthesis may be slowed down as indicated by the reduction in the pool of active ribosomes. Thus fasting lowers both protein synthesis capacity and efficiency in skeletal muscle.

To characterize myofibrillar protein degradation status in rabbit skeletal muscle during fasting, daily urinary 3-MH excretion was measured in control and fasted rabbits during the 8 d fast. This has often been expressed as a fraction of creatinine excretion (Afting et al., 1981). However, it is not yet established which is a more appropriate index: daily urinary 3-MH excretion or the ratio of 3-MH to creatinine (Hillgartner et al., 1982). For this reason, we evaluated both indices. As shown in Figure III.1, daily urinary 3-MH excretion increased gradually as the fasting period progressed and rose to about 200% of the control level on day 8. Creatinine excretion was slightly higher, but not significantly different ($P>0.05$) in fasted rabbits over control ones on d 2 and sustained this pattern thereafter. This increase in creatinine level in response to fasting may reflect a change in creatinine metabolism or its filtration efficiency by the kidney. The pattern of 3-MH/creatinine ratio for fasted rabbits and control ones during the 8 d experimental period is essentially similar to that for absolute rates of excretion of 3-MH but with a slightly lower magnitude of difference between control and fasted rabbits over the

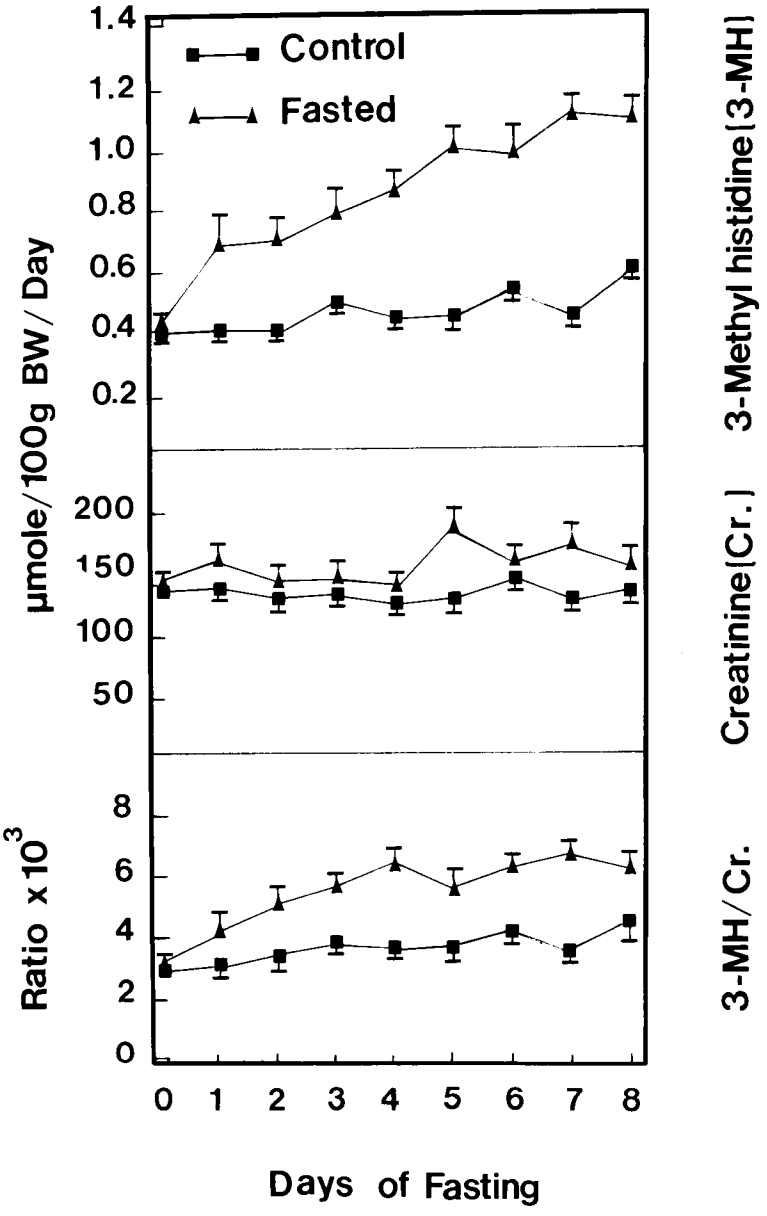

Figure III.1. Effect of fasting on daily urinary excretion of 3-methyl histidine (3-MH) and creatinine. Results are means ± SEM (n = 6).

experimental period. These results indicate that fasting dramatically increased myofibrillar protein degradation in rabbits and reached 200% of the control level on d 8 of fast.

Effect of fasting on mRNA levels of various proteases

To investigate the characteristics of the transcriptional regulation of expression of calpains and calpastatin in skeletal muscle during normal (i.e. control treatment) and accelerated myofibrillar protein degradation (i.e. fasting condition), we determined steady state mRNA levels of calpain I, calpain II and calpastatin in skeletal muscle of control versus fasted rabbits. Furthermore, to shed light on the nature of response (i.e. specific versus general) of mRNAs of the calpain system to fasting, we studied the effects of fasting on mRNA levels of cathepsin D, proteasome C2 and β-actin in skeletal muscle.

To establish the utility of using cDNA probes of the calpain system, cathepsin D, proteasome and β-actin to perform Northern analysis quantitatively in this study, a dose response curve and cross-species validation analysis were performed for every probe. Results of dose-response curves for calpain I, calpain II, calpastatin, cathepsin D, proteasomes and β-actin, using 12.5, 25 and 50 μg of total RNA, showed linear response of image density to gradation in RNA concentrations (Figures 2 and 3). Consequently, 50 μg of total RNA was routinely processed for Northern blot analysis to enhance detection sensitivity.

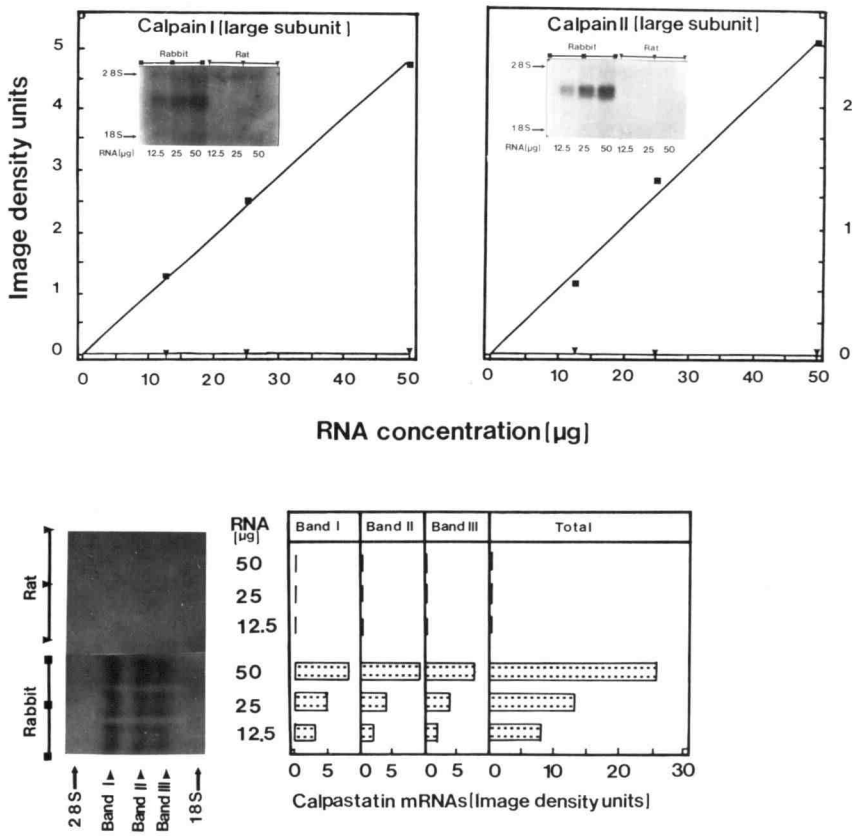

Figure III.2. Dose response curve and validation results for cDNA probes of calpain I and calpain II large subunits and calpastatin used in this study against RNA samples of rabbit and rat skeletal muscle.

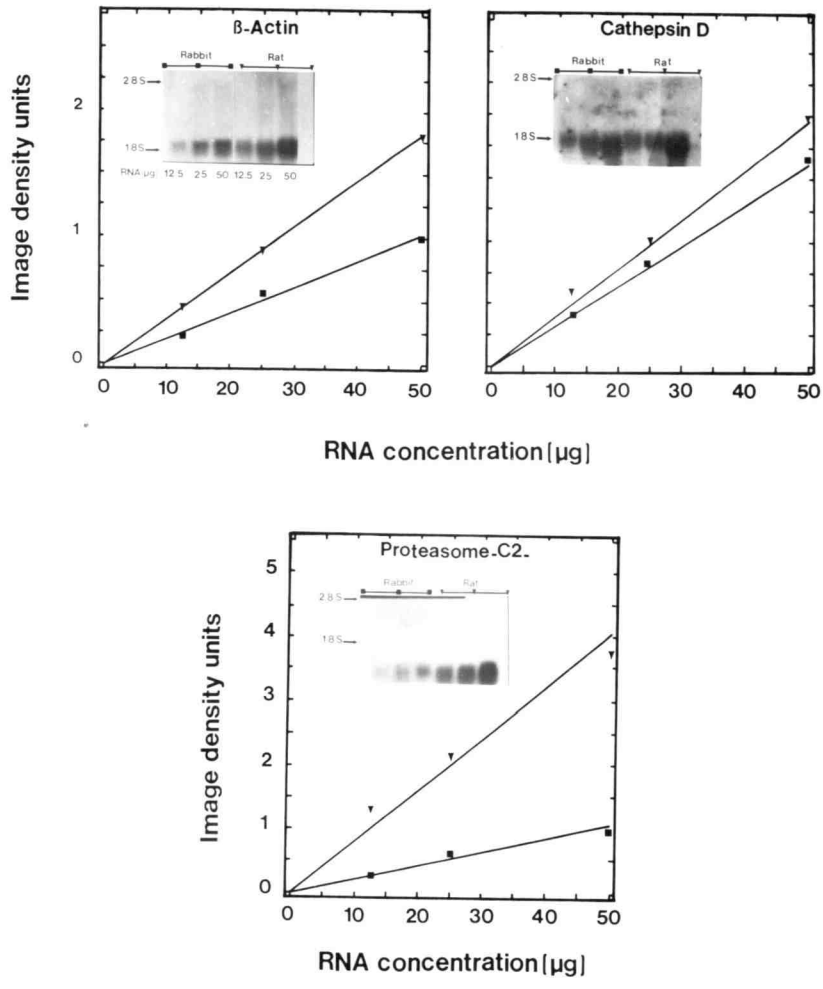

Figure III.3. Dose response curve and validation results for cDNA probes of cathepsin D and proteasome C2 and β-actin against RNA samples from rabbit and rat skeletal muscle.

Cross-species validation study indicated that the 3' non-coding region cDNA probes prepared from pLU1001 and pLM28 are isozyme specific, as they hybridized to RNA isolated from rabbit muscle but not from rat muscle (Figure 2). Coding-region cDNA probes from human cathepsin D, rat proteasome C2 and rat ß-actin hybridized to both rat and rabbit mRNAs (Figure 3). These corresponded to bands of molecular sizes of 2.2, 1.35 and 2.1 kb, respectively. These results closely match predicted sizes of mRNAs encoding these proteins in human liver (Faust et al., 1985), rat liver (Fujiwara et al., 1989) and rat kidney, respectively. Dose-response curves to gradations of RNA indicated that image density increased linearly to 50μg total RNA.

The effect of fasting on the expression of mRNAs of calpain I, calpain II and calpastatin was examined by Northern blot analysis (Figure 4). Fasting caused dramatic up-regulation of mRNA expression of calpain I, calpain II and calpastatin in rabbit skeletal muscle. Levels of mRNA of calpain I, calpain II and calpastatin (total forms) were increased 4.2, 4.5 and 3.2 folds, respectively, in skeletal muscle of fasted rabbits relative to values in tissues of control animals.Northern blot analysis of total RNA of skeletal muscle using calpain I and calpain II cDNA probes revealed single bands of about 3.5 kilobases (Kb) in size (Figure 5), similar to those reported previously in rabbits (Emori et al., 1986a). Analysis of total RNA of skeletal muscle samples for calpastatin mRNA, however, revealed three bands as I, II, and III(Figure III.4) of 3.8, 3.0 and 2.5 kb, respectively, as has been previously reported(Emori et al.. 1987). Calpastatin mRNA multiple bands arise from the same gene and differ only in processing of their 3'non-coding regions (Emori et al., 1987).

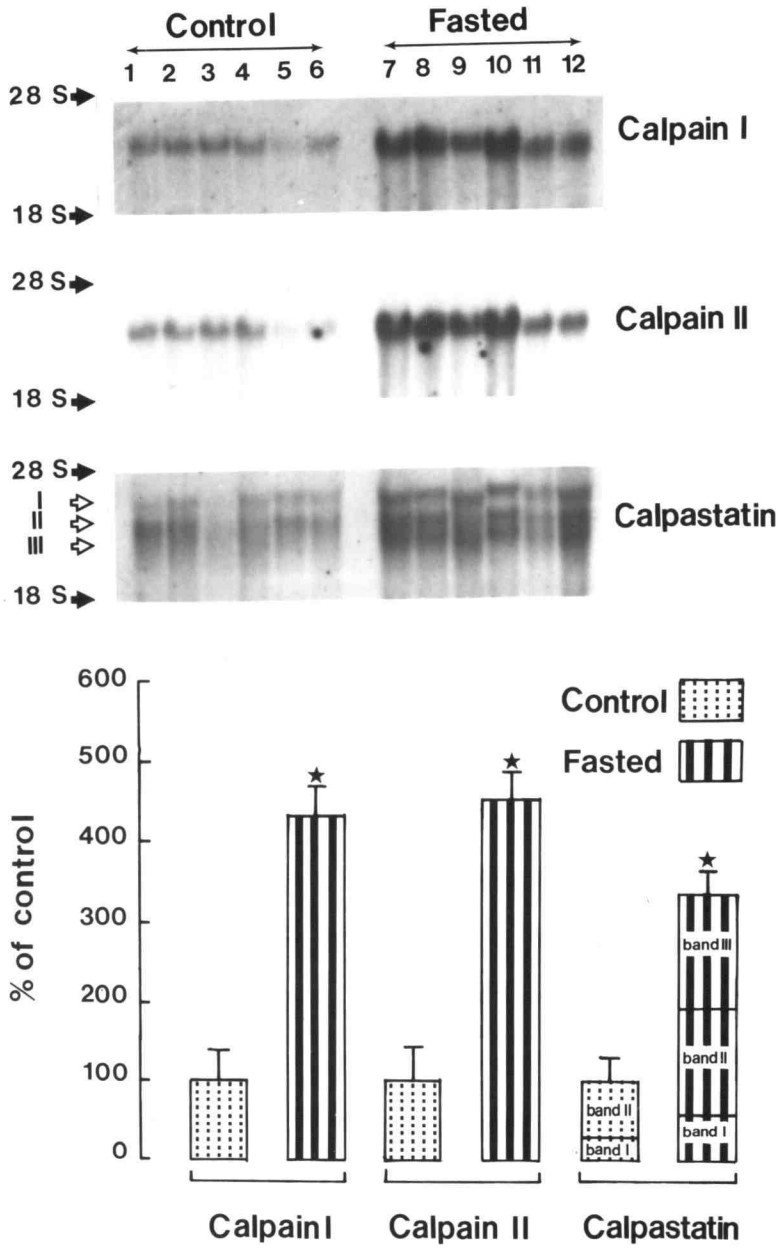

Figure III.4. Effect of fasting on steady-state mRNA concentration encoding calpain I and calpain II large subunits and calpastatin. The window panels present Northern blots. Washing conditions for all probes were 1 x SSC/0.1% SDS four times at room temperature followed by 0.1 x SSC/0.1% SDS two times at 50° C. The positions of ribosomal RNAs (28S and 18S) are indicated by solid arrows. Positions of calpastatin mRNA multiple bands are indicated by open arrows. Lower panel represents results of quantitation of mRNAs by scanning densitometry as percent of control. Each bar is the mean ± SEM (n = 6) and differences ($P < 0.05$) between control and fasted animals are indicated with a "star" located above densitometry scans.

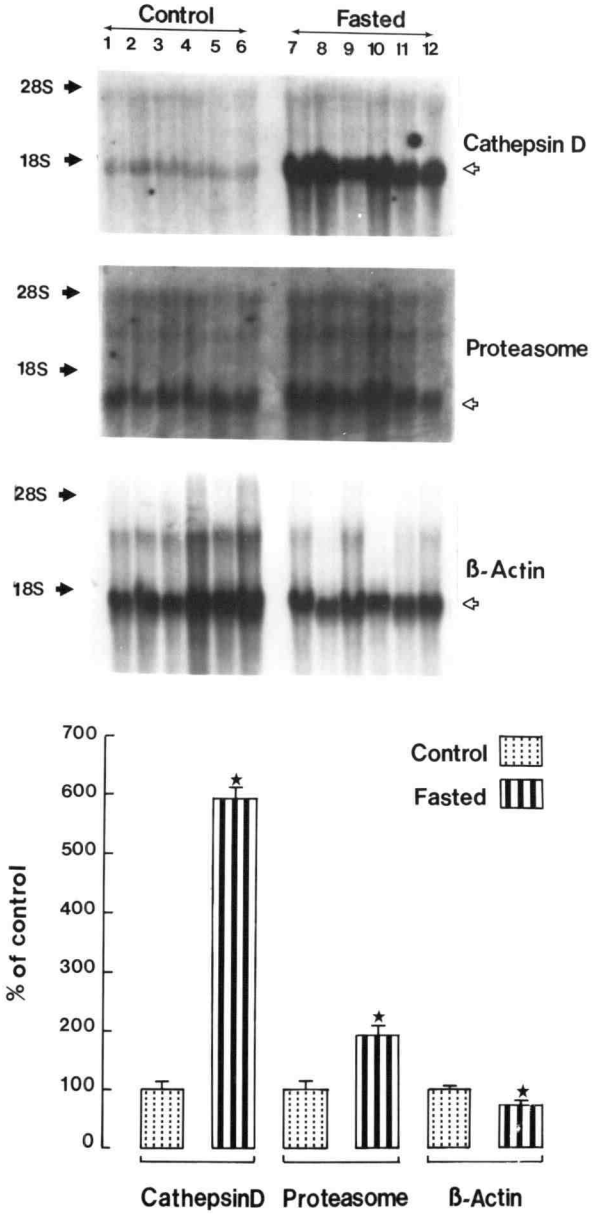

Figure III.5. Effect of an 8 d fast on steady state mRNA concentrations encoding cathepsin D, proteasome C2 and β-actin (upper window panels). Filters hybridized to cathepsin D and proteasome C2 cDNAs were washed four times with 1 x SSC/0.1% SDS at RT. Washing conditions for β-actin membrane was 0.5 x S SSC/0.1% SDS at 60° C four times. The positions of ribosomal RNAs (285 and 18S) are indicated by solid arrows. Positions of putative mRNAs of cathepsin D, proteasome C2 and β-actin are shown by open arrows. Lower panel represents results of quantitation of mRNAs by scanning densitometry as percent of control. Each bar is the mean $\pm$ SEM ($n=6$) and differences ($P<0.05$) between control and fasted animals are indicated with a "star" located above densitometry scans.

