AN ABSTRACT OF THE THESIS OF

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Title: Effects of Age, Castration and Gestation on Calpains and Calpastatin in Sheep and Rabbit Skeletal Muscle

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Objectives of this study were to examine effects of age, castration and gestation on actvities of calpains and calpastatin in skeletal muscle. Also, the regulation of calpains and calpastatin at the molecular level was investigated. Two experiments were designed. Experiment 1 was designed to evaluate effects of age and castration on calpains and calpastatin in sheep skeletal muscle. Experiment 2 was designed to examine the effects of age and gestation on calpains, calpastatin and their molecular regulation in rabbit skeletal muscle.

In Experiment 1, ten newborn male lambs, six weaned wethers (92 days), six weaned rams (92 days), six market weight wethers (89 days post-weaning) and six market weight rams (89 days post-weaning) were slaughtered and gluteobiceps femoris was taken for assay of calpain I, calpain II and calpastatin. Body weights were not different between wethers and rams in weaned animals; however, post-weaning, body weight gain of rams was greater ($p < .05$) than that of wethers. Ram gluteobiceps femoris weight at market weight was greater than that of wethers ($p < .05$). Activities of muscle calpain I, calpain

II and calpastatin in newborn lambs were higher than those of weaned and market weight lambs. The age-dependent reduction of calpain and calpastatin activities was complete at weaning and may be associated with or cause age-dependent attenuation of muscle protein degradation. Although muscle weights were greater in rams compared to wethers, no differences in activities of muscle calpains and calpastatin were detected between these two groups at weaning and at market weight. Hence, castration does not influence lamb muscle growth by changing muscle total calpain or calpastatin activities.

In Experiment 2, biceps femoris muscle was collected from newborn, weaned (1 month), market weight (2 months), non-gestational adults (5 months) and gestational (20 day) adult rabbits and for assay of calpains, calpastatin activities, muscle N^{τ}-methylhistidine (NMH) and mRNA encoding calpains and calpastatin. Muscle protein content (mg/g tissue) increased as animals aged indicating proliferation of myofibrillar protein. Total RNA content and ribosomal capacity (mg/g protein) declined ($p < .05$) as the rabbits aged; however, no differences in protein content and ribosomal capacity were detected in muscles taken from gestational versus non-gestational adult rabbits. Concentration of muscle NMH decreased as animal aged; however, no differences were detected in 20-day gestational muscle versus non-gestational muscle. Activities of calpains and calpastatin declined ($p < .05$) as animal aged. Most of the loss of activity occurred before 4 weeks of age (weaning). No differences in calpain and calpastatin activities were detected in gestational versus non-gestational animals. Reductions in activities of calpains and calpastatin were associated with reduced RNA concentration (ribosomal capacity) and reduced concentrations of muscle calpains and calpastatin mRNA (expressed as densitometry units per g tissue protein). The reduction of mRNA appeared to be

associated with an overall loss of RNA from skeletal muscle because mRNA/g protein was greatly reduced whereas mRNA/total RNA for both calpains and calpastatin did not change greatly; however, mRNA/total RNA in adult rabbits compared to newborn, weaned and market weight animal declined significantly. In addition, from the Northern blot assay, it was determinated that newborn and weaned rabbits only expressed an intermediate length calpastatin mRNA isoform (II). However, the longest calpastatin mRNA isoform (I) was expressed in market weight and adult rabbits. The reason why muscle cells express different forms of calpastatin mRNA at different ages remains unknown.

In pregnant rabbits, neither calpains nor calpastatin changed significantly. Also, muscle NMH concentration did not change. However, at the mRNA level, the steady-state concentration of mRNA encoding calpain II increased and calpastatin band II mRNA decreased in pregnant rabbits. This implies that in late catabolic stage of gestation, increased calpain II activity and decreased calpastatin activity may facilitate proteolysis.

Effects of Age, Castration and Gestation on Calpains and Calpastatin in Sheep and Rabbit Skeletal Muscle

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INTRODUCTION

Skeletal muscle makes up a larger proportion of body tissue than of any other and is the single largest protein pool in the body. Rates of protein deposition during growth are dependent on the differences between protein synthesis and protein degradation (Millward et al., 1975). Rates of protein synthesis and degradation of muscle protein are very high in young animals but become attenuated as animals approach maturity. Age-dependent attenuation of muscle protein degradation has been reported in several animals such as rats (Millward et al., 1976), pigs (Mulvaney et al.,1985), chickens (Klasing and Calvert, 1987), turkeys (Kang et al., 1985) and sheep (Oddy et al., 1986).