Levels of both band I and II of calpastatin mRNA increased in rabbit skeletal muscle due to fasting. The most intriguing observation, however, is the specific induction of band III (short species) which is mostly responsible for the significant increase in total calpastatin mRNA of skeletal muscle due to fasting.

To determine whether effects of fasting on mRNAs of the calpain system were unique or whether fasting effected changes in other proteinase mRNA concentrations, effects of fasting on cathepsin D and proteasome C2 subunit mRNA concentrations were examined. Cathepsin D and proteasome were selected in this comparison because they represent lysosomal and ATP-dependent proteolytic systems, respectively (Faust et al., 1985; Fujiwara et al., 1989) and because both were implicated in myofibrillar protein degradation (Arakawa et al., 1983; Kettelhut et al., 1988). Northern analysis of total RNA of skeletal muscle in control and fasted rabbits indicated that fasting caused 6 and 2 fold increase in levels of mRNA of cathepsin D and proteasome C2, respectively (Figure 5). Thus, fasting stimulates mRNA expression of multiple proteolytic systems in skeletal muscle, which suggests the integrated role(s) of these proteases in the physiological response of this tissue in response to food deprivation. This effect is unique for the various proteases studied as levels of β-actin mRNA decreased about 30% ($P<0.05$) in skeletal muscle of fasted rabbits as compared to the control group.

Activities of calpains and calpastatin in skeletal muscle of control and fasted rabbits

Table 2 shows the calcium-dependent proteinase and inhibitor activities determined in *biceps femoris* tissue of control and fasted rabbits. Activities were expressed as total value per gram of muscle tissue and as specific activity (units/g of extractable protein). Total activities of calpain I, calpain II and calpastatin showed no significant changes between control and fasted rabbits. Expression of activities of calpain I and calpain II in specific activity units revealed a slight numerical increase, though not significantly different, in activities of these proteins in skeletal muscle of fasted rabbits as compared to control. Calpastatin activity, expressed in specific activity units, showed larger differences, though not significant ($P > 0.05$), between control and fasted treatments. The calpastatin:calpain I plus calpain II ratios were essentially similar in tissues of control and fasted rabbits no matter how the results were expressed.

Polysomal mRNAs of calpain I and II

Although fasting increased steady state mRNA levels of calpain I, calpain II and calpastatin (Figure 4), enzymatic activities of these proteins were unchanged (Table 2). This discrepancy cannot be explained by the possibility that calpain activity did not provide an estimate of calpain content of tissues as I have determined in other studies (Chapter IV) using Western analysis that muscle calpain I and II activities are correlated with calpain I and II concentrations. This observation

Table III.2. Effect of fasting on activities of rabbit muscle calpain I, calpain II and calpastatin.

Parameter*	Control	Fasted	% Change
Calpain I			
- (Units**/g tissue)	1.5[a] ± 0.14	1.5[a] ± 0.1	-
- (Units/g protein)	21.2[a] ± 2.2	25.1[a] ± 3.4	18
Calpain II			
- (Units/g tissue)	2.58[a] ± 0.08	2.45[a] ± 0.20	-5
- (Units/g protein)	36.50[a] ± 2.7	40.10[a] ± 3.10	10
Calpastatin			
- (Units/g tissue)	8.8[a] ± 0.82	8.36[a] ± 0.31	-5
- (Units/g protein)	111.3[a] ± 11.5	149.9[a] ± 31.5	34.6

* Values are means of six observations ± SEM. Values within same row with similar superscripts are not significantly different ($P>0.05$).

** Calpain activity is defined as one unit in the amount of enzyme which caused an increase of 1 absorbance unit at 280 nm and 25° C when incubated with Hammersten casein for 30 min. One unit of calpastatin is the amount of inhibitor which inhibits 1 unit of calpain.

tempted me to study the composition of the translated mRNA population of calpain I and II in skeletal muscle of control and fasted rabbits. The levels of calpain I and II in skeletal muscle polysomes of control and fasted rabbits are shown in Figure 6. Notably, fasting caused about 2-fold increase in the number of RNA messages for calpain I and calpain II loaded on polysomes of skeletal muscle of fasted rabbits as compared to those of the control.

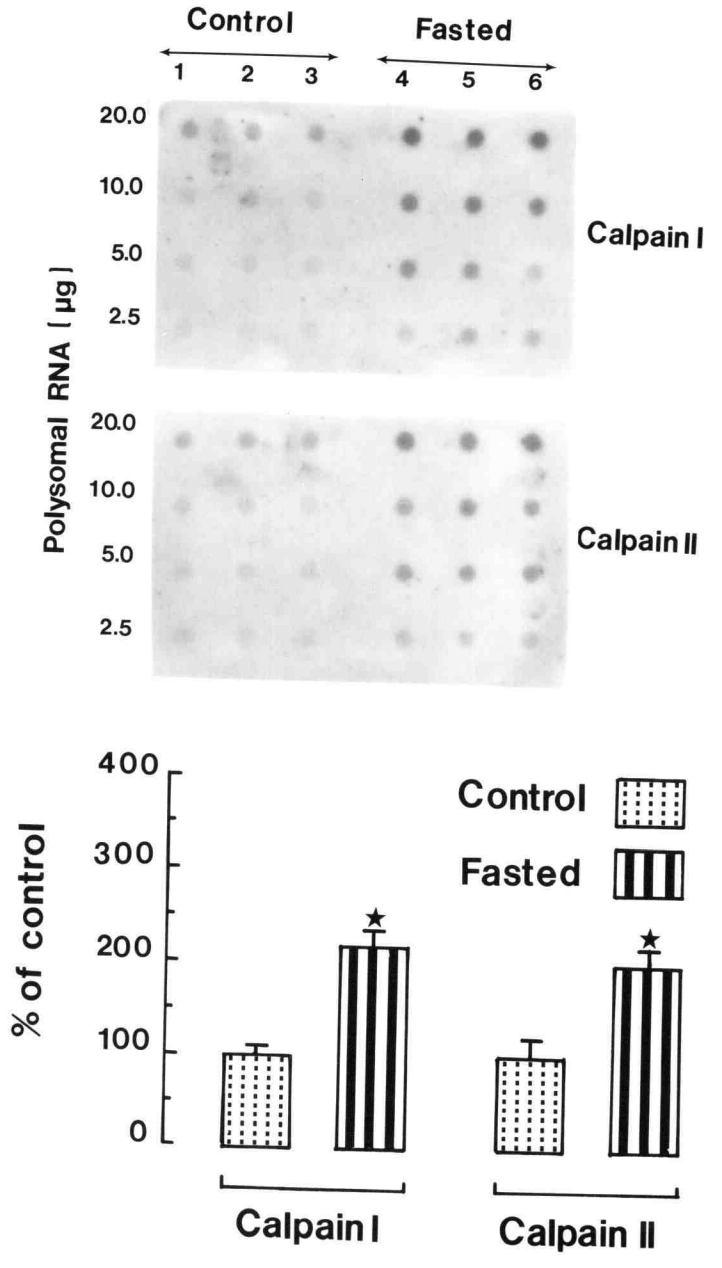

Figure III.6. Dot blot hybridization analysis of calpain I (upper panel) and calpain II (middle panel) mRNAs charged to ribosomes in skeletal muscle of control and fasted rabbits. Serial dilutions of polysomal RNA (2.5-20 μg) were spotted onto nitrocellulose filters for each animal. Lower panel represents results of quantitation of mRNAs by scanning densitometry as percent of control. Each bar is the mean ± SEM (n = 3) and differences ($P < 0.05$) between control and fasted animals are indicated with a "star" above densitometry scans.

DISCUSSION

Characteristics of fasted rabbits

Successful metabolic adaptation to fasting depends, in great part, on the ability of the animal to mobilize body energy reserves, mainly myofibrillar proteins in skeletal muscle, to support its energy requirements, and, in particular, to maintain required levels of blood glucose for proper functioning of brain and erythrocytes (Owen et al., 1967). Many studies have been conducted on the effect of fasting on muscle protein metabolism in the rat (Long et al., 1988; Emery et al., 1986). Results of these studies and others (Li and Wassner, 1984; Lowell et al., 1986a,c) revealed that fasting causes preferential acceleration in the degradation of myofibrillar proteins over saracoplasmic proteins in skeletal muscle. A comprehensive survey of literature indicated the lack of studies on effects of fasting on muscle protein metabolism in rabbits. Results in this study on the effects of fasting on urinary 3-MH metabolism and RNA content in skeletal muscle in rabbits are essentially similar to results observed for the rat (Lowell et al., 1986a,c; Emery et al., 1986). The present results indicate that fasting in young rabbits causes marked effects on protein metabolism in skeletal muscle. In this tissue, a fall in muscle protein synthesis and a significant rise in myofibrillar protein degradation are observed for rabbits fasted for 8 days, as indicated by a significant decrease in total and polysomal RNA (Table 1) and the considerable rise in urinary 3-MH excretion (Figure 1).

Gene expression of calpain system during fasting

Knowledge on regulation of expression of calpain I, calpain II and calpastatin during physiological functioning or in response to certain treatments is rudimentary. To date, only one article has been published which describes the regulation of expression of calpain II alone during pregnancy and involution (Samis et al., 1991). Based on their results, Samis and his associates (1991) concluded that calpain II mRNA is constitutively expressed in the rat uterus regardless of its physiological condition (i.e. non-pregnant, pregnant or post-partum).

Regulation of enzymes at the transcription level is usually employed only for enzymes that are needed in special circumstances or at a particular stage of organismal development. Enzymes that perform general "housekeeping' functions, essential to every cell at all times, are generally not regulated in this manner (Mathews and Van Holde, 1990). Ubiquitous distribution of the calpain-calpastatin system in animal cells seems to lend support to the notion that calpains are housekeeping enzymes with general uniform functions in various cells (Miyake et al., 1986; Suzuki et al., 1987). On the other hand, distinct unevenness of the absolute and relative amounts of calpain I and II and calpastatin in different cells (Murachi et al., 1989) and large variation in mRNA levels of calpain I and II among various tissues (Emori et al., 1986a) can be a clear indication of the highly diversified significance of the calpain system in different cells. Proposed proteolytic down regulation of protein kinase C by calpain (Kishimoto et al., 1983) can be one of the strong candidates in the former category of general, uniform functions while the

calpain-catalyzed initiation of myofibrillar protein degradation in muscle may belong to the latter category of more tissue-specific functions. With this in mind, we believed that examination of the regulation of expression of calpains and other proteinases, implicated in myofibrillar protein degradation, in skeletal muscle in normal and fasted animals would provide insight into the molecular determinants of proteolysis in skeletal muscle.

Results of mRNA analysis of calpain I, calpain II and calpastatin indicated that expression of these genes in skeletal muscle is up-regulated by fasting (Figure 4). We believe that the current study is the first of its kind to describe significant changes in the mRNA of calpain I, calpain II and calpastatin during some physiological process and in response to certain treatments. The implication of this observation is that fasting may be used as a biological model to characterize regulatory mechanisms controlling expression of the calpain system. Another interesting result pertains to the specific induction of calpastatin (III) expression in skeletal muscle of fasted rabbits but not in tissue of control animals. The structural element responsible for the appearance of three forms of mRNA for calpastatin has become clear after the cloning and sequencing of calpastatin cDNA of rabbits (Emori et al., 1987). This study revealed that calpastatin gene contains three 3'-processing sites (AAATTA) which account for expression of three mRNA species of varying size but identical in the coding region and thus in the protein product produced from their translation. The physiological significance for expression of three calpastatin mRNA isoforms is unknown at the present time, although it has been suggested that the size of the 3'-noncoding region of mRNA influences its

stability (Sheiness and Darrell, 1973; Brawerman, 1976) or transport from nucleus to cytoplasm (Brawerman and Diez, 1975). The significance of specific induction of the small species of calpastatin mRNA and reasons why muscle cells express three forms of calpastatin mRNA under stressful contions are worthy of additional studies. It is suggested that the specific induction of calpastatin mRNA (III) in skeletal muscle of fasted rabbits may represent a mechanism for control of calpastatin expression during stress.

Several studies indicated that muscle atrophy involves coordinated changes in several proteolytic systems, each of which may function in the breakdown of different cell components (Furano et al., 1990). To determine whether effects of fasting on mRNAs of calpain system were unique to calpain I, calpain II and calpastatin or whether fasting effected changes in mRNA concentrations of other proteinases, we determined mRNA levels of cathepsin D and proteasome C2 in skeletal muscle of control and fasted rabbits. Both enzymes showed up-regulation in the expression of their mRNAs (Figure 5). The up-regulation of mRNAs of calpain system, cathepsin D and proteasome C2 in response to fasting in skeletal muscle is novel and specific as shown from β-actin mRNA response pattern (Figure 5). Observations in our study that fasting increased cathepsin D, proteasome and calpain system mRNA concentrations indicate that fasting invokes control of several proteolytic systems in skeletal muscle and underscores the likelihood that each proteolytic system plays a role(s) in the adaptation of skeletal muscle to the fasted state. An important adaptation to fasting in animals is the mobilization of body protein reserves, mainly myofibrillar proteins in muscle, to support the organism's

energy requirements. Since myofibrillar proteins must be degraded completely to amino acids to provide energy and support gluconeogenesis during fasting, the complete hydrolysis of myofibrillar proteins into amino acids cannot be performed by calpain system as this proteinase system is an endopeptidase. Thus it is not out of the ordinary to expect the concerted up regulation of other proteolytic systems side by side with calpain system in response to fasting.

The highly coordinated response of expression of mRNAs of calpain I, calpain II and calpastatin in skeletal muscle of rabbits during normal (control treatment) and stressful (fasting) conditions, as shown in Figure 4, is of considerable interest. Such a pattern of response indicates that common regulatory elements may coordinate expression of calpain system genes in muscle.

Hata et al. (1989) have determined that the human calpain II gene is under control of four negative enhancer-like cis-elements and have proposed that relative tissue concentrations of calpains are determined, in part, by concentrations of an uncharacterized negative trans-acting element. If this is true, concentrations of this element would be lower in skeletal muscle of fasted animals, compared to those of the control ones. For example, fasting may reduce concentration of this trans-acting factor in skeletal muscle and result in release of the negative control on gene expression. Although the sequence of the 5'-flanking region of calpain I and calpastatin genes have not been elucidated, based on the similarity of the gene expression response to fasting among calpain I, calpain II and calpastatin, it may be

possible that calpain I and calpastatin genes possess common features to the calpain II promoter region.

Proteolytic activity of calpain system during fasting

The results of measuring proteolytic activity of calpain I, calpain II and the inhibitory activity of calpastatin in skeletal muscle of control and fasted rabbits were surprising and unexpected. These results cannot support a major role for calpains in the turnover of myofibrillar protein degradation in skeletal muscle since no correlation was observed between enzyme activity of calpain system and the considerable rise in urinary 3-MH concentration at d 8 of fast. Thus, it is fair to suggest either that calpain system has no major role in myofibrillar protein degradation in skeletal muscle or that urinary 3-MH is not a reliable index for the status of myofibrillar degradation in skeletal muscle or that the response of different types of skeletal muscle to fasting is not homogeneous. I believe that a combination of both the second and third possibilities may be responsible for the discrepancy between activities of skeletal muscle calpain system and urinary 3-MH results. In our study, we analyzed a single muscle, "*biceps femoris*", for activities of calpain system. Thus, results of enzyme activities in this muscle may not represent the activities of calpain system in skeletal muscle of the whole animal.

Previous studies (Jefferson et al., 1980; Emery et al., 1986) suggested that skeletal muscles, which contain a high proportion of dark oxidative fibers, are more

resistant to the effect of fasting than are muscles of a more mixed, dark-pale, fiber type composition. Since *biceps femoris* in rabbits is of the pale fiber type, it is expected that it will respond rather early to fasting by mobilizing myofibrillar proteins rapidy. If that is the case, an 8 d fast may be detrimental to the survival of *biceps femoris* if myofibrillar protein degradation remains high. Thus, results of activities of calpain system in *biceps femoris* after 8 d of fasting rabbits does not rule out the role of calpains in myofibrillar protein degradation as this day may have not coincided with the maximum rate of myofibrillar protein degradation in this muscle.

Polysomal mRNAs of calpain system during fasting

The most extraordinary result observed in this study is the lack of correlation between response of mRNAs of calpain system and their activity due to fasting. Significant increases of mRNA levels of calpain I and calpain II in skeletal muscle due to fasting were accompanied by no differences in activity of these enzymes between control and fasted rabbits. This observation tempted us to analyze the composition of the translated mRNA population for calpain I and calpain II by isolating polysomes from skeletal muscle and analyzing translated mRNA levels of calpain I and calpain II in control and fasted animals.

Results of this analysis (Figure 6) revealed significant differences (2-fold) between polysomal mRNA levels of calpain I and calpain II in control and fasted rabbits. Thus, up-regulation of mRNA expression of calpain I and calpain II in

skeletal muscle due to fasting is needed to boost message loading on ribosomes. This mechanism could be important for regulating protein synthesis in skeletal muscle during stressful conditions such as fasting. As stated earlier, fasting is characterized not only by enhanced protein degradation rate but also by a drastic drop in protein synthesis rate (Harmon et al., 1984). Hence, to explain our observations, the following theory is proposed. During fasting, muscle translational activity is reduced. However, during the fast it is important that muscle maintains adequate levels of proteinases such that capability for mobilization of muscle protein reserves is maintained throughout the fast. To circumvent the effects that the reduction in translation would have on muscle proteinase concentration, transcription of proteinase genes is selectively induced to increase mRNA levels to a level that offsets the drop in protein synthesis rate by increasing message loading on ribosome. In support of this theory, Calzore et al. (1983) reported that message loading is the major factor limiting the rate of protein synthesis, and large and rapid changes in the general rate of protein synthesis occur by regulation of the number of loaded messages.

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CHAPTER IV

A COMPARATIVE STUDY ON GENE EXPRESSION OF CALPAINS AND CALPASTATIN IN VARIOUS TISSUES OF FED AND FASTED RABBITS

by

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CONTRIBUTIONS

Mohammad A. Alyan participated in developing the experimental design, development and streamlining of analytical techniques utilized to perform this study, performing the animal trial, sampling tissues, analyzing the data and calculating the results, performing the statistical analysis and writing the manuscript. Dr.Neil E. Forsberg participated in the experimental design, supervising progress of study and editing the manuscript.

A COMPARATIVE STUDY ON GENE EXPRESSION OF CALPAINS AND CALPASTATIN IN VARIOUS TISSUES OF FED AND FASTED RABBITS

ABSTRACT

Regulation of activity of the calpain system at the transcriptional level in skeletal muscle has not previously been studied. Accordingly, we investigated effects of fasting, which increases myofibrillar protein degradation, on steady-state mRNA levels encoding calpain I and II large subunit, calpain small subunit and calpastatin in skeletal muscle, liver, kidney, nad lung using Northern blot analyses. Parallel to this, we determined changes in mRNA levels of cathepsin D, proteasome and β-actin for comparative purposes. Results indicated that fasting induced expression of calpain large subunits and small subunit, calpastatin, cathepsin D and proteasome mRNAs and reduced expression of β-actin mRNA in skeletal muscle. To determine whether the regulation observed in skeletal muscle was also present in other tissues, we examined effects of fasting on proteinase mRNA concentrations in liver, lung and kidney. Results revealed that fasting either caused no effect or reduced mRNA levels for the proteinases investigated. Based on our results and those of others, a model for calpain activation, the "Triarchy Model", was proposed.

A COMPARATIVE STUDY ON GENE EXPRESSION OF CALPAINS AND CALPASTATIN IN VARIOUS TISSUES OF FED AND FASTED RABBITS

INTRODUCTION

Considerable progress in knowledge on calpain (EC 3.4.22.17), a typical intracellular proteinase, has been made during the past fifteen years (Suzuki et al., 1987; Goll et al., 1989; Mellgren et al., 1989; Pontremoli and Melloni, 1986; De Martino and Croall, 1987; Murachi, 1989). As a result, calpains are now among the best well-characterized proteinases, at least as to the structure-function relationship. However, the rapidly growing and expanding knowledge of structure-function of calpain system is not paralleled with an equal rate of progress in understanding of the biological function and activity regulation of the calpain system in vivo. Since calpains are housekeeping enzymes (Hata et al., 1989; Suzuki and Ohno, 1990) and co-exist in the cytosol together with various proteins which must be securely protected from the uncontrolled action of proteases, both calpain activity and gene expression are under very tight control (Suzuki and Ohno, 1990). It was, therefore, very difficult to find a suitable experimental model in which significant changes in calpain mRNA levels occur upon certain treatments.

In Chapter III of this thesis, we reported that expression of calpains and calpastatin mRNAs in skeletal muscle is up-regulated by fasting. In this chapter, we extend our investigation to study the co-regulation of calpain system genes. This includes the regulation of calpain I large subunit (LI), calpain II large subunit (LII), calpain small subunit (S) and calpastatin in various tissues of control and fasted animals. Furthermore, in this study, we compared responses of genes of the calpain system in a variety of other tissues to gain insight into whether skeletal muscle reponse during fasting is tissue specific or represents a general response.

MATERIALS AND METHODS

Animals

Details of animal care and treatment are presented in the Materials and Methods section of Chapter III. At the conclusion of the fasting period, rabbits were anesthetized as indicated earlier (chapter III). Blood was collected in heparinized vacutainer tubes from the heart. Plasma was separated by centrifugation and was replaced by the same volume of distilled water to lyse the blood cells. Samples were frozen at -20° C until analysis. After blood collection, the animals were euthanized in CO_2 tanks and the following tissues were then excised, blotted and weighed: skeletal muscle (*vastus lateralis)*, liver, kidneys and lung. After their removal, tissues were frozen in liquid nitrogen and stored in -89° C deep freezer until nucleic acid and enzyme determination.

Calpain and calpastatin assays

Analysis of tissue calpain activity was performed by utilizing phenyl-sepharose chromatography as described by Gopalakrishna and Barsky (1985). Details of the analytical procedure are outlined in the Materials and Methods section of Chapter III. One unit of calpain activity was defined as the amount of enzyme which produced an increase of one absorbance unit at 280 nm after 30 min incubation at 25° C, corrected by subtracting the activity found in the presence of 1 mM EDTA.

Calpastatin activity was assayed as described (chapter III). One unit of calpastatin inhibited one unit of rabbit calpain II.

Messenger RNA analysis

Determination of steady state mRNA concentrations of calpain LI, calpain LII, calpastatin, cathepsin D, proteasome C2 and β-actin were performed by Northern blotting, essentially as outlined in Chapter III.

To analyze mRNA levels of calpain small subunits, a 30-base oligonucleotide probe designated RCal-S1 was synthesized chemically at the Center for Gene Research and Blotechnology of OSU. The probe sequence: 5' CCG GCG AGG CGG AAG GGG GCG GCT CTG GGG 3' is complementary to nucleotides 809 to 838 of the 3' non-coding region of published sequence of rabbit calpain small subunit (Emori et al., 1986). The basis for selecting the 3' non-coding region 809-838 in RCal-S1 is twofold: 1) structural analysis of the 3'-non-coding region is markedly different from the 3' non-coding region of other calpain subunits (LI and LII) both in length and sequence. Thus RCal-S1 is highly specific for calpain S mRNA, and 2) RCal-S1 structure has 83% G+C content. This high G+C content is very beneficial in Northern blotting as it translates into higher melting temperature (Tm) of RNA-DNA duplex and, consequently, the ability to apply strong washing conditions if background or non-specific binding problems exist.

After chemical synthesis was completed, RCal-S1 preparation was purified from contaminants of synthesis by using urea denaturing polyacrylamide gel electrophoresis according to Vorndam and Kerschner (1986) with some modifications. The probe RCal-S1 was radiolabeled to specific activity (~ 5.0 x 10^7 dpm/μg DNA)
with γ^{32}P-ATP (6000 Ci/mmol, New England Nuclear, Boston, MA) using 5'-end labeling method, after which it was purified from free γ^{32}P-ATP using P2 gel chromatography.

Western blotting analysis

Preparation of crude extract was performed as follows. Tissue samples from skeletal muscle (*vastus lateralis*), liver, kidney, lung and hemolyzed blood were each homogenized in 5 vol of ice cold homogenization buffer (50 mM Tris HCl, pH 7.5, 1 mM EDTA, 10 mM 2-mercaptoethanol and 150 nM pepstatin A) by using a Polytron at half maximum setting, three times for 15 s each while keeping the tube immersed in ice. Afterward, samples were centrifuged at 13,000 g for 30 min and the supernatant was decanted through glass wool to separate lipids. The supernatant was assayed for protein concentration. Determination of protein concentration was performed with the Bio-Rad Bradford protein assay solution according to the supplier instructions, and a standard curve was constructed using BSA (Fraction IV).

Tissue homogenates (100 μg protein per lane) were subjected to SDS-PAGE according to the procedure of Laemmli (1970) in a 7.5% polyacrylamide slab gel. Molecular weight markers were included in every gel to determine size of immunostained bands. After completion of the gel run, the molecular weight markers lane was cut out and visualized with Coomassie brilliant blue P250 stain. Visualized bands were used to construct a standard curve by plotting distance migrated on gel (cm) against log molecular weight of standard proteins. The remaining gel was transferred to nitrocellulose membrane electrophoretically according to the method of Towbin et al. (1979). Immunoblotting was carried out as follows: After transfer, the membrane was blocked with 3% (w/v) gelatin in Tris-buffered saline (TBS) for 60 min at room temperature and then washed 2 times for 5 min each in TBS containing 0.05% (v/v) Tween-20 (TTBS). Then the membranes were incubated with anti-calpain monoclonal antibodies (clone $3C_{11}B_{10}$ against calpain I and clone $1G_5C_8$ against calpain II; Kasai et al., 1986) diluted with TTBS and 1% (w/v) gelatin to 1/1,000. The monoclonal antibodies were kindly donated by Dr. Seichi Kawashima, Department of Biochemistry,Tokyo Metropolitan Institute of Gerontology, Tokyo 173. The incubation period for primary antibody treatment was 12 h to enhance sensitivity, followed by two washes wiith the 1 x TTBS for 5 min each at RT. Then, the membranes were incubated with horse radish peroxidase-conjugated rabbit anti-mouse immunoglobulin as the second antibody. The incubation period was 2 h followed by washing two times with 1 x TTBS for 5 min each at RT. The peroxidase staining was developed using 0-dianisidine as the substrate (Hawkes et al., 1982).

Partially purified calpain I and calpain II preparations by phenyl sepharose chromatography were used to establish the specificity of monoclonal antibodies. Here we immunoblotted a membrane carrying 2.5, 5.0 and 7.5 μg protein of partially purified calpain I and calpain II samples with Ab 3C11B10 to confirm its monospecificity against calpain I. Also, we immunoblotted a membrane carrying 2.5, 5.0 and 7.5 mg protein of partially purified calpain II, 7.5 μg protein of partially purified calpain I and 100 and 200 μg protein of hemolyzed blood sample against calpain II Ab. Quantitation of calpain proteins was performed by producing transparencies from immunoblotted membranes and scanning them by using a Hoefer-350H scanning densitometric program.

Statistical analysis

Differences ($P<0.05$) between the two experimental treatments were examined by using an unpaired Student's t-test (Steel and Torrie, 1980).

RESULTS

Characterization of the animal model

The effect of fasting on body condition, water intake and urine output is described in Figure IV.1. At 8 days of fasting, the fasted rabbits lost about 30% of their initial body weight, whereas the fed group (control) gained about 14%. Response of animals, in terms of water intake and urine output, to fasting was intriguing. Fasted animals continued to raise their water intake as fasting progressed to reach at the end of the trial about 3 fold that of their intake at the start of the experiment. This pattern of water consumption was paralleled with a similar pattern of urine output. Control animals showed a constant level of water intake and urine output throughout the experimental period.

The effect of fasting on individual organs was quite variable (Table IV.1). For example, the liver lost a substantial part of its weight (59.3%). Kidney, skeletal muscle and lung lost 34.9, 31.0 and 29.8 percent of their sizes in response to 8 d fast, respectively.

The effects of fasting on skeletal muscle, hepatic, kidney and lung RNA levels are shown in Table IV.2. After 8 d of fasting, skeletal muscle, lung and kidney lost 35.7, 32.0 and 24.8% of RNA ($P<0.05$). However, liver response was much less profound, as RNA decreased only 8.5% due to fasting. Among tissues, liver had

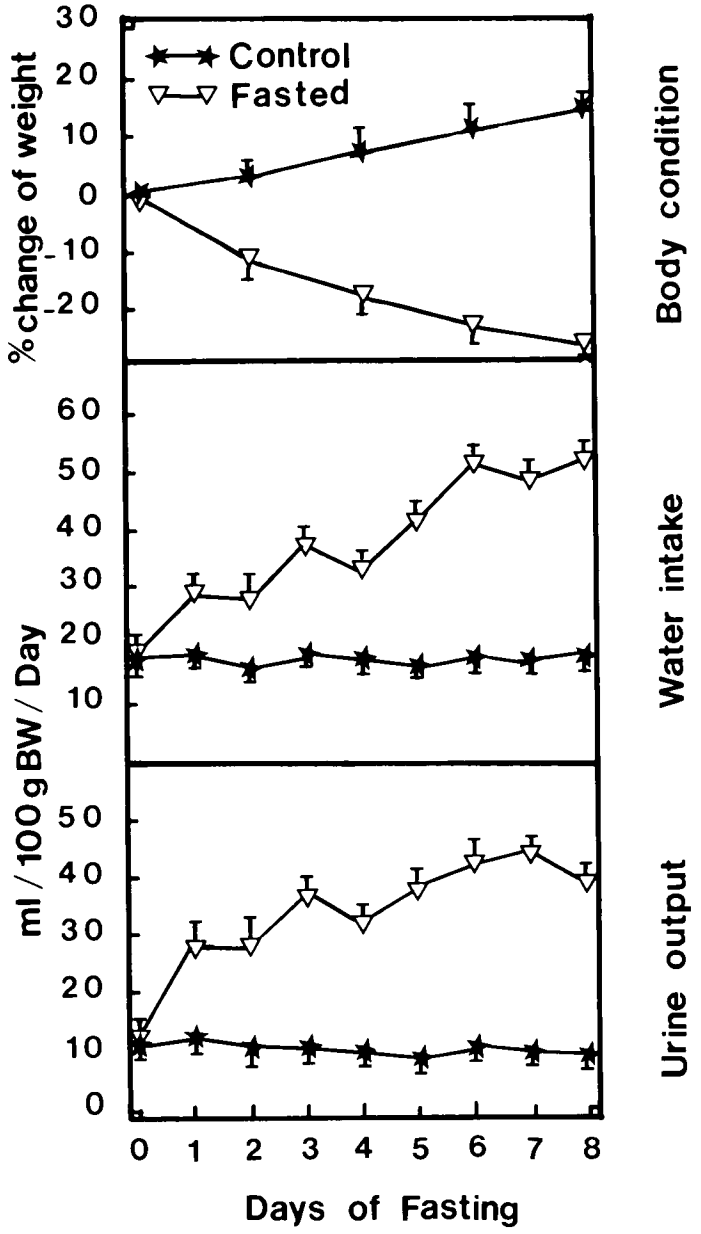

Figure IV.1. Effect of fasting on body weight, water consumption and urine output in rabbits.

Table IV.1. Effect of fasting on body and organ weights.

Parameter (g)	Control	8-day Fasted	Change from Control (%)
Body wt*	2284.0 ± 65.7^{a**}	1420.0 ± 19.7^{b}	-37.8
Skeletal muscle	10.0 ± 0.5^{a}	6.9 ± 0.2^{b}	-31.0
Liver	99.5 ± 6.3^{a}	40.5 ± 1.7^{b}	-59.3
Kidney	18.9 ± 1.0^{a}	12.3 ± 0.5^{b}	-34.9
Lung	10.4 ± 0.5^{a}	7.3a^{b} ± 0.5^{b}	-29.8

* Initial body weight for both groups was 2000 g.

* Each datum is an average ± SEM of 6 observations and values followed by different superscripts within rows are significantly different at $P<0.05$.

Table IV.2. Effect of fasting on tissue RNA* (μg/g).

Organ	Control	8 Day Fasted	Change from Control (%)
Skeletal muscle	933 ± 42^{a**}	600 ± 36^{b}	-35.7
Liver	4861 ± 164^{a}	4497 ± 290^{a}	-8.5
Kidney	1856 ± 112^{a}	1395 ± 98^{b}	-24.8
Lung	1604 ± 182^{a}	1090 ± 156^{b}	-32.0

* Total RNA in tissues was determined as the yield of RNA calculated from u.v. absorption.

** Each datum is an average ± SEM of 6 observations. Values followed by a different superscript within rows are statistically different at $P>0.05$.

the highest concentration of RNA, followed by lung and kidney, then skeletal muscle.

Since concentration of total RNA per unit weight of tissue is an index of protein synthetic capacity in that tissue, results presented in Table IV.2 indicate that the protein synthetic capacity most affected by fasting was that of muscle, followed by that of lung and kidney. However, liver, in spite of its drastic reduction in size (Table IV.1), maintained its protein synthetic capacity very efficiently during fasting.

Activity of calpain system

The determination of calpain I, calpain II and calpastatin activities in various tissues of control and fasted rabbits are shown in Table IV.3. The highest levels of calpains were detected in the lung and kidney while other organs (skeletal muscle, blood cells and liver) had lower levels of the enzymes. With regard to the effect of fasting on calpain activities in various tissues, no dramatic changes were observed in any tissue; however, there was slight, though not significant ($P > 0.05$), increase in calpain I and calpain II activities in skeletal muscle and liver of fasted rabbits as compared to controls. The levels of calpastatin in different tissues followed that of calpains rather closely. The total activity of calpastatin was highest in lung, followed by liver, kidney, blood cells and muscle. Again, fasting did not cause significant changes in calpastatin activities among all tissues investigated.

Table IV.3. Distribution of calpain I, calpain II and calpastatin in various tissues of control and fasted rabbits.

*Enzyme**	Skeletal Muscle (*vastus lateralis*)	Liver	Kidney	Lung	*Blood Cells* (1 g packed cells)
Calpain I					
- Control	2.62 ± 0.48	1.22 ± 0.18	2.70 ± 0.45	4.22 ± 0.3	2.18 ± 0.3
- Fasted	2.91 ± 0.41	2.27 ± 0.51	2.32 ± 0.47	4.55 ± 0.26	1.82 ± 0.15
Calpain II					
- Control	2.12 ± 0.12	0.93 ± 0.2	3.41 ± 0.55	4.97 ± 0.31	ND
- Fasted	2.34 ± 0.21	1.28 ± 0.2	3.87 ± 0.58	5.09 ± 0.33	ND
Calpastatin					
- Control	3.77 ± 0.24	7.10 ± 0.2	5.82 ± 2.1	19.15 ± 1.70	4.00 ± 1.17
- Fasted	4.17 ± 0.15	5.23 ± 0.8	6.62 ± 0.83	17.92 ± 1.88	3.97 ± 0.73
Calpains/Calpastatin**					
- Control	1.25	0.30	1.05	0.40	0.55
- Fasted	1.25	0.67	0.94	0.53	0.46

* Activity is expressed in units/g tissue. The values represent mean ± SEM from six observations for six animals. ND = no activity detected.

** Calpains/Calpastatin = calpain I + calpain II + calpastatin.

Calculation of the ratio between calpains and calpastatin indicated that in the skeletal muscle the total levels of calpains exceeded slightly that of calpastatin in both control and fasted animals. Kidney had approximately the same level of total calpains compared to calpastatin for both control and fasted rabbits. The liver, lung and blood cells in both control and fasted treatments showed a higher total level of the inhibitor compared to the total amounts of calpains.

Immunoblotting of calpain I and calpain II

For quantitative determination of calpain concentrations, monoclonal antibodies to the 80 kDa subunits of calpain I ($3C_{11}B2_{10}$) and calpain II ($1C_5C_8$) were used to perform Western analysis on various tissues. To establish the specificity and the validity of these antibodies for quantitative determination of calpains by immunoblotting, graded levels of partially purified calpain I and calpain II from rabbit muscle were subjected to SDS-PAGE, immobilized on nitrocellulose and detected by immunoblotting. As shown in Figures IV.2 and IV.3, both monoclonal Abs were found to be isozyme specific as they bound to their respective 80 kDa subunits but not 30 kDa subunit and were found to give linear values with gradations in partially purified protein antigen concentrations (2.5-7.5 μg protein). Consequently, these antibodies were used to analyze calpain I and calpain II concentrations in various tissues of control and fasted rabbits.