The mechanisms of muscle protein degradation are still poorly understood. It was traditionally believed that lysosomal proteases were resposible for intracellular protein degradation and that age-related changes in the activities of such enzymes may be expected to parallel developmental changes in protein degradation. It has been shown that the activities of some lysosomal proteases, cathepsin B and D, but not cathepsin H, consistently change in parallel with the rates of protein degradation in rat muscle (Goldspink and Lewis, 1985). In 1989, Saunderson and Leslie reported that in fast and slow growing chicks, cathepsin D and H in muscle decreased with increasing age, but

cathepsin B did not change (Saunderson and Leslie, 1989). These observations suggest that a small difference in muscle proteolytic mechanisms between birds and mammals may exist. However, recent studies suggest that lysosomal enzymes are not involved in the control of myofibrillar protein turnover (Lowell et al., 1986).

At present, a mechanism for explaining myofibrillar protein degradation begins with the action of extralysosomal proteases that selectively cleave myofibrillar proteins. Individual myofibrillar proteins or small fragments of these proteins can then be taken up by lysosomes and degraded to free amino acid by cathepsins (Allen, 1986). Calpains (calcium-dependent proteases), play a critical role in the initiation of myofibrillar protein degradation (Dayton et al., 1976a; van der Westuyzen et al., 1981; Suzuki et al., 1988). Because calpains are responsible for initiating myofibrillar protein degradation, their activities may be involved in the change of protein degradation associated with aging. In addition, several reports suggest that calpastatin (calpain-specific inhibitor) may be important to regulation of calpain activities in various tissues (Otsuka and Goll, 1987). Experiments were planned to examine whether age-dependent attenuation of protein degradation in sheep and rabbit was associated with changes in calpain and calpastatin activities. Also, effects of castration and gestation on calpains and calpastatin activities were examined.

LITERATURE REVIEW

MECHANISMS OF MYOFIBRILLAR PROTEIN DEGRADATION

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

The turnover of myofibrillar protein in skeletal muscle occuring during growth can be regulated by changing in the rates of both protein synthesis and degradation. Protein synthesis in muscle is almost certainly an important site of regulation, being particularly

sensitive to dietary and hormonal changes. However, it appears that a substantial degree of change of muscle protein levels can be achieved through the control of the degradation process. Overall protein degradation may be manipulated under conditions of nutritional or hormonal environment (Hershko and Ciechanover, 1982). Promoting an increase in growth rate, for example, by administration of trenbolone acetate or clenbuterol in rats, causes rates of muscle growth to increase by decreasing the rate of muscle protein degradation with little or no change in rate of muscle protein synthesis (Vernon and Buttery, 1978; Reeds et al., 1986; Forsberg et al., 1989).

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

So far, several proteolytic systems have been identified. These include (1) lysosomal proteases, which include cathepsins B, D, H, L, (2) nonlysosomal proteases, which include calpains (calcium-dependent proteases), ATP-dependent ubiquitin-dependent proteinase, ATP-dependent proteinase (protesome) and neutral serine protease.

Traditionally, it is believed that lysosomal proteases were responsible for muscle protein degradation (Schwartz and Bird, 1977; Matsukura, 1981). However, evidence

indicating that lysosomal proteases are not involved in the initial degradation of myofibrillar protein has accumulated. Libelius et al. (1979) and Gerard and Schneider (1979) have shown that products of myofibrillar degradation, such as myofilament fragments or myofibrillar protein fragments, could be detected in the lysosome, but intact myofibrils did not exist in the lysosome. This implied that lysosomal proteases can only degrade individual myofibrillar protein fragments and are not involved in the degradation of intact myofibrils. Furthermore, lysosomal proteases are contained in lysosomes and are active at acidic pH; in the other words, lysosomal proteases can not degrade myofibrils at sarcoplasmic pH. In addition, treatment of cells with lysosomal protease inhibitors failed to suppress calcium-induced protein degradation (Furuno and Goldberg, 1986).