Results of Western analysis of calpain I in skeletal muscle, blood, kidney and lung are shown in Figure IV.4.

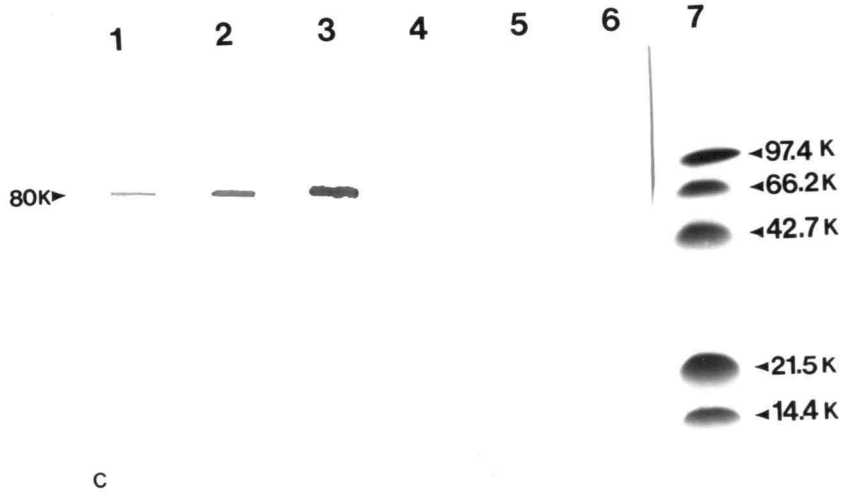

Figure IV.2. Immunoblotting of partially purified calpain I and calpain II with monoclonal antibody against calpain I. Preparations of calpain I and calpain II were loaded as 2.5, 5.0 and 7.5 μg of total protein per well to establish a dose response curve. Membrane was treated as in Materials and Methods section.

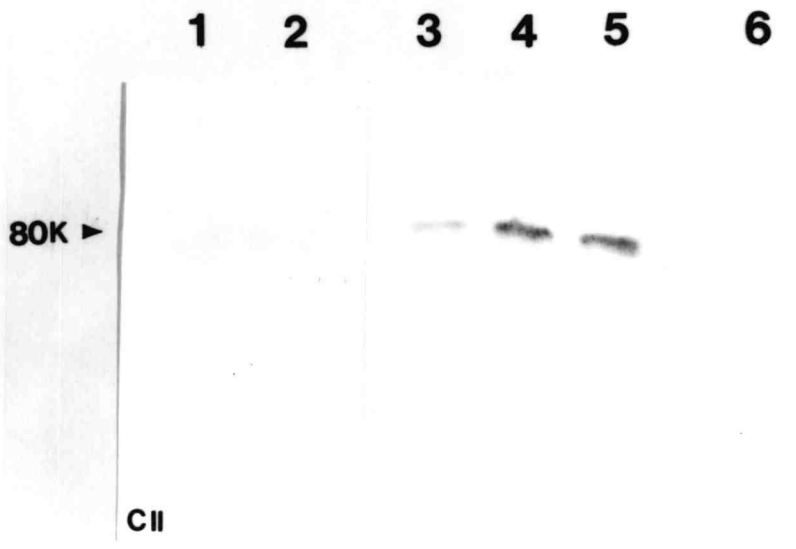

Figure IV.3. Immunoblotting of partially purified calpain I and calpain II and RBC lysate against calpain II. Preparations of calpain I and calpain II were loaded as 2.5, 5.0 and 7.5 μg of total protein per well to establish a dose response curve. RBC lysate (100 μg) was loaded as a regulative control. Membrane was treated as in Materials and Methods section.

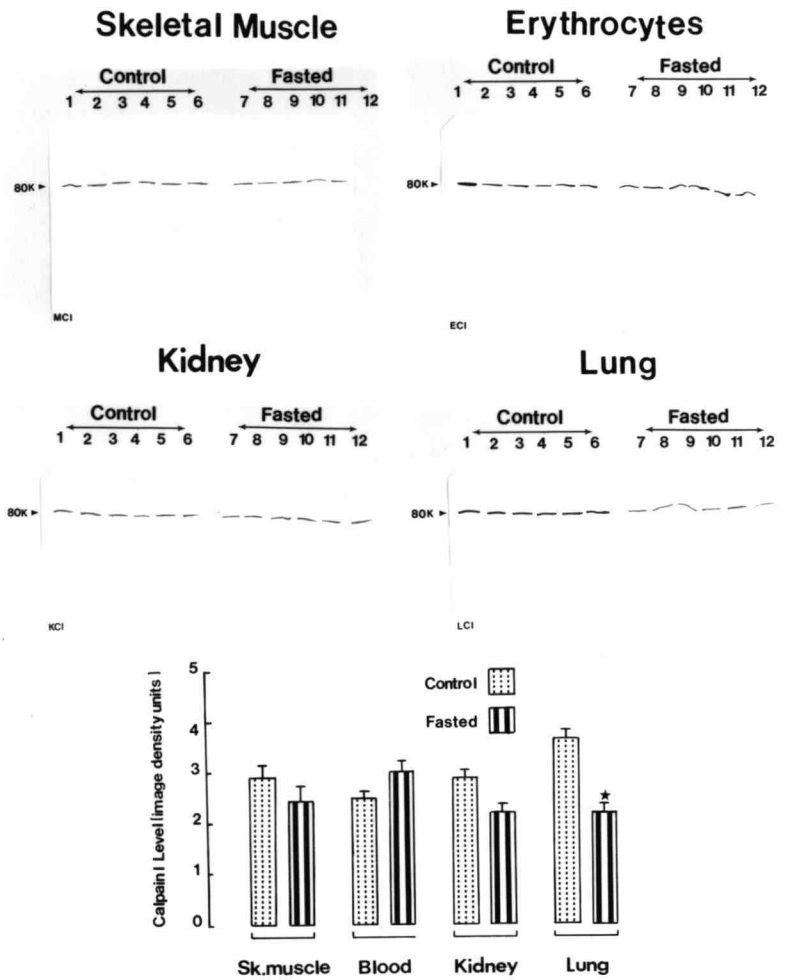

Figure IV.4. Western blot analysis on calpain I of various tissues of control and fasted rabbits. Tissue homogenates of various samples (100 μg/well) were subjected to SDS-PAGE, and then transferred to a nitrocellulose filter which was stained with anti-rabbit calpain I monoclonal antibody. Molecular weights of various bands are indicated by solid arrows (K = kDa).

The data showed that there were no significant changes in calpain I concentration in skeletal muscle, blood and kidney due to fasting. On the contrary, lung calpain I level was lower ($P<0.05$) in fasted animals as compared to controls. Among the tissues of control rabbits, lung possessed the highest calpain I concentration. Blood also exhibited high calpain I concentrations. Skeletal muscle and kidney contained similar quantities of calpain I concentration.

Western analysis of calpain II in various tissues is also presented in Figure IV.5. The immunoblot data show no change in calpain II levels due to fasting in skeletal muscle, liver, kidney and also lung. When comparing levels of calpain II among tissues, lung was found to have the highest level, followed by kidney, skeletal muscle and liver, respectively. It is interesting to detect with calpain II Ab multiple bands in liver (252 and 30 kDa) and kidney (252, 56 and 30 kDa) in addition to the putative calpain II band (80 kDa). The physiological significance of this observation is not clear.

Expression of mRNAs of calpain system in tissue of control and fasted rabbits

To investigate tissue specificity of the transcriptional up-regulation of expression of calpains and calpastatin in skeletal muscle (*biceps femoris*) due to fasting which was reported earlier (chapter III), we determined steady state mRNA levels of calpain and calpastatin in skeletal muscle *(vastus lateralis*) in conjunction with liver, kidney and lung.

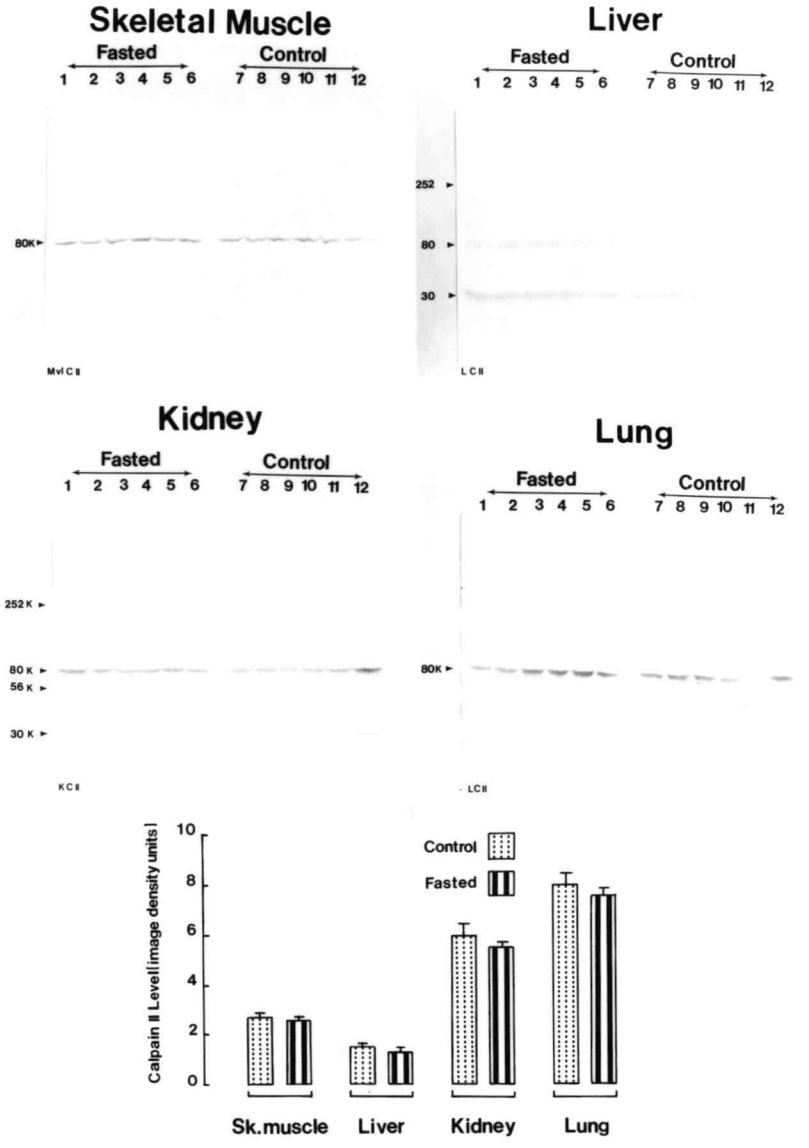

Figure IV.5. Western blot analysis on calpain II of various tissues of control and fasted rabbits. Tissue homogenates of various samples (100 μg/well) were subjected to SDS-PAGE and then transferred to a nitrocellulose filter which was stained with anti-rabbit calpain II monoclonal antibodies. Molecular weights of various bands are indicated by solid arrows (K = KDa).

Another major objective of the study was to investigate the co-regulational expression of calpain system genes. Thus we determined mRNA concentration for LI, LII, S and calpastatin in tissues of control and fasted rabbits.

The utility of using cDNA probes for calpain LI, calpain LII, calpastatin, cathepsin D and β-actin for specificity and quantitative Northern analysis was established earlier (Figures IV.6 and IV.7). Validation studies for calpain small subunit oligonucleotide probe (RCalS1) were performed in this study (Figure IV.6). A dose-response curve and cross-species validation analysis were performed. Results of the dose-response analysis using 12.5, 25 and 50 μg of total RNA showed linear response of image density to gradations in RNA concentration for the rabbit.

Cross-species validation analysis indicated that the 3' non-coding region selected for the design of RCal-S1 is specific to the calpain small subunit mRNA in rabbit as no signal was observed for rat RNA and a single hybridization band of about 1.5 Kb was detected (Figure IV.6). This result closely matched the predicted size of calpain small unit mRNA reported by Emori et al. (1986).

Expression of mRNAs for calpain LI in tissues of control and fasted rabbits is shown in Figure IV.8. Among tissues of control animals, the highest level of mRNA concentration was found in lung, followed by kidney and muscle, with the lowest found in liver. Effects of fasting on expression of calpain LI differed among tissues. Whereas fasting increased calpain LI mRNA level 2-fold ($P<0.05$) in skeletal

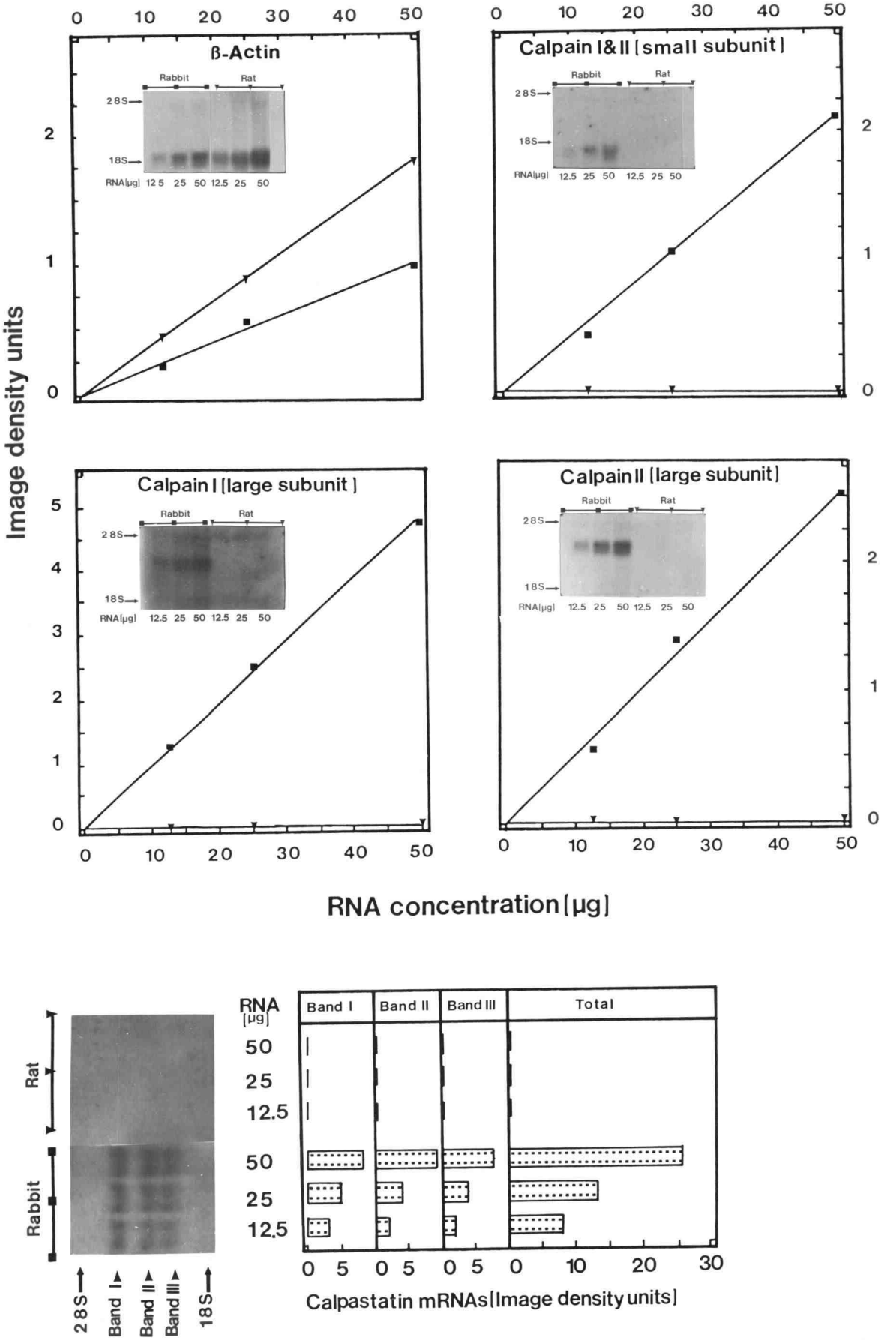

Figure IV.6. Dose response curves and validation results for cDNA probes of calpain I and II large subunits. Calpain small subunit, β-actin and calpastatin used in this study against RNA samples of rabbit and rat skeletal muscle.

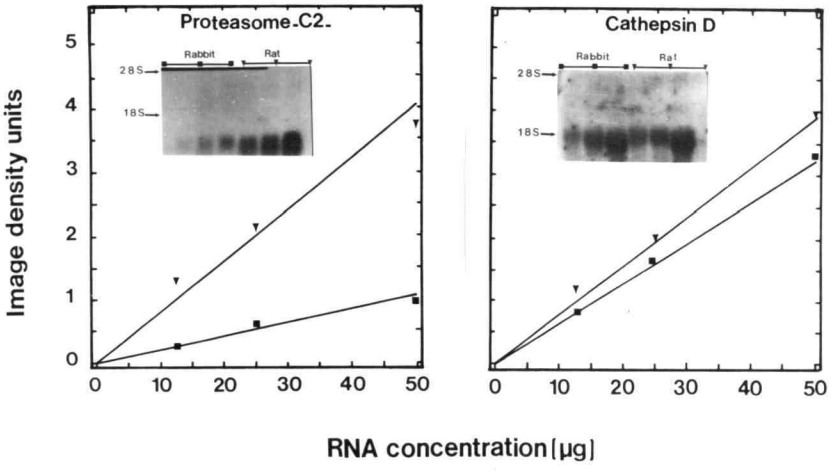

Figure IV.7. Dose response curve and validation results for cDNA of cathepsin D and proteasome C2 used against rabbit and rat RNA from skeletal muscle.

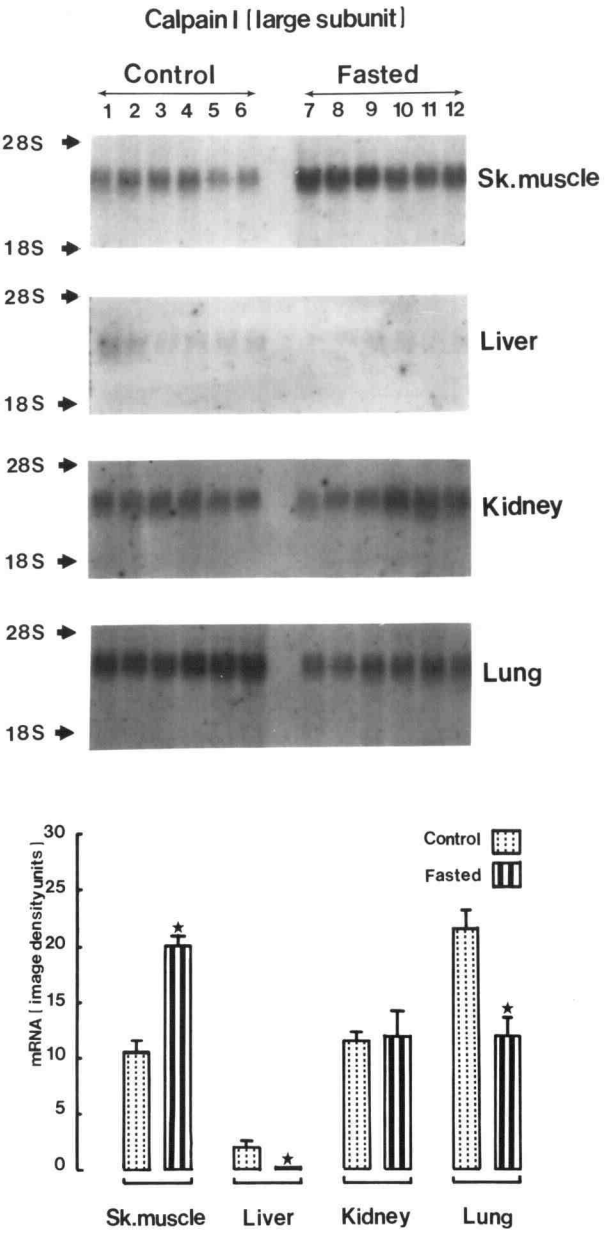

Figure IV.8. Expression of calpain I large subunit mRNA in skeletal muscle (*vastus lateralis*), liver, kidney and lung of control and fasted rabbits. Washing conditions were four times with 0.1 x SSC/0.1% SDS at 50° C. Scanning densitometry (mean ± SEM, n = 6) is presented in the second panel. Differences ($P < 0.05$) between control versus fasted animals are indicated by a "star" above scan bars.

muscle, it decreased expression of calpain I mRNA significantly in liver and lung and produced no change ($P>0.05$) in calpain I mRNA in kidney.

The expression of mRNAs of calpain LII in various tissues of control and fasted rabbits is shown in Figure IV.9. Essentially a similar distribution pattern for tissues of control animals and a similar response to fasting among tissues to that of calpain LI mRNA was observed.

Analysis of mRNA expression for the small subunit of calpain enzymes revealed very interesting results (Figure IV.10). The pattern of calpain S mRNA concentrations among tissues and in response to fasting was very similar to the expression profile of calpain LI and calpain LII mRNAs.

Calpastatin mRNA expression in skeletal muscle, liver, kidney and lung of control and fasted rabbits is shown in Figure IV.11. Results of Northern analysis of calpastatin in various tissues revealed three bands as I, II and III (Figure IV.11) of 3.8, 3.0 and 2.5 Kb, respectively, as has been previously reported (Emori et al., 1987). These bands arise from the same gene and differ only in processing of their 3' non-coding regions (Emori et al., 1987). The relative distribution and mode of expression of calpastatin mRNA multiple bands were different in different tissues and in response to fasting. Skeletal muscle expressed bands I and II constitutively in control and fasted rabbits, but with slightly higher levels for the fasted animals. Fasting, however, induced expression of Band III in skeletal muscle. Thus muscle total calpastatin mRNA (summation of Bands I-III) increased significantly ($P<0.05$)

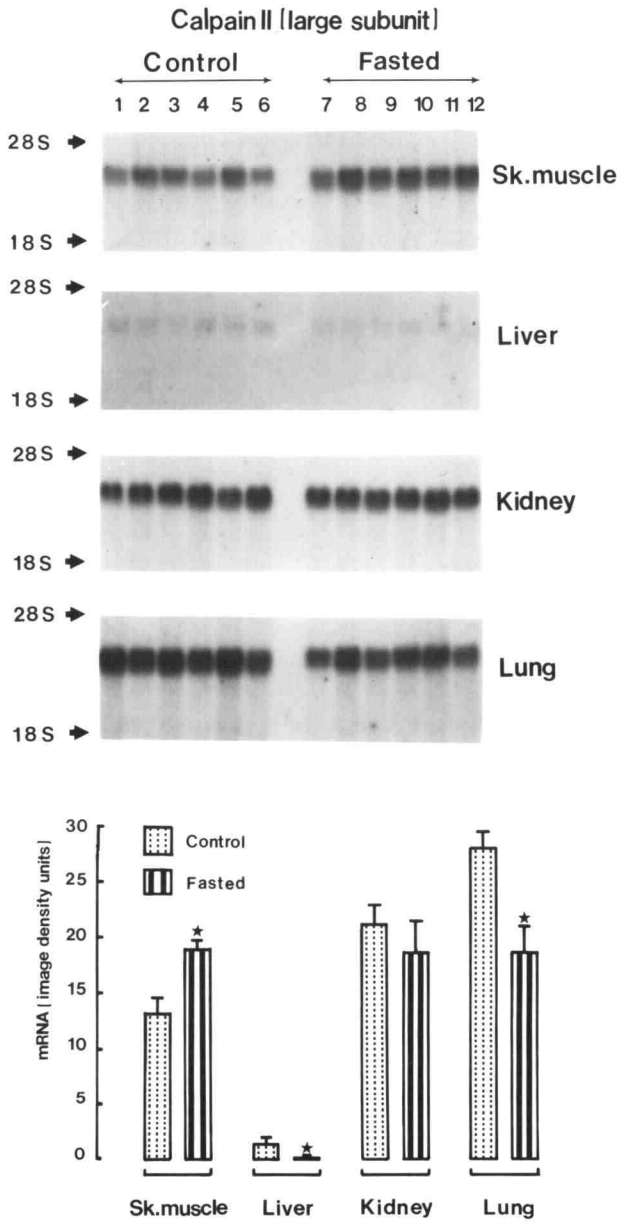

Figure IV.9. Expression of calpain II large subunit mRNA in skeletal muscle (*vastus lateralis*), liver, kidney and lung of control and fasted rabbits. Washing conditions were as for Figure 4. Scanning densitometry (mean ± SEM, n = 6) is presented in the second panel. Differences ($P < 0.05$) between control versus fasted animals are indicated by a "star" above scan bars.

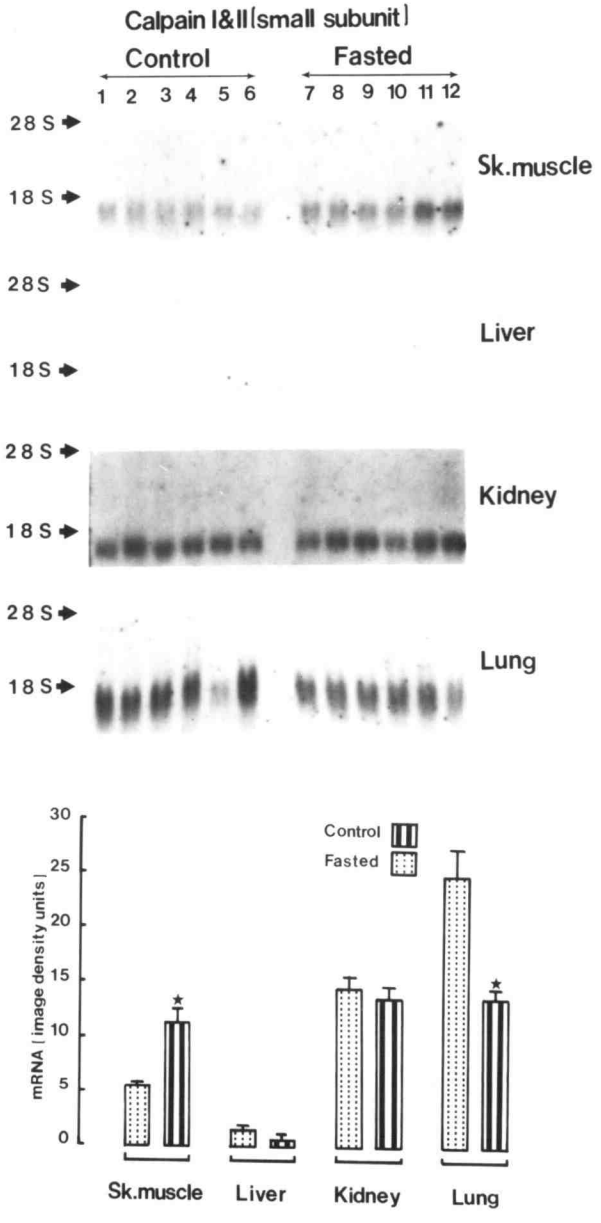

Figure IV.10. Expression of calpain small subunit mRNA in skeletal muscle (*vastus lateralis*), liver, kidney and lung of control and fasted rabbits. Washing conditions were for 20 min each in: 2 x SSC/0.1% SDS) 2 times at RT; 0.1 x SDS/0.1% SDS 2 times at RT and 0.1 x SSC/0.1% SDS at 60%. Scanning densitometry (mean ± SEM, n = 6) is presented in the second panel. Differences ($P < 0.05$) between control versus fasted animals are indicated by a "star" above scan bars.

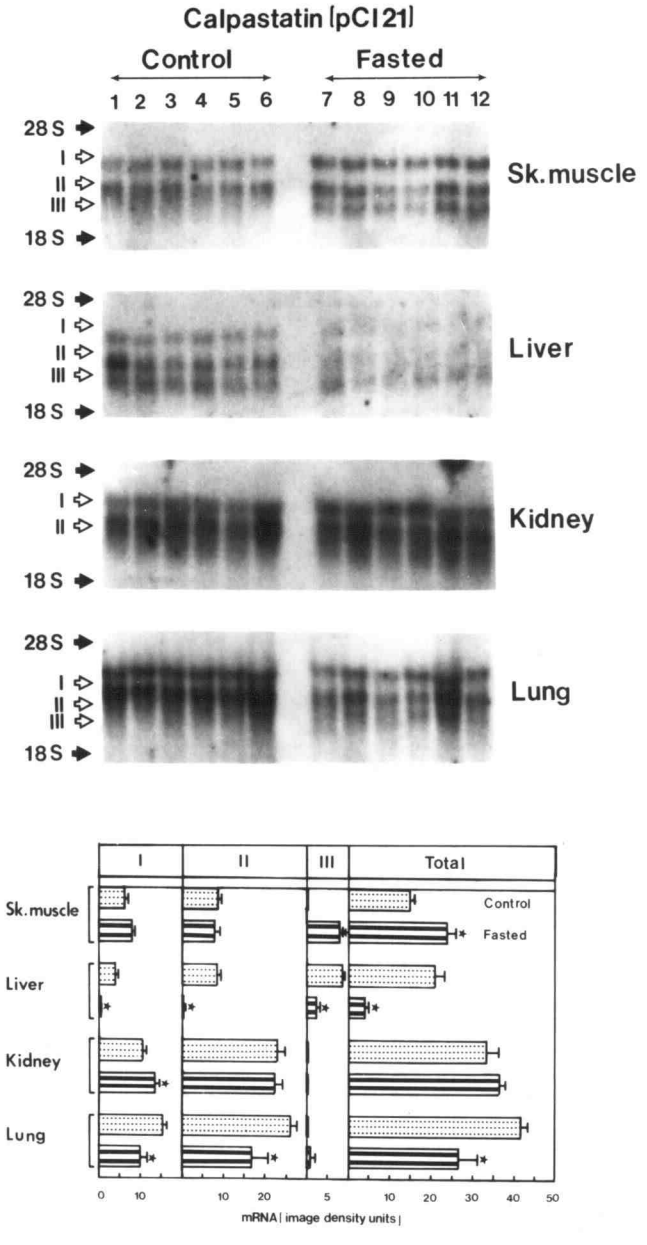

Figure IV.11. Expression of calpastatin mRNAs (probed by pCI 21 cDNA) in skeletal muscle (*vastus lateralis*), liver, kidney and lung of control and fasted rabbits. The window panels present Northern blots. Washing conditions for cell membranes were 1 x SSC/0.1% SDS four times at 25° C followed by 0.1 x SSC/0.1% SDS two times at 50° C. Positions of ribosomal RNAs (28 S and 18 S) are indicated by solid arrows. Position of calpastatin mRNA multiple bands are indicated by open arrows. The lower panel represents results of quantitation of mRNAs by scanning densitometry of individual mRNA species and their cumulative value in each tissue from either control or fasted. Each hbar is the mean ± SEM ($n=6$) and differences ($P<0.05$) between control and fasted animals are shown with a "star" located above densitometry scans.

in response to fasting. Liver, on the other hand, expressed the three forms of calpastatin constitutively in the control and fasted animals. Fasting reduced total calpastatin mRNA concentration in liver significantly ($P<0.05$) by decreasing the expression of the three forms. Kidney experienced no change in level and mode of expression of calpastatin mRNAs due to fasting and expressed only bands I and II in tissues of control and fasted animals. Lung behaved uniquely with regard to the mode and level of expression of various calpastatin mRNA forms in response to fasting. In lung, similar to skeletal muscle, fasting induced the expression of calpastatin band III mRNA in some of the experimental animals (Figure IV.11, lane 8, 10, 11, 12) in addition to the constitutively expressed bands I and II. However, total calpastatin mRNA levels, in lung, similar to liver, decreased in response to fasting. Because the pattern of expression of calpastatin mRNAs in tissues of control and fasted rabbits is very distinctive, we confirmed it by re-probing the membrane with probe pCI 413 (Figure IV.12), which covers the first half of the coding region in calpastatin mRNA. Exactly the same pattern was observed with pCI21. Thus all bands observed belong to calpastatin mRNAs.

To evaluate the co-regulation of the calpain system genes, the correlation matrix for the effect of fasting among various tissues on mRNA levels of calpain I and II large subunits, the small subunit and calpastatin was calculated (Table IV.4). The results indicated strong correlation coefficients among all components of the calpain system. The strongest correlation is between the small subunit and the large subunit ($r^2=0.997$). The large subunits LI and LII were also highly correlated with regard to the effect of fasting on their mRNA expression. The response of

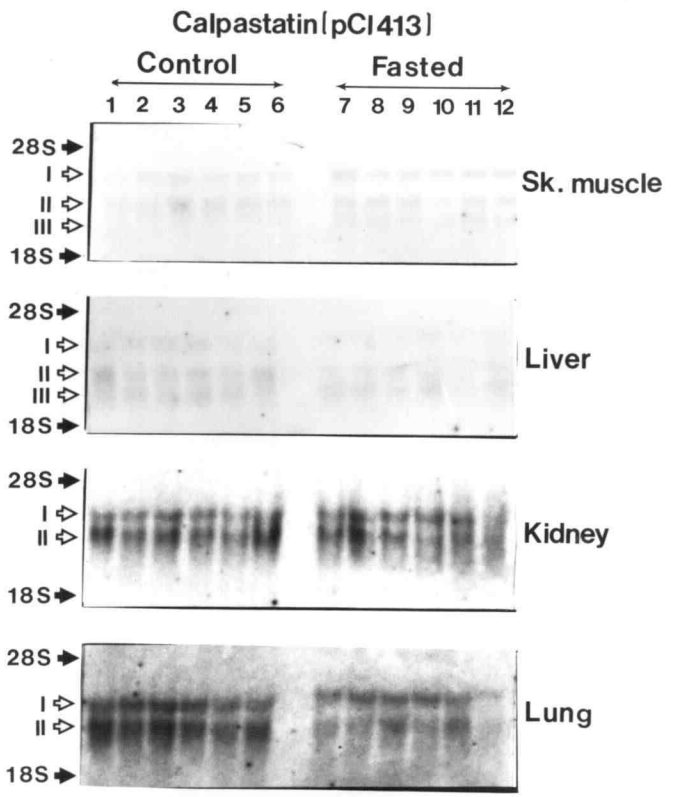

Figure IV.12. Expression of calpastatin mRNAs (probed by pCI 413 cDNA) in skeletal muscle (*vastus lateralis*), liver, kidney and lung of control and fasted rabbits. The windows present Northern blots. Washing conditions were as for Figure 7. Positions of ribosomal RNAs (28 S and 18 S) are indicated by solid arrows. Position of calpastatin mRNA multiple bands are indicated by open arrows.

calpastatin mRNA expression to fasting was also directly correlated to the response of LI, LII and S mRNAs ($r^2=0.77$). These results suggest that the genes of calpain Li, LII and S share common regulatory elements.

Table IV.4. Correlation matrix for the effect of fasting among various tissues on mRNA expression of calpain I and calpain II large subunits (LI, LII), calpain small subunit and calpastatin.

Parameter	Li	LII	S	Calpastatin Total
LI	1.00	0.980	0.997	0781
LII	0.986	1.000	0.997	0.769
S	0.997	0.996	1.000	0.787
Calpastatin	0781	0.769	0.787	1.000

To determine whether the fasting-dependent increase in calpain mRNAs was unique, we examined expression of mRNAs encoding cathepsin D, and proteasome C2 was determined in skeletal muscle, liver, kidney and lung of control and fasted rabbits.

Effects of fasting on cathepsin D mRNA levels in various tissues of control and fasted rabbits are shown in Figure IV.13. Fasting increased skeletal muscle cathepsin D mRNA 6-fold. The highest tissue in cathepsin D mRNA detected in normal animals was kidney, followed by lung, liver and skeletal muscle, respectively (Figure IV.13).