Currently, a mechanism for explaining myofibrillar protein degradation suggests that calpains initiate metabolic turnover of the myofibrillar proteins by releasing thick and thin filaments from the surface of myofibrils. Then, individual myofibrillar proteins can be degraded to free amino acid by lysosomal proteases (Allen, 1986). Evidence supporting this belief includes (1) Ca^{++}-dependent proteolytic activity was isolated from myofibrils and was shown to degrade myofibrillar proteins at the pH and ionic strengths of the intracellular environment (Busch et al., 1972). The Ca^{++}-dependent degradation was initiated at the Z-disk, a site where myofibrillar disassembly is initiated. (2) Immunohistochemical studies show that the calpains are located inside muscle and some calpains are associated with the Z-disk (Dayton et al., 1981; Goll et al., 1983; Yoshimura, et al., 1986; Kleese et al., 1987). (3) The release of myofilaments was

Ca^{++}-dependent and was reduced by inhibitors of calpains (van der Westhuyzen et al., 1981; Zeman et al., 1985). (4) Treatment of muscle cells with high extracellular Ca^{++} or Ca^{++}-ionophore A23187 increased myofibrillar protein degradation (Publicover et al., 1978; Lewis et al., 1982; Zeman et al., 1985). (5) It has been shown using SDS-PAGE gels that the proteolytic changes caused by calpain treatment of myofibrils resemble the changes seen in myofibrils prepared from patients with Duchenne muscular dystrophy (Sugita et al., 1980; Obinata et al., 1981).

If the belief that myofibrillar protein degradation initiated by calpains is accurate, the process of initial degradation should be the rate-limiting step. Though the regulatory details of these proteases have not been eluciated, a specific inhibitor of these proteases, calpastatin, coexists with calpains in various tissues (Suzuki et al., 1988), and may play an important role in the regulation of calpain activities.

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

An ATP-dependent proteolytic system was initially found in reticulocytics (Etlinger and Goldberg, 1977). The ubiquitin-dependent ATP-dependent protease required ATP for covalent conjugation of the substrate to ubiquitin by ubiquitin-conjuate-degrading enzyme (UCDEN). UCDEN then could be degraded by the protease. It is believed that the role of this proteolytic system is in the degradation of abnormal proteins, proteins whose functions are lost during cell maturation and short-lived proteins (Dice, 1989; Fagan et al., 1987).

Several reports have suggested that an ATP-dependent ubiquitin-independent system may also exist in the mammalian liver, skeletal muscle and other tissues (Tsukahara et al., 1988; Driscoll and Goldberg, 1989; Fujiwara et al., 1989). This proteolytic system, called proteosome or multicatalytic proteinase complex, is consists of at least 13 nonidentical components (Fujiwara et al., 1989). When proteosome was isolated rapidly from rabbit skeletal muscle in presence of glycerol, its activity showed a large reversible activiation by physiological concentrations of ATP. Although proteosome has high activity in skeletal muscle (Driscoll and Goldberg, 1989), its precise role in vivo and its regulation are not understood.

CALPAINS AND CALPASTATIN

INTRODUCTION A generally proposed function of calpains in skeletal muscle is for the disassembly of the three-dimensional architecture of myofibrils initiated by removal of the Z-disk, then release individual myofibrillar proteins for further degradation.

Calpains (EC 3.4.22.17) also refered to as calcium-activated neutral protease (CANP) and calcium-dependent protease (CDP), were purified from porcine skeletal muscle in 1976 by Dayton and his coworkers. Recently, calpains in chicken, rabbit, pig, rat, sheep and human have been found (Ohno et al., 1984; Emori et al., 1986; Sakihama et al., 1985; Aoki et al., 1986; Ohno et al., 1986). Two homologous isozymes with different calcium sensitivities exist, calpain I and calpain II, active at micromolar and millimolar concentrations of Ca^{++}, respectively. The structure of calpain I and calpain II have been analyzed. These two isozymes consist of different large (80 KDa) subunits and identical small (30 KDa) subunit. Additionally, recently, two other calpains (p94 and high-calpain II) have been found in human, rat (Sorimachi et al., 1989) and chicken muscle (Wolfe et al., 1989). p94 consists of 820 amino acids and shows significant sequence homology with both human calpain I (54%) and calpain II (51%) large subunit. To date, p94 has been detected only in human and rat skeletal muscle by Northern blot analysis (Sorimachi et al., 1989) and was recently shown to have proteolitic activity (Suzuki; personal communication). The special function of this protease is still under investigation. High-calpain II has been found in chicken muscle (Wolfe et al., 1989). It requires 3.8 mM Ca^{++} for one half maximal activity. Because of the high Ca^{++} required for activation of

this proteinase, the physiological function of high calpain II remains unclear.

STRUCTURES AND PROPERTIES OF CALPAINS Both calpain I and calpain II consist of two subunits: 80 KDa subunit and 30 KDa subunit. The 80 KDa subunit is composed of four distinct domains. Domain I is called the propeptide domain which is similar to propeptides of various cysteine proteinases in various respects, i.e. length of region, lack of tryptophan residues, intron break point (Suzuki et al., 1988). However, the sequence of domain I is not homologous to those of propeptides (Suzuki et al., 1988). The function of this domain is not clear.

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

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

The C-terminal fourth domain (IV) is a calmodulin-like Ca^{++}-binding domain with four consecutive "EF hand" structures. Ca^{++} binds to the EF hand region. It is believed that the differences in Ca^{++}-sensitivity between calpain I and calpain II probably arise from differences in domain I and domain IV.

The 30 KDa subunit is composed of at least two domains. The N-terminal domain

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

The upstream region of human calpain II large subunit lacks typical promoter elements such as TATA and CAAT boxes and is characterized by its high GC content (-300 to -20) and contains multiple transcription initiation sites which cluster between -142 and -103 (Hata et al., 1989). The gene for the small subunit of calpain also shares similar structural characteristics in its 5'-flanking region (Miyake et al., 1986). Their structural features common to many gene of houskeeping proteins. These features imply that calpains are involved in essential cellular functions.

MECHANISMS OF REGULATION OF CALPAIN ACTIVITY Several mechanisms have been proposed for the modulation of calpain activity. First, obviously, Ca^{++} concentration is the most important factor for regulation of calpain activities. Native calpains are completely inactive and it is suggested that the native calpains should be called a proenzymes or zymogens (Suzuki et al, 1988). In the cytosol, the concentration of Ca^{++} is not sufficient for pro-calpain activation. When Ca^{++} concentrations increase to above 1-2 μM, pro-calpain's conformation changes and causes translocation of the pro-enzymes to the inner cell membrane. Autolysis of these proteases occurs at this site. Autolysis consists of cleavage of small fragments of the N-terminal of domain I of the

large subunit and the C-terminal of domain V of the small subunit, subsequently. Proteases are then released to the cytosol as soluble, physiologically-active proteases (Pontremoli and Melloni, 1986; Suzuki et al., 1988). Also, autolysis of calpain I may occur in cytosol without participation of the inner cell membrane (Suzuki et al., 1988).

Normally, pro-calpain II requires millimolar concentrations of Ca^{++} for autolysis. However, the interaction of domain V of the small subunit with membrane phosphotidylinositol reduces the Ca^{++} requirement of calpain II to μM levels (Suzuki et al., 1988). In addition, several laboratories have reported that Mn^{++}, Mg^{++}, or other metal ions reduce the Ca^{++} requirement of calpain II to μM concentrations of Ca^{++} (Pontremoli et al., 1985).

The calpain specific inhibitor, calpastatin, undoubtedly plays an important role in the regulation of calpain activity. It inhibits translocation of pro-calpains to plasma membrane and removes both active and inactive forms of calpains from cell inner membrane (Suzuki et al., 1988). It inhibits the autolytic activation of pro-calpains and it inhibits actual proteolysis of substrates catalyzed by membrane-bound and cytosolic active enzymes (Suzuki et al., 1988). Calpastatin co-exists with calpains in cytosol and specifically inhibits both calpain I and calpain II. In the primary structure of calpastatin, four highly-homologous repeating sequences (Glu-Lys-Leu-Gly-Glu-Xaa-Ile-Pro-Pro-Xaa-Tyr-Arg), referred to as the central conserved region, are assumed to be the active sites for the inhibition of calpain. One molecule of calpastatin contains at least four inhibitory sites (Imajoh et al., 1987; Suzuki et al., 1988). Ca^{++} is required for calpastatin to bind the calpains (Ishiura et al., 1982; Otsuka and Goll, 1987). Proteolytic activity of calpains

occurs at different Ca^{++} concentrations which is different from the Ca^{++} requirement for binding of calpain and calpastatin. This suggests that more than one set of Ca^{++}-binding sites exists in calpains. One set of Ca^{++}-binding sites is associated with calpastatin binding. A second set of Ca^{++}-binding sites may be involved in proteolytic activity (Kapprell and Goll, 1989). Although the role of calpastatin in vitro has been identified, its exact role in the modulation of protease activity in vivo remains unclear.

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

AGE-DEPENDENT ATTENUATION OF MUSCLE PROTEIN DEGRADATION

Domestic animal skeletal muscle is a major source of high quality protein in human diets. It makes up a larger proportion of the body tissue than any other tissue and is the single largest protein pool in the body. During animal growth, the quantitative balance between protein synthesis and protein degradation determines the rate of accumulation of protein in muscle. In normal growing animals, synthesis and degradation of protein tend to move in parallel. As maturity is approached, both synthesis and degradation rates decrease and ultimately reach a low and equal rate.