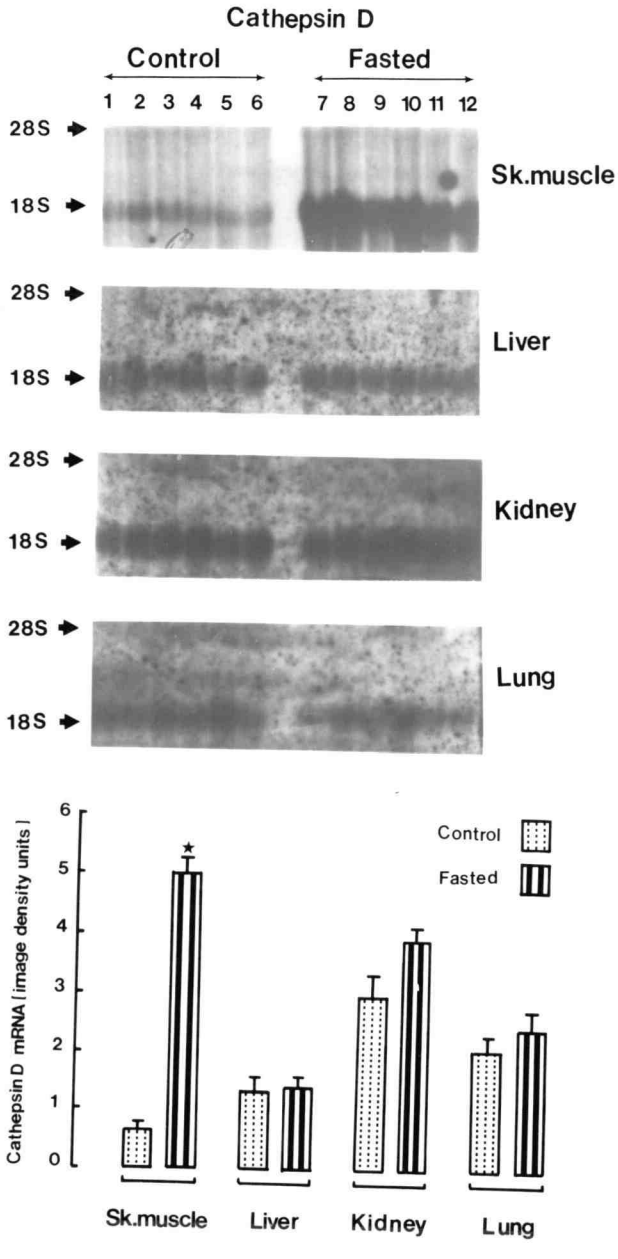

Figure IV.13. Expression of cathepsin D mRNA in skeletal muscle (*vastus lateralis*), liver, kidney and lung of control and fasted rabbits. The windows present Northern blots. Washing conditions were four times with 1 x SSC/0.1% SDS at RT. Positions of ribosomal RNAs (28 S and 18 S) are indicated by solid arrows. The lower panel represents scanning densitometry (mean ± SEM, n = 6). Differences ($P < 0.05$) between control versus fasted animals are indicated by a "star" above scan bars.

Proteasome C2 mRNA expression in various tissues of control and fasted rabbits was also examined (Figure IV.14). Expression of proteasome C2 subunit mRNA increased 2-fold in skeletal muscle due to fasting. Other tissues responded to fasting either by decreasing mRNA ($P<0.05$) of proteasome C2 (lung) or with no change ($P>0.05$) (liver and kidney). Thus, fasting caused a unique response in skeletal muscle as compared to other tissues with regard to expression of the calpain system, cathepsin D and proteasome mRNAs. The response of skeletal muscle to fasting by up-regulating expression of mRNAs of various proteases is novel, as levels of β-actin mRNA decreased about 30% ($P<0.05$) in all tissues studied (Figure IV.15).

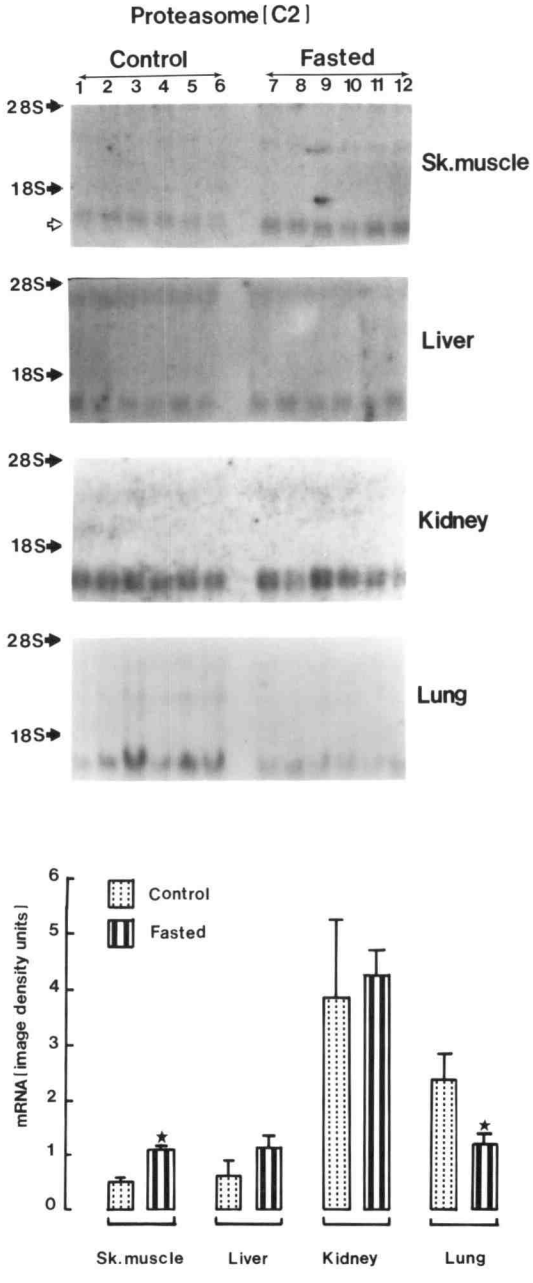

Figure IV.14. Expression of proteasome C2 mRNA in skeletal muscle (*vastus lateralis*), liver, kidney and lung of control and fasted rabbits. The windows present Northern blots. Washing conditions were as in Figure 9. Position of ribosomal RNAs (28 S and 18 S) are indicated by solid arrows. The lower panel represents scanning densitometry (mean ± SEM, n = 6). Differences ($P < 0.05$) between control versus fasted animals are indicated by a "star" above scan bars.

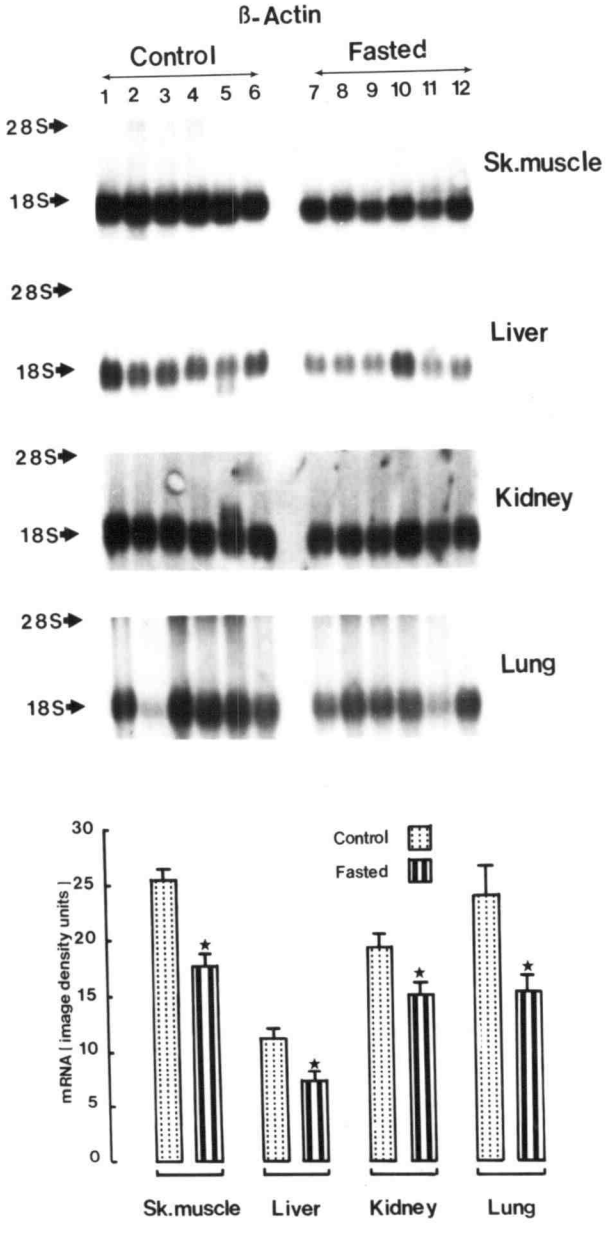

Figure IV.15. Expression of β-actin mRNA in skeletal muscle (*vastus lateralis*), liver, kidney and lung of control and fasted rabbits. The windows present Northern blots. Washing conditions were 0.5 x SSC/0.1% SDS at 60° C four times. Position of ribosomal RNAs (28 S and 18 S) are indicated by solid arrows. The lower panel represents scanning densitometry (mean ± SEM, n = 6). Differences ($P < 0.05$) between control versus fasted animals are indicated by a "star" above scan bars.

DISCUSSION

Characterization of Animal Model

Several studies reported on the effect of fasting on rat body size, organ weights and RNA (Goodman and Ruderman, 1980; Li and Wassner, 1984; Kadowaki et al., 1989). Response of animals to fasting depends on various parameters such as age, body condition and duration of fast. Taking these parameters into consideration, the observations in this study are in accord with published results (Goodman and Ruderman, 1980).

Expression of calpain system

Considerable interest centers on the biological roles and activity regulation of the calpain system. In order to deduce the function and activity regulation of the calpain system, determination of the expression of the calpain system in tissues and cells is of primary importance. For this purpose, we analyzed expression of the calpain system by determining enzymic activity, protein concentration and steady-state mRNA levels of the calpain system proteins in tissues of fed (control) and fasted rabbits.

With regard to enzyme activity measurements, many papers have reported the distribution of calpains in tissues. In 1981, Murachi et al. and Kishimoto et al. determined calpain activity in rat tissues. Their results indicate that calpain I and

calpain II as well as calpastatin are very widely distributed, and that their absolute as well as relative abundance vary from one tissue to the other. Our finding in this study furnishes basically the same conclusion with regard to the distribution of calpain activity in various tissues of the rabbit. One additional feature of this study, however, is that we measured calpain activity distribution in normal and stressed animals by subjecting them to an 8 d fast. The fact that we observed no changes in calpain I, calpain II and calpastatin activities in response to fasting underscores the unique regulatory system involved in maintaining activity level under diverse conditions. Since fasting is characterized by dramatic reduction in protein synthesis rate (Harmon et al., 1984), then calpain system proteins must be protected from the effect of fasting.

Results of calpain I and calpain II concentrations in various tissues of control and fasted rabbits closely corresponded to the results of activity measurements. This observation may indicate that the majority of the calpain proteins in vivo are functional proteins and that aged or denatured calpain proteins are effectively disposed of. Another very important result emerges from the Western analysis in that only a single band is detected, with molecular weight of 80 kDa, for both calpain I and calpain II in all tissues studied regardless of treatment. This means that autolyzed calpain I and calpain II do not constitute a significant population in vivo.

Analysis of mRNA levels of calpain system proteins revealed many unique features for the calpain system. First, results of steady state mRNA levels of

calpain LI, LII and calpastatin in tissues of control animals were found to correlate very closely with their activity measurements in various tissues. Secondly, the expression of mRNAs of calpain system proteins LI, LII, S and calpastatin among tissues and in response to fasting was very closely co-regulated (Table IV.4). Co-regulation of calpain large subunits and small subunit has been suggested before (Emori et al., 1987). Our study, however, is the first to investigate the expression of mRNAs of LI, LII and S in the same tissues of two experimental treatments to show that the pattern of response is also highly co-regulated. Another important feature in this study is the observation that calpastatin mRNA expression is also highly co-regulated with mRNA of calpain proteins. Thus, we propose to designate the functional unit in the calpain system as the calpain/calpastatin complex. Thirdly, the calpain system possesses constitutive and tissue specific regulatory elements. This unique observation is clearly shown by skeletal muscle where the co-regulated up-regulation in expression of mRNAs of the calpain system due to fasting was seen.

Mechanism of calpain activation: the "Triarchy Model"

Existing knowledge implicates calpains in numerous cellular functions through limited proteolysis of a wide variety of substrate proteins (Murachi, 1989). The ramifications of this position for calpain in the cell are twofold; calpain activity is constructive if exerted discriminatingly and destructive if utilized randomly. We propose here that calpain was created to exert two major types of functions in the cell: primary or life functions, and secondary or death functions. The distinction lies

with whether the intracellular Ca^{2+} concentration is being kept within the range of physiological limits or whether it has become abnormally high. We further propose that both functions are utilized by the cell but through different activation mechanisms.

For calpain to perform discriminate catalysis, it must exist in the living cell in an inactive state and become active only when its activity is required. Active calpain must become inactive again immediately once its function is performed. For the secondary function to be performed in the cell (i.e. during death and disease), calpain activation is irreversible as the enzyme is terminally activated.

At the present time, the most widely accepted model for the regulation of calpain activity is the membrane/activation theory proposed by Suzuki et al. (1987). This theory suggests that the calpains are activated intracellularly by attaching, through the N-terminal region of their small subunit, to the plasma membrane by interaction with phospholipids. This interaction lowers the Ca^{2+} concentration required for autolysis. Autolysis breaks apart the N-terminal region of the 30 kDa subunit, thereby releasing the autolyzed calpains from the membrane. It is proposed that the autolyzed calpains are then active proteinases able to cleave their in vivo substrates.

Considerable efforts have gone into understanding the mechanics of the membrane activation theory within the boundaries of the living cell by trying to

extrapolate the observations made in the test tube, but with no success (Nagainis et al., 1988).

The characterization of the various limitations in the "membrane activation theory" is mostly credited to the diligent work of the Darrell Goll lab (Nagainis et al., 1988; Kapprell and Goll, 1989; Cong et al., 1989; Thompson et al., 1990). Limitations ofthis model include:

(1) The membrane-activation theory implies that the unautolyzed calpains are, in an analogy to trypsinogen, proenzymes. Careful investigation of this postulate revealed that both calpain I and calpain II are active enzymes in their native state (Cong et al., 1989).

(2) Schematic presentation of the membrane activation theory suggests that autolysis of the 80 kDa and 30 kDa unblocks an active site to become available for the substrate. Results of inhibiting autolyzed and native enzymes with E-64 (Thompson et al., 1990) or iodoacetate (Suzuki, 1983) showed no effect on proteolytic activity of calpains if these irreversible inhibitions were removed before adding Ca^{2+} indicating that the active site is masked in both forms. However, both E-64 and iodoacetate rapidly inhibit calpains irreversibly if added in the presence of 1 mM Ca^{2+}. The conclusion is that autolysis of the calpains does not make an active site available. This makes the analogy between calpains and trypsin not valid. Rather such data suggest that a specific interaction with Ca^{2+} is required to

"unblock" the active sites of the calpains and that autolysis has some role other than activation.

(3) After the realization that calpains are active enzymes in their native state (Cong et al., 1989), and that autolysis if it occurs does not make the active site available (Thompson et al., 1990). Kapprell and Goll (1989) investigated the possibility that autolysis may be a means to release calpain from the Ca^{2+}-induced inhibition by calpastatin. The results were perplexing. Autolysis did not alter the Ca^{2+}- induced control of calpain activity by calpastatin.

Clearly, for calpains, especially calpain II, to be functional at the 300-1200 nM Ca^{2+} concentrations present in the cell, some other, yet undiscovered mechanism must be present.

Based on our results and those of others (Takeyama et al., 1986; Pontremoli et al., 1987, 1988; Nagainis et al., 1988; Kapprell and Goll 1989; Cong et al., 1989; Thompson et al., 1990), a new model is proposed to explain the activation of calpains in vivo. This model involves native calpains, calpastatin and a putitive endogenous activator thus called the "Triarchy Model" (Figure IV.16).

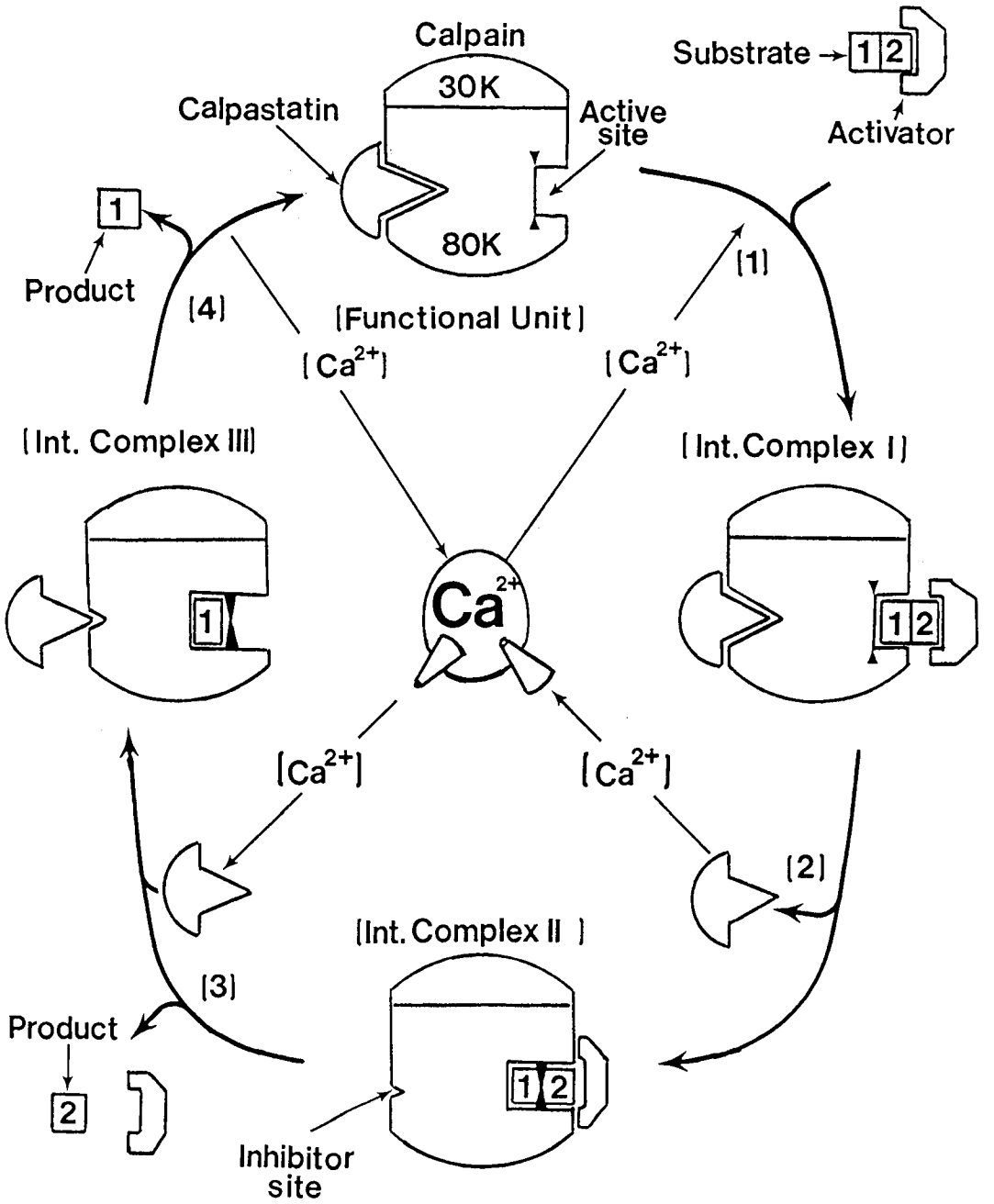

Figure IV.16. Proposed model of calpain activation in vivo, the "Triarchy Model". For details of steps 1-4, refer to text.