In order to improve yields of domestic protein products, most attention by animal scientists during the past several decades has focused on increasing the rate of protein synthesis. Much is known about mechanisms of muscle protein synthesis and how it is controlled. However, the mechanism of muscle protein degradation is still poorly understood.

In 1975, it was estimated that about 15-25% of the energy ingested by domestic animals was used for replacement of muscle protein that was degraded during metabolic turnover (Young et al., 1975). Decreasing rate of protein degradation would decrease the amount of energy needed for protein resynthesis and net rate of protein deposition would be accelerated. Therefore, the rate of protein degradation may be an important factor in animal growth.

In studies by Millward et al. (1976) and Goldspink (1985), attenuation of muscle protein degradation was age-dependent in well-fed rats. In 1985, Mulvaney et al. found

that muscle protein degradation was lower in pigs with larger body weights. In 1986, Oddy et al. found that age-related decrease in muscle protein degradation in sheep. Some investigators worked with chickens and turkeys and reported similar results (Maeda, 1984; Kang et al., 1985; Ballard, 1988; Muramatsu et al., 1988). It has been shown that the activities of some lysosomal proteases, cathepsin B and D, but not cathepsin H, consistently change in parallel with the rates of protein degradation in rat muscle (Goldspink and Lewis, 1985). In 1989, Saunderson and Leslie reported that in fast and slow growing chicks, cathepsin D and H in muscle decreased with increasing age, but cathepsin B did not change (Saunderson and Leslie, 1989). However, recent studies suggest that lysosomal enzymes are not involved in the control of myofibrillar protein degradation (Lowell et al 1986). Some investigators have shown that age-related changes in calpain and calpastatin activities. Varnum et al (1989) reported that rat lens calpain II activity declined and calpastatin remained unchanged during aging. Blomgren et al (1989) reported that the activities of rat thymus calpain and calpastatin declined with aged. Whether calpains and calpastatin are involved in the age-related changes muscle protein degradation are not understood. Understanding of the mechanism may ultimately permit scientists to effectively control this process and improve muscle growth. Therefore, it is important to learn what proteases are involved in degradation of muscle proteins and how the activities of these proteinases are controlled in vivo.

MUSCLE PROTEIN DEGRADATION IN CASTRATED AND GESTATIONAL ANIMALS

Generally, post-weaning body and muscle growth of intact male animals exceeds that of castrated animals (Mulvaney et al., 1988). Hence, androgenic agents are used as growth promoting compounds to enhance the efficiency of protein deposition in animals. It has been reported that adminstration of testosterone and androgens to castrated male pigs and other meat animals increases growth rate of nonadipose tissues. The mechanisms by which androgens increase growth are not fully understood, although there is strong evidence that they increase satellite cell contribution of DNA to muscle (Mulvaney et al., 1988). Lobley et al (1987) have also shown that testosterone infusions into wethers reduced muscle protein synthesis and concluded that a larger reduction in muscle protein degradation must occur. In 1978, Vernon and Buttery found that trenbolone acetate, a synthetic androgen, reduced myofibrillar protein degradation in rats. Also, adminstration of testosterone to rats had similar results (Hager and Kalkhoff, 1989). Presumbly, there are two ways anabolic agents can effect protein degradation, they may have direct effect on protein degradation, by reducing protease activities or indirectly, modifying endogenous hormones, which in turn affect muscle protein degradation.

If androgens affect muscle growth through regulation of protein degradation, specific mechanisms have not been investigated. Because calpains may be rate-limiting to this process in skeletal muscle, we investigated effects of castration on calpain activities in sheep muscle to gain insight into mechanisms underlying androgenic control of muscle

growth.

During pregnancy, skeletal muscle protein mass changes dramatically. An initial anabolic phase, in which protein is deposited within maternal tissue, mainly muscle, is followed by a catabolic phase during which the deposited protein is catabolized and the amino acid products are used for protein synthesis in the fetus and placenta (Mayel-Afshar et al., 1982; 1983a). Muscle may therefore be an important provider of amino acids for fetal and placental growth in late pregnancy (catabolic phase). For this proposal, proteolysis of protein must occur. Some proteases such as cathepsin D and calpain have been evaluated in rats muscle during pregnancy (Mayel-Afshar et al., 1983b). Enzyme measurements in their work were performed on crude homogenate and subcellular fractions of mixed thigh muscle. In normal pregnancy, there was no evidence that the changes in muscle protein mass which occurred were assisted by changes in the activities of cathepsin D and calpain (Mayel-Afshar et al., 1983b). However, due to the limitation of calpain assay in their work, improved methods for calpain and calpastatin assays should be used to clarify whether calpains and calpastatin are involved in protein degradation during pregnancy.

CONCLUSION

Intracellular protein degradation in muscle can have important effects on the rate of muscle growth in domestic animals. At present, nutritional and physiological experiments in vivo and biochemical experiments in vitro have provided an important descriptive base regarding general mechanisms by which proteins are degraded and regarding regulation at the enzyme activity level. However, future progress in our ability to control this process will depend upon our understanding of the molecular determinants of proteinase expression. Examples of needed information include mechanisms by which hormones and second messengers control proteinases and control of proteinase gene expression.

MATERIALS AND METHODS

Two experiments were conducted. In the first, muscle samples were taken from rams and wethers at various ages to investigate effects of age and castration on expression of calpain and calpastatin activities. To gain further insight into molecular aspects of calpain and calpastatin expression, a second study in which muscle samples were taken from rabbits at different ages and from pregnant versus non-pregnant rabbits was conducted.

EXPERIMENT 1. SHEEP: Thirty-four male Coopworth x Polypay lambs were used. Ten newborn male lambs were euthanatized at day 1 of age and the gluteobiceps femoris was taken from animals immediately then weighed and frozen at -90°C until analysis. Twelve male Coopworth x Polypay castrates (wethers) and twelve intact ram lambs were taken following weaning (92 days), weighed and six from both groups (wethers and rams) were slaughtered and gluteobiceps femoris samples were taken.

The remaining twelve lambs (6 wethers and 6 rams) were allowed ad libitum intake of a pelleted diet (Table 1) which met all requirements for animal growth (NRC 1985). Lambs were slowly brought onto this diet over two weeks by initially offering hay free-choice and gradually increasing amounts of pelleted diet. Animals were maintained on this diet until 89 days post-weaning, at which time they were weighed and slaughtered. Gluteobiceps femoriss were obtained and stored at -90°C immediately following slaughter in the meats facility.

EXPERIMENT 2. RABBIT: Female New Zealand White rabbits of various ages 1 day

(newborn; n=6), 1 month (weaned; n=6), 2 months (market weight; n=6), 5 months (adult; n=6) and 20-day pregnant adult rabbits (5 months; n=4) were ordered and euthanatized by injecting T61 euthanasia solution. The biceps femoris muscle was taken from the hindlimb, weighed and stored in -90°C until analysis. Rabbit diets are shown in Table 2.

ASSESSMENT OF TOTAL RNA. Total RNA was determined by the method of Munro and Fleck (1969). A muscle sample (.5 g) was homogenized in 3 ml distilled deionized water. Half of this homogenate (1.5ml) was placed in a 15 ml centrifuge tube with 1.5 ml ice-cold 20% TCA (trichloroacetic acid; W/V). The mixture was mixed immediately and stood on ice for 1 hour. After standing, the mixture was centrifuged at 5000xg for 15 min at 4°C. The pellet was resuspended in 2 ml ice-cold 10% TCA (W/V) and centrifuged as previously described. The supernatant was discarded. This washing procedure was repeated one time. The pellet was resuspended with 2 ml of 5% TCA (W/V) and heated at 90°C for 15 min. After centrifugation at 2000xg for 5 min, .5 ml of the supernatant was mixed with 3 ml of Orcinol reagent (Sigma; O-1875) then heated at 95°C for 30 min. The mixture was cooled to room temperature (RT) and its absorbance (695 nm) versus blank was determined by using a spectrophotometer (Shimadzu UV-160). Standard curves of RNA obtained by using Escherichia coli (E. Coli.) RNA (Sigma; R-1753).

ASSESSEMENT OF PROTEIN CONTENT. Muscle protein content was determined by

the method of Bradford (1976), using bovine serum albumin as a standard. Briefly, muscle samples were homogenized in 5 volumes of distilled deionized water, disolved in an equal volume of 0.1 N NaOH and incubated at 37°C for 30 min. The mixture was neutralized with 0.1 N HCl and Bradford's solution was added (Bio Rad; 500-0006; 1:4 water dilution). After incubation at RT for 10 min, absorbance was measured by spectrophotometry at a wavelength of 595 nm.