Activation of calpain by the "Triarchy Model" may best be described as an activation-deactivation cycle. The detailed steps are as follows:

A. Activation half cycle

1. The activation cycle starts by anchoring the native substrate protein to the enzyme active site domain by an endogenous activator. The step is modulated by Ca^{2+} interacting with the calmodulin domain on the enzyme. The functional unit in this step is the calpain/calpastatin complex. Thus, the activation cycle starts with an inactive enzyme.

2. Once the substrate is firmly anchored at the active site domain, the activation starts to insert the substrate into the active site by causing some conformational modification in the structure of the substrate that is transmitted as a signal to form a hallway to the cysteine active site, Hence, the processing of the substrate does not begin until the substrate is fully positioned at the active site. As the substrate starts to take its position into the active site, farther conformational changes start to develop around the binding site of calpastatin, which is on a different domain (III) from the active site domain (II) and calpastatin begins to depart from the functional unit. The departure of calpastatin from the binding site in the enzyme is modulated by the release of the Ca^{2+} involved in calpain/calpastatin interaction. We base this postulate on careful examination of the primary structure of calpain and calpastatin, both proteins have potential sites for Ca^{2+} binding as γGla sites similar to blood clotting factors. Thus Ca^{2+} is

involved in the interaction of calpain with its substrate and with its inhibitor in a calmodulin domain IV and γGla domain III, respectively. The complete release of calpain from the inhibition of calpastatin allows the cysteine active site to perform its proteolytic function at the cleavage site. At this point, the activation cycle is completed and the activator together with the outer fragment of the substrate are ready to disperse. This process signals the beginning to the deactivation half cycle.

B) Deactivation half-cycle

3. The hydrolysis of the peptide bond at the cleavage site in the substrate is an exergonic process. The liberated energy initiates restructuring of the conformational changes of the activation cycle by beginning to disperse the activator and the outer part of the substrate. The inner part of the substrate is locked in at the active site. As the activator begins to disperse, calpastatin starts to develop affinity to its binding site on the enzyme (domain III) as this domain starts to regenerate in response to a Ca^{2+} influx. Once calpastatin is anchored at its binding site on calpain, the activator and the outer part of the substrate (part 2) are dispersed completely.

4. This step is the end of the deactivation cycle. It starts by the destabilization of the Ca^{2+} bound to calmodulin domain with the concomitant build-up of calpain/calpastatin interaction. The propelling of calpastatin into its binding site is accompanied by the extruding of "part 1" as in Figure IV.16 of the substrate from

the active site. The complete anchoring of calpastatin onto the binding site causes the removal of part 1 substrate from the active site and the closure of the hallway and release of Ca^{2+} from calmodulin domain IV. Now the functional unit is available for a second turn of catalysis.

Basic features of the "Triarchy Model"

1. The functional unit is the calpain/calpastatin unit.

Experimental evidence:

a. Tissue distribution studies show that calpain is always accompanied by calpastatin, both are ubiquitously distributed in all tissues studied (Murachi, 1989).

b. Immunolocalization studies have found that calpain I, calpain II and calpastatin all are widely distributed throughout the cytoplasm in all cells examined (Kleese et al., 1987). Furthermore, stress caused by starvation or denervation does not alter the intracellular co-localization of calpains and calpastatin.

c. Calpastatin co-elutes with calpain in gel chromatography analysis unless all the Ca^{2+} is chelated by EDTA (Murachi, 1989).

d. In our study, the genes for calpains and calpastatin are very closely co-regulated.

2. An endogenous activator is responsible for the discriminate nature of the enzyme.

Experimental evidence:

a. A variety of endogenous calpain activators have been characterized. These include:

i. A protein activator from human neutrophils (Pontremoli et al., 1988).

ii. A protein activator associated with brain microsomal insoluble elements (Takeyama et al., 1986).

iii. An amino acid metabolite "isovaleryl carnitine" (Pontremoli et al., 1987).

These activators interact reversibly with calpain and release the enzyme from calpastatin inhibition.

b. The discriminate functioning of calpain necessitates the existence of a "recognition process" for its substrate. Here the activator is proposed to escort the

substrate to the enzyme. This mechanism is analogous to the utilization of ubiquitin by the ATP dependent protease for selective proteolysis of substrates (Rechsteiner, 1987) and the interaction of heat shock proteins with KFERQ containing proteins for the targeting to lysosomes.

3. Calpain has two binding sites (inhibitor binding site and active site)

Experimental evidence:

Kinetic studies indicated that calpastatin is a noncompetitive inhibitor of the calpains (Takahashi et al., 1981).

4. Calpastatin is a Ca^{2+} binding protein.

Careful structural examination of human, pig, rabbit and rat calpastatin cDNA sequence revealed that these proteins are very rich in the Glu-Glu sequences, the binding site for vitamin K-dependent carboxylase; thus we propose that calpastatin binds Ca^{2+}, similar to thrombin via γGla residues.

Table IV.5. Glu-Glu residues in calpastatin primary structure.

Type	# of Glu-Glu site	Reference
Human	9	Asada et al. (1989)
Pig	11	Takano et al. (1988)
Rabbit	9	Emori et al. (1987)
Rat	11	Ishida et al. (1991)

These Glu-Glu sequences are the unique sequences required by vitamin K-dependent carboxylase for the carboxylation of Glu to γGla for Ca^{2+} binding (Suttie, 1985).

5. Calpain has two types of Ca^{2+} binding domains.

Experimental evidence:

a. Calpain has one calmodulin binding domain on each subunit designated as domain IV (Suzuki et al., 1988).

b. Careful examination of primary structure of calpains revealed Glu-Glu sequences in large and small subunits as shown in Table IV.6.

Table IV.6. Glu-Glu residues in calpain primary structure.

Type	# of Glu Glu Sites	Reference (cDNA Sequence)
Chicken 80 kDa	(2) domain II, (4) domain III	Ohno et al., 1984
Human I 80 kDa	(1) domain I (1) domain III	Aoki et al., 1986
Human II 80 kDa	(2) domain II, (6) domain III	Imajoh et al., 1988
Human 30kDa	(1) domain IV, (I) domain V	Ohno et al., 1986
Rabbit 30 kDa	(1) domain IV (1) domain V	Emori et al., 1986
Pig 30 kDa	(1) domain IV (1) domain V	Sakihama et al., 1985

Thus both the large subunit and small subunit have potential sites for γGla residues. Furthermore, the number of Glu-Glu sequences is proportional to the Ca^{2+} requirement for half maximal activity that is human calpain II > chicken calpain > calpain I. The majority of Glu Glu sequences are present in domain III, which supports the idea that calpastatin interacts with a binding site different from the active site. Kapprell and Goll (1989) studied the effect of Ca^{2+} on binding of the calpains to calpastatin. They noticed the interaction of calpastatin with calpain is mediated by a set of Ca^{2+} binding sites different from those Ca^{2+} binding sites involved in proteolysis.

6. Calpain is a heterodimer composed of 80 kDa and 30 kDa in the native state.

Experimental evidence:

a. The general consensus among scientists is that calpains are heterodimers (Suzuki et al., 1987). The reason for some articles reporting the isolation of calpain as monomeric may be because of partial degradation of 30 kDa subunit which makes it not detectable on SDS-PAGE.

b. Autolyzed calpains are not detectable in the cellular extracts of muscle, liver, kidney, lung and erythrocytes as indicated from Western analysis.

c. Expression of mRNAs of large subunits and small subunit is highly correlated in both normal and stressful conditions (Chapter III)

7. Comparison between "Triarchy Model" and "Membrane Model".

Feature	Triarchy Model	Membrane Model
Nature of Activation	Reversible	Irreversible
Nature of catalysis	Discriminate	Nondiscriminate
Type of enzyme	Native	Autolyzed
Cellular loci	General to all cell compartments having activations	Membranes
Proposed physiological significance	Substrate processing	Protein degradation

8. Does the "Triarchy Model" discredit the "Membrane Model" physiologically?

Based on available literature, we believe that the common model for activation of calpain in vivo is the Triarchy model; however, under some pathological or special physiological conditions, the membrane activation model becomes functional in vivo (Croall, 1989). The distinction lies with whether the intracellular Ca^{2+} concentration is being kept within the range of physiological fluctuation or whether it has become abnormally high.

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CHAPTER V

EFFECTS OF GROWTH HORMONE ON CALPAIN AND CATHEPSIN D GENE EXRESSION IN PORCINE SKELETAL MUSCLE

by

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CONTRIBUTIONS

Mohammad A. Alyan participated in developing the experimental design, development and streamlining of analytical techniques utilized to perform this study, performing the animal trial, sampling tissues, analyzing the data and calculating the results, performing the statistical analysis and writing the manuscript. Dr. Neil E. Forsberg and Dr. Steven Davis participated in the experimental design, supervising progress of study and editing the manuscript.

EFFECT OF GROWTH HORMONE ON CALPAIN AND CATHEPSIN D EXPRESSION IN PORCINE SKELETAL MUSCLE

ABSTRACT

The effect of growth hormone (GH) on expression of calpains, calpastatin and cathepsin D in skeletal muscle, as major proteases involved in myofibrillar protein degradation, were investigated. GH reduced total RNA in skeletal muscle ($P<0.05$) but had no effect on cytoplasmic protein level. Furthermore, GH caused a 40% reduction in mRNA levels encoding cathepsin D in skeletal muscle. However, GH did not affect catehpsin D activity. Calpain I and calpain II and their specific inhibitor calpastatin activities as well as calpain I mRNA levels in skeletal muscle were unaffected by GH treatment. In conclusion, GH may be exerting its anabolic activity in part by down-regulating protease activities. Further work needs to be done to investigate this possibility.

EFFECTS OF GROWTH HORMONE ON CALPAIN AND CATHEPSIN D GENE EXPRESSION IN PORCINE SKELETAL MUSCLE

INTRODUCTION

Although the growth-promoting properties of GH on skeletal muscle are well documented (Kostyo and Regan, 1976), the detailed mechanisms by which GH coordinates the complex metabolic and cellular events associated with growth are not understood. Allen (1988) postulated that GH effect on muscle growth could be the result of action at several sites; Firstly on adipose tissue, where GH exerts an antilipogenic effect; Secondly, GH could also have part of its effect by stimulating differentiation of satellite cells; Thirdly, GH may reduce muscle protein degradation in muscle. Since proteolytic enzymes are responsible for degradation of proteins during metabolic turnover, we wanted to investigate the effect of GH administration on expression of intracellular proteases in skeletal muscle of pigs. I selected to study the calcium-dependent proteinase system (calpain I, calpain II and calpastatin) and cathepsin D, because both have been implicated in myofibrillar protein degradation (Goll et al., 1989; Schwartz and Bird, 1977; Okitani et al., 1981).

In the present study, we report on the effect of administering GH to pigs on expression of calpains and cathepsin D at the activity and mRNA levels.

MATERIALS AND METHODS

Animals

This is a collaborative study with Dr. Rosemary Wander of the Food and Nutrition Department. The details of animal care are given in Susan Hayes MS thesis (1991). In brief, sixteen meat-type barrows (breed type) were assigned according to weight to two groups, treatment and control. The average weights at start of study for control and GH treated pigs were 120 ± 9 and 119 ± 6 lb, respectively. Animals were allowed access to food and water ad libitum. A standard corn-soybean meal diet was fed to both groups (diet composition is included in Appendix Section).

The administration of GH was via daily subcutaneous injections at the base of the ear. Animals in the treatment group were injected with 3 mg of recombinant porcine GH (Pitman-Moore, Terre Haute, IN), dissolved in 50 mM bicarbonate buffer, pH 10.34. The GH dose administered was 0.73 IU/kg at start and 0.37 at end of the experiment. Control pigs were given a placebo injection of bicarbonate buffer. Pigs were slaughtered when they reached market weight (about 235 lb) at the Clark Meat Science Laboratory, OSU. Half of the animals from each treatment were slaughtered on day 50 and the remaining were slaughtered on day 57.

Muscle sampling

After slaughtering, a sample (20 g) of the thigh skeletal muscle was rapidly dissected out, immediately put between dry ice cubes to freeze, and stored at -80° C.

Biochemical procedures

Methodologies for RNA and protein determination for RNA extraction of total cellular RNA from muscle for Northern analysis for preparation of hybridization probes and for hybridizations to estimate cathepsin D, calpain I and II, and β-actin are given in previous chapters.

Methodologies for determining calpain activities and their specific inhibitor calpastatin are described in Chapter III. Measurement of cathepsin D activity was performed according to Barrett and Heath (1977) with some modification. The tissues (2 g) were homogenized in 10 ml each of 0.25 M sucrose containing 200 g l EDTA by using a Polytron for 3 x 15 sec at maximum setting. All the extraction steps of cathepsin D were performed at 4° C. After homogenization, Triton X -100 (0.2% w/v) was added and homogenates were stirred for 30 min. Determination of cathepsin D activity was performed using denatured hemoglobin (as substrate at pH 3.8. The reaction mixture, in a final volume of 3 ml, consisted of 0.5 g denatured hemoglobin, 0.5 ml of 1M acetate buffer, pH 3.5, and 2 ml of tissue homogenate. Each sample was run in triplicate. The samples were incubated in a water bath

(37° C) for 20 min. The reaction was stopped by adding 0.5 ml of 50% (w/v) trichloroacetic acid and samples were placed at 4° C overnight. Afterward, samples were centrifuged at 10,000 g for 30 min and supernates were aspirated into clean tubes. The amount of aromatic amino acids liberated into the supernate was measured spectrophotometrically at 280 nm wavelength. Cathepsin D activity was defined as one A_{280} is unit above blank. Protein determination was conducted by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Statistical Analysis

Differences ($P<0.05$) between the two experimental treatments were examined by using an unpaired Student's T test (Steel and Torrie, 1980).

RESULTS

Analysis of skeletal muscle for total RNA and protein content of control and GH treated pigs is shown in Table V.1. Animals treated with GH had lower total RNA levels in their skeletal muscles than the controls by 29.7%. The observation indicates that GH treatment produced a considerable reduction in the protein synthetic capacity in skeletal muscle of treated animals as compared to controls. The effect of GH on total protein content in skeletal muscle of control and GH treated pigs is also listed in Table V.1. Results indicate that GH treatment did not affect protein level in skeletal muscle.

The determination of cathepsin D, calpain I, calpain II and calpastatin in skeletal muscle of control and GH treated pigs are shown in Table V.2. In GH treated animals, the activity of cathepsin D in skeletal muscle was lowered by 16.0% compared with the control. Furthermore, in GH treated animals, the activities of both calpain I and calpain II were decreased by 14.8 and 12.6%, respectively. However, calpastatin activity in both GH treated and control animals was similar.

To study the effect of GH on the transcriptional regulation of the calpain system and cathepsin D in porcine skeletal muscle, tissues from GH treated and untreated (control) animals were subjected to Northern analysis to determine concentrations of mRNA encoding cathepsin D, calpain I and β-actin. Results of Northern analysis for calpain I, cathepsin D and β-actin are presented in Figures V.1., V.2

Table V.1. Total RNA and protein in skeletal muscle of control and GH-treated pigs.

Item	Experimental Treatment		Treatment Response Over Control (%)
	Control	GH	
Total RNA* (μg/g)	$1064^{a} \pm 105.5$**	$748.5^{b} \pm 135.4$	-29.7
Protein (mg/g)	$225^{a} \pm 20$	$238^{a} \pm 14$	+5.8

* RNA concentrations were determined spectrophotometrically at wavelength 260/280 nm following its purification as described in the Materials and Methods section.

** Values are means ± SEM of 6 observations. Means in the same raw with different superscripts are significantly different($P < 0.05$)

and V.3, respectively. As shown in Northern blots, cDNA probes used in this study for cathepsin D, calpain I and β-actin detected single bands with molecular size of approximately 2.2, 3.5 and 2.1, respectively. Thus cathepsin D, calpain I and β-actin mRNAs of pigs were highly homologous to their counterparts in human, rabbit and rat, respectively. The quantitation of cathepsin D, calpain I and β-actin mRNAs in skeletal muscle of GH treated pigs and controls is shown in Figure V.4.

Results indicted that GH decreased expression of cathepsin D mRNA by 40% ($P < 0.05$) but did not alter ($P > 0.05$) mRNA expression of calpain I or β-actin.

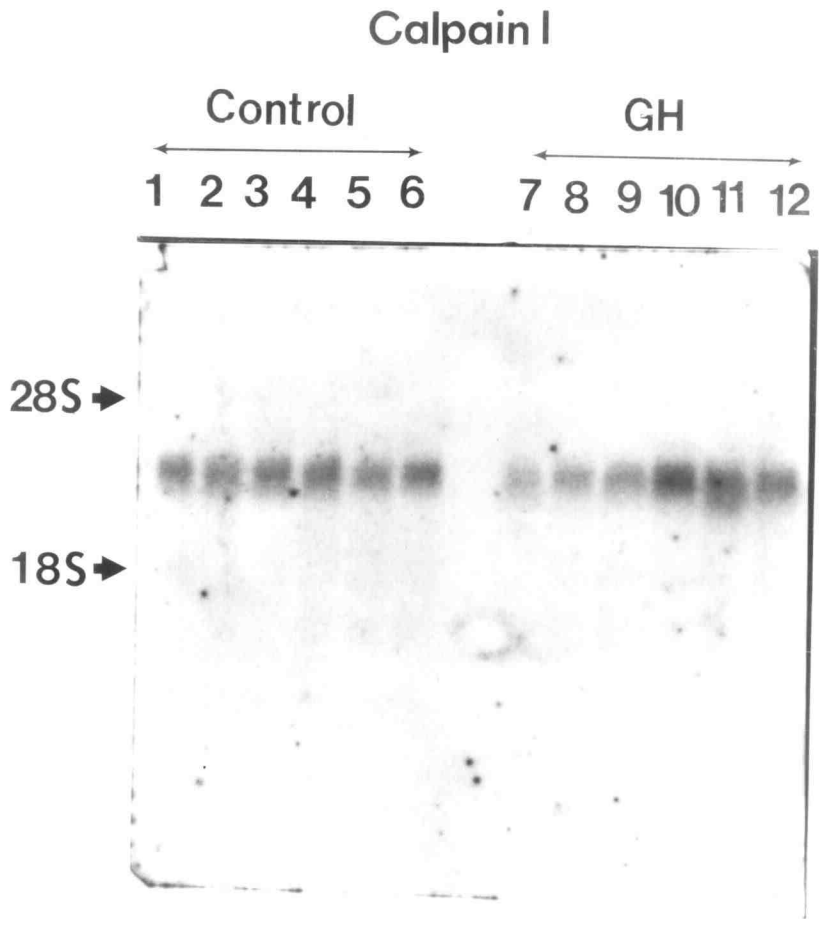

Figure V.1. Effect of GH on expression of calpain I mRNA in skeletal muscle. Washing conditions were 2 x SSC + 0.1% SDS two times at RT for 15 min followed by 1 x SSC + 0.1% SDS two times at 42° C for 15. Positions of ribosomal RNAs (28S and 18S) are indicated by solid arrows.