ASSESSEMENT OF MUSCLE NMH. Muscle NMH was measured as described by Forsberg and Liu (1989) with some modifications. Muscle (.5g) was homogenized in 3 ml 5% ice-cold TCA (W/V) solution. The homogenate was centrifuged at 5000xg for 30 min at 4°C. A portion of the supernatant (1.5 ml) was combined with 3 ml 10 N HCl and heated at 100°C for 2 hours to deacetylate NMH. The supernatant .5 ml was lyophilized in 10 x 75 mm disposable glass tubes in a virtus Uni-Top 600 SL freeze-dryer for 16 hours, then resuspended in .5 ml of 7:1:1:1 mixture of ethanol: phenylisothiocyanate (Pierce; 26922):triethylamine (Aldrich; 239623): distilled deionized water, and allowed to stand at RT for 20 min. The sample was lyophilized again and resuspended in .5 ml of a 5 mM phosphate buffer (PH 7.4) containing 5% (V/V) acetonitrile (J.T. Baker; 9011-03). A 2.5 mM NMH standard (Sigma; M-3879) was processed identically to the muscle sample.

The derivatized sample was placed in a limited volume insert for injection of 50 μl into a HPLC system which consisted of two Model 510 solvent delivery pumps, one Model 680 gradient controller, one WISP 710B auto-injector, one Model 730 integrating

recorder, one C18 PICO-TAG amino acid analysis column, and one Model TCM column heater. All equipment was manufactured by Waters Associates. Both Eluent 1 and Eluent 2 were obtained from Waters Associates, and delivered by pump A and pump B, respectively. Solvent delivery conditions and gradients used for separation of NMH from other muscle PITC derivatized components are shown in Table 3. Column temperature was maintained at 44°C.

ASSESSMENT OF CALPAIN ACTIVITY. (Appendix 1) Details of the partial purification of calpains prior to assay of their acivity was conducted as described by Gopalakrishna and Barsky (1985). Briefly, 2 g of sheep or rabbit muscle was homogenized in 10 ml of buffer containing 50 mM Tris-HCl (pH 7.5); 1mM EDTA; 10 mM 2-mercaptoethanol and 150 nM pepstatin A (Sigma; P-4265). The homogenate was centrifuged at 10000xg at 4°C for 30 min. To the supernatant (10 ml), 20μl of 1 mM leupeptin (Boehringer Mannhein Biochemicals; BMB; 1017128), 0.6 ml of 5M NaCl and 1 ml of phenyl sepharose CL-4B (Sigma; P-7892), which was pre-washed with Buffer A (20mM Tris-HCl, pH 7.5; 0.1 mM $CaCl_2$, 10 mM 2-mercaptaethanol, 20μM leupeptin) containing .25M NaCl was added. After shaking the mixture for 5 min, .4 ml of .1M $CaCl_2$ was added and the mixture was agitated for 10 min. Then, the gel suspension was poured into a column (0.8x4 cm; Bio-Rad; 731-1550). The packed column was washed successively with 2 ml of Buffer A with 0.25 M NaCl, Buffer A without NaCl, then Buffer A without leupeptin. Calpain II was eluted with 4 ml of buffer B (20 mM Tris-HCl, pH 7.5; 1 mM EGTA; 10 mM 2-mercaptoethanol) containing .1

M NaCl. After collection of calpain II, the column was washed with 2 ml of Buffer B with NaCl. Then, calpain I was eluted by 4 ml of Buffer B. All above procedures were carried out at 4°C.

Calpain activities were determined by using Hammarsten casein (EM Science; 2242) as substrate. The reaction mixture contained 2 mM $CaCl_2$, 3 ml tissue extract and 1 ml casein solution (8 mg/ml casein in 20 mM Tris-HCl, pH 7.5; 10 mM 2-mercaptoethanol). After incubation at 25°C for 30 min, 1 ml of 36% ice-cold TCA was added to stop the reaction. The mixture was centrifuged at 3500xg for 15 min at 4°C. TCA-soluble digested products were measured by the dye-binding method of Bradford (1976). One unit of calpain activity was defined as the amount of the enzyme which caused an increase of .1 absorbance unit at 595 nm after incubating 30 min at 25°C.

ASSESSMENT OF CALPASTATIN ACTIVITY. (Appendix 2) The calpastatin assay was adapted from Nakamura et al. (1988). The skeletal muscle homogenate was centrifuged at 10000xg for 20 min at 4°C. The supernatant was heated at 100°C for 10 min to inactivate calpains and other proteases and after heat treatment, the mixture was centrifugated at 10000xg for 10 min at 4°C and the supernatant was taken for assay of calpastatin. The supernatant was added to a fixed amount of calpain II which was partially purified from rabbit or sheep skeletal muscle and contained 2 mM $CaCl_2$. The mixture was incubated at 25°C for 5 min to allow calpastatin to react with calpain II. Then, digestion was started by adding the casein solution. The remaining calpain II activity was measured as described above. One unit of calpastatin was defined as the

amount of which inhibited one unit of calpain II.