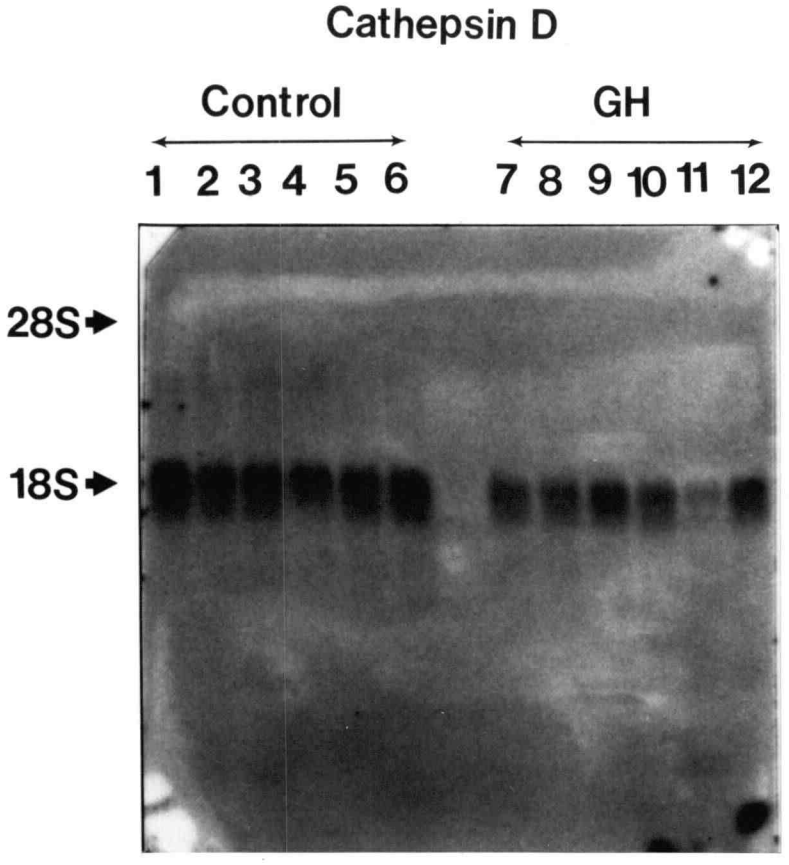

Figure V.2. Effect of GH on expression of cathepsin D mRNA in skeletal muscle. Washing conditions were 0.5 x SSC + 0.1% SDS two times at 45° C for 15 min each. Positions of ribosomal RNAs (28S and 18S) are indicated by solid arrows.

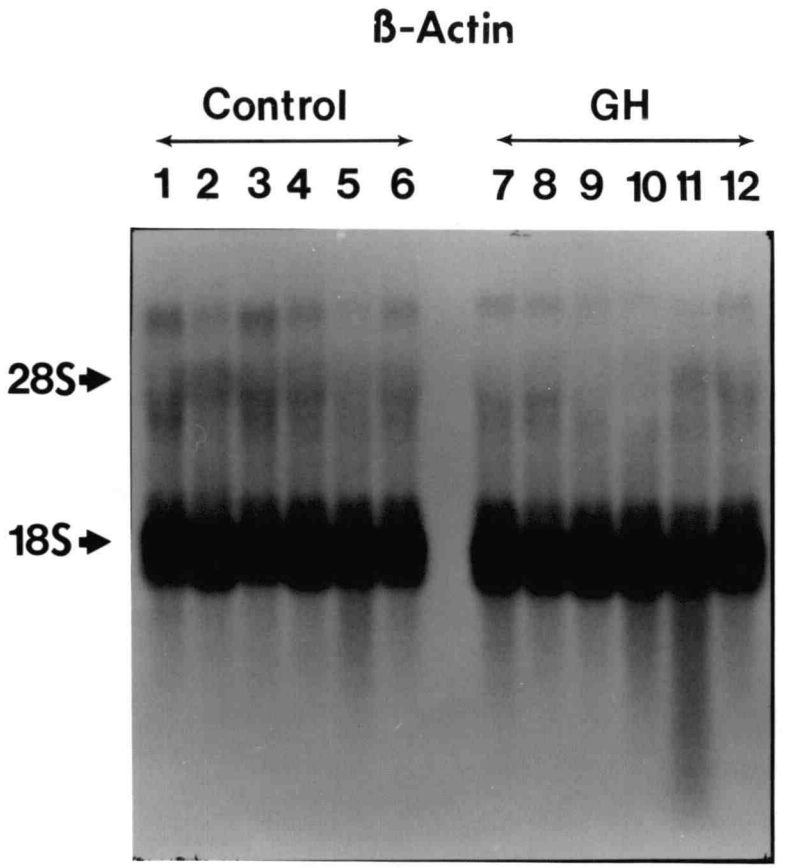

Figure V.3. Effect of GH on expression of β-actin mRNA in skeletal muscle. Washing conditions were 0.5 x SSC + 0.1% SDS 3 times at 60° C for 15 min. Position of ribosomal RNAs (28S and 18S) are indicated by solid arrows.

Table V.2. Effect of GH administration on activities of proteases in pig skeletal muscle.

Enzyme (μ/g protein)*	Experimental Treatment		% Change
	Control	GH	
Cathepsin D**	0.45 ± 0.02	0.38 ± 0.07	16.0
Calpain I***	1.10 ± 0.36	0.94 ± 0.31	-14.8
Calpain II***	1.34 ± 0.18	1.17 ± 0.25	-12.6
Calpastatin****	12.59 ± 3.27	12.72 ± 6.49	+1.0

* Values are means ± SEM of 6 observations. All comparisons were not statistically significant($P > 0.05$)

** Cathepsin D activity is defined as one unit will produce OD of 1.0 in 20 min at pH 3.5 at 37° C.

*** Calpain activity is defined as one unit will produce OD_{280} of 1.0 in 30 min at pH 7.5 and 25° C.

**** One unit of calpastatin activity will inhibit one unit of calpain activity.

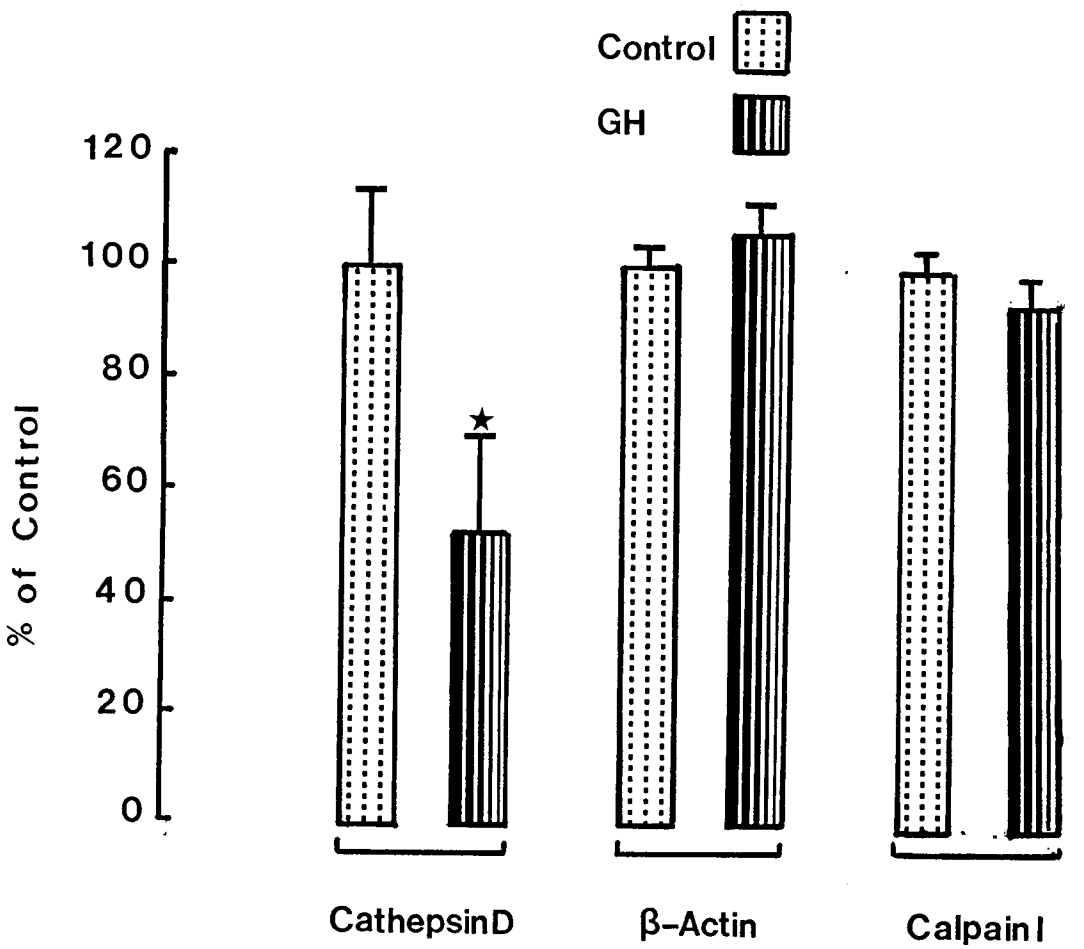

Figure V.4. Quantitation of mRNA levels of calpain I, cathepsin D and β-actin in skeletal muscle of control and GH treated pigs. Results are (average ± SEM, n=6). Differences ($P<0.05$) between control versus GH treated animals are indicated by a "star" above scan bars.

DISCUSSION

A number of reports of GH action on isolated muscles from hypophysectomized animals would appear to support the view that GH has direct effects on muscle in addition to the actions mediated by the insulin like growth factors (Florini, 1987). However, the effect of GH on cathepsin D and calpain system expression is not characterized. In this study, we observed a tendency for GH to reduce cathepsin D, calpain I and calpain II activities, though the differences were not significant. Furthermore, GH reduced levels of cathepsin D mRNA in treated skeletal muscle by 40% ($P<0.05$), but did not affect calpain I mRNA. The molecular basis for these observations and how GH alters cathepsin D mRNA is not clear at this point. Kostyo and Nutting (1973) reported that GH stimulated amino acid uptake by skeletal muscle. It is well documented that leucine inhibits protein degradation in skeletal muscle. Whether GH mediates its effect via leucine or directly through its membrane receptor is not understood. It is hoped that in the near future more studies will be conducted to investigate the effect of GH on protease activity in skeletal muscle.

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APPENDICES

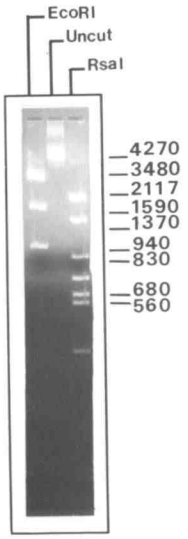
APPENDIX I

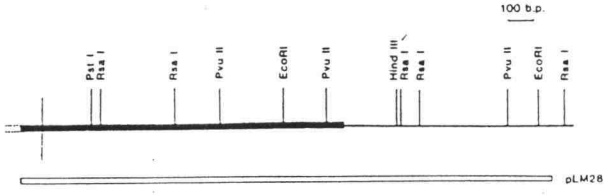
Composition of Rabbit Diet.

Ingredient	%
Dehydrated Alfalfa	54.00
Soybean Meal	20.00
Wheat Mill Run	21.00
Vegetable Oil	1.25
Molasses	3.00
Salt	0.50
Dicalcium Phosphate	0.25

APPENDIX II

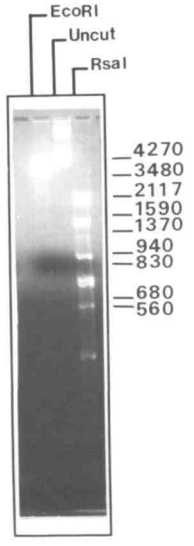
Restriction endonuclease digest of pLM28
(cDNA of calpain II cloned in pBR322)

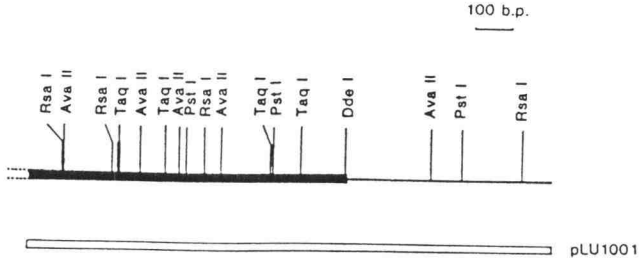




APPENDIX III

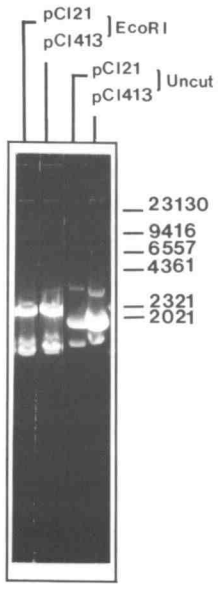
Restriction endonuclease digest of pLU1001
(cDNA of calpain I cloned in pBR322)

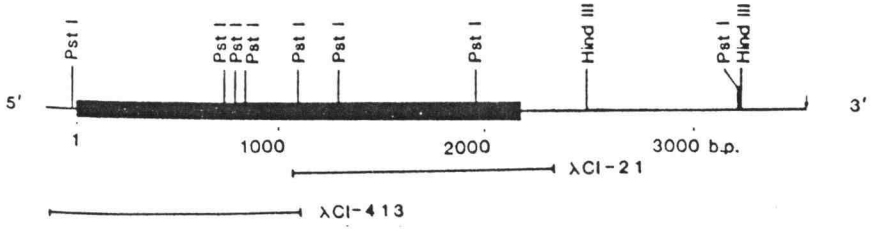




APPENDIX IV

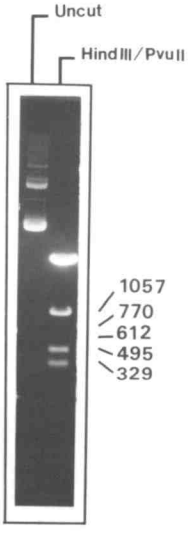
Restriction endonuclease digest of pCI413 & pCI21
(cDNAs of calpastatin cloned in pUC18)

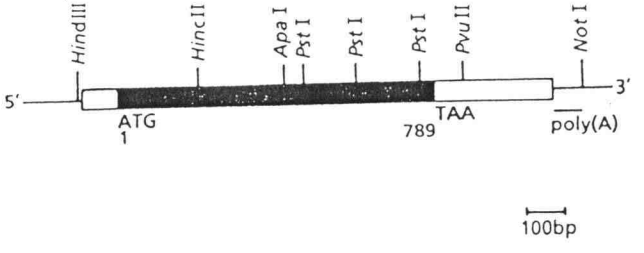




APPENDIX V

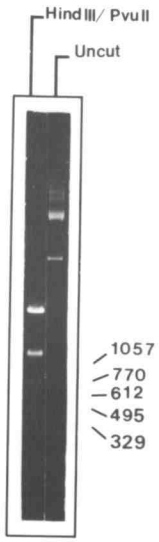
Restriction endonuclease digest of pC2 alpha (cDNA of C2 proteasome subunit cloned in blue script KS+)





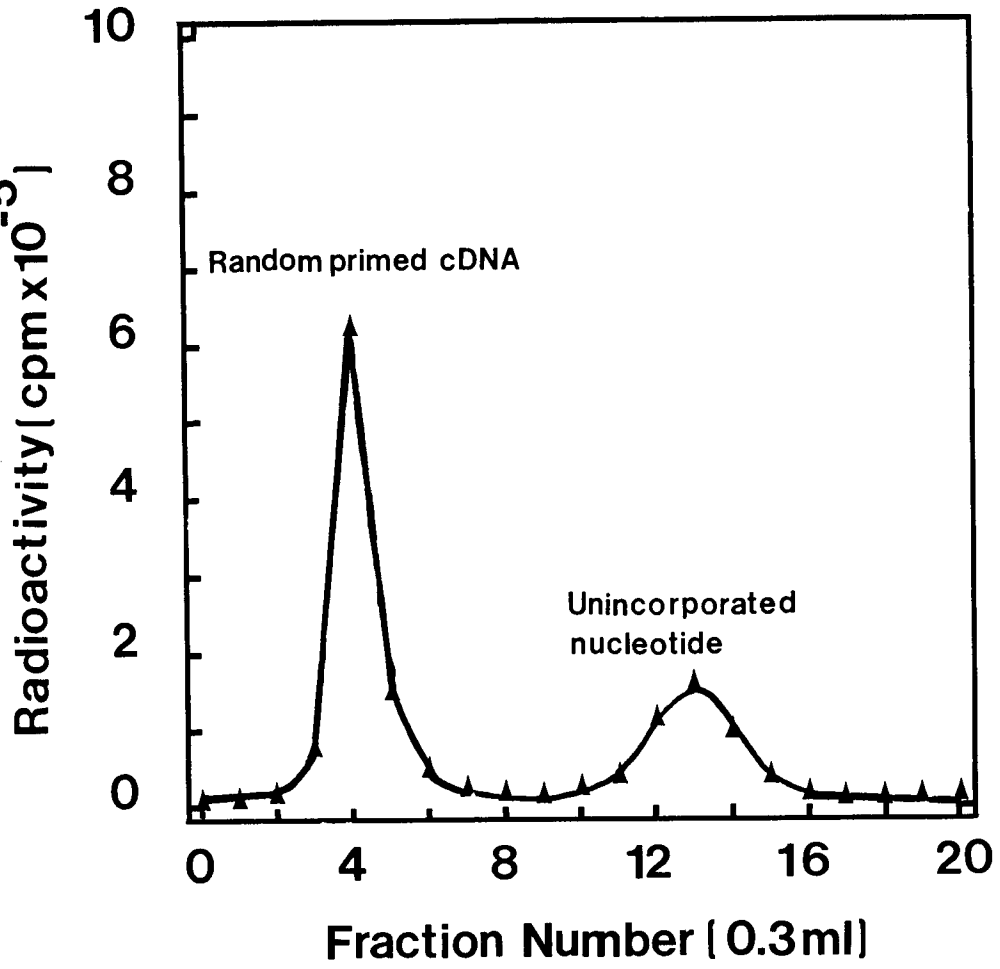
APPENDIX VI

Restriction endonuclease digest of pRB. A1
(cDNA of B-actin cloned in pBR322)



APPENDIX VII

Elution profile of random primed cDNA



APPENDIX VIII

A representative picture of electrophoresed RNA
used to conduct Northern analysis