EXTRACTION OF TOTAL RNA. (Appendix 3) The protocol of extraction of total RNA was described in Chomczynski and Sacchi (1987). Muscle .5g was homogenized at RT with 5 ml of solution A (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7.0; 0.5% sarcosyl; 10 mM 2-mercaptoethanol). To this .5 ml of 2 M sodium acetate, pH 4.0, 5 ml phenol (Appendix 4), and 1 ml of chloroform:isoamyl alcohol mixture (49:1) were added. The mixture was mixed well by inversion after each addition and centrifuged at 10000Xg for 20 min at 4°C. After centrifugation, the aqueous layer (upper layer) was transfered to a fresh tube and 5 ml of isopropanol (Sigma; I-4057) were added. The mixture was stored at -20°C for 1 hour. The mixture was then centrifuged at 10000xg for 20 min at 4°C, the supernatant was discarded and the RNA pellet was resuspended in .5 ml solution A. One volume of isopropanol was added and the mixture was stored at -20°C for 1 hour. The RNA pellet were precipitated at 12000xg for 10 min at 4°C, washed with 75% ethanol and dried under vacuum. The dried RNA pellet was dissolved in diethylpyrocarbonate-treated (DEPC)-water and quantitated by spectrophotometry at a wavelength of 260 nm.

PREPARATION OF PLASMIDS. E. Coli (HB101) were transformed with pLU1001 (cloned from rabbit DNA library; for calpain I; Emori et al 1986a; Appendix 5) , pLM28 (cloned from rabbit DNA library; for calpain II; Emori et al, 1986a; Appendix 6) and pCI413 (cloned from rabbit DNA library; for calpastatin; Emori et al, 1987; Appendix

7), respectively; and cultured in 25 ml Luria-Bertani (LB) medium (Maniatis et al., 1982). E. Coli transformed with pLU1001 or pLM28 were cultured with presence of tetracycline whereas pCI413-transformed E.Coli were ampicillin-resistant. Cultures were incubated at 37°C with shaking until the bacterial culture reached late log phase (OD_{600} = 0.6). The late log phase bacterial cultures were transfered into 500 ml of LB medium with appropriate antibiotics. After shaking at 37°C for 2.5 hours, chloramphenicol was added to make the final concentration reach 170 μg/ml. The bacterial mixture was incubated at 37°C for another 12 hours. After which cells were recovered by centrifugation at 4°C. For lysis of bacteria, the bacterial pellet was resuspended in 10 ml of freshly-prepared ice cold lysozyme containing solution (50.0 mM glucose, 25.0 mM Tris-HCl, 10.0 mM EDTA and 5mg/ml lysozyme (Sigma; L-6876)) and stood at RT for 5 min. After standing, 20 ml of freshly-made SDS solution (.2 N NaOH, 1 % SDS) was added and inverted gently. The mixture stood on ice for 10 min, after which 15 ml of ice cold 5 M potassium acetate (pH 4.8) was added. This was mixed by inverting sharply and stood on ice for another 10 min. Bacterial debris and cell DNA were removed by centrifugation. The supernatant was transfered to glass Corex tubes. Isopropanol (.6 vol) was added and this stood at RT for 15 min. Plasmid DNA was precipitated by centrifugation at 12000xg for 30 min at RT. The DNA pellet was rinsed with 70% ethanol then dried in a vacuum desicator.

The dried DNA pellet was re-dissolved in a small volume of Tris-EDTA (10 mM Tris-Cl and 1 mM EDTA; pH 8.0) buffer. RNase (.1 mg/ml; BMB; 1119-915) was added and incubated at 37°C for 30 min to remove RNA contamination. After

incubation, an equal volume of phenol and chloroform were added to re-extract lipid and protein contamination. Plasmid DNA was precipitated with ethanol and dried in a vacuum desicator. Dried plasmid DNA was further purified on a sepharose-4B column (Phamacia; 17-0120-01). Plasmid-containing fractions were pooled and precipitated with ethanol. Quantity of plasmid DNA was determined by the absorbance measurement at 260 nm (1.0 Abs_{260} = 50μg dsDNA).

RNA BLOT HYBRIDIZATION. Total RNA was extracted from skeletal muscle by the acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). The RNA sample was denatured at 55°C for 15 min and electrophoresed on a 1.1% agarose-formaldehyde gel at 30 V for 12 hr (Maniatis et al., 1982). RNA was transferred onto a nitrocellulose membrane (Schleicher & Schuell; 21640) and immobilized by baking for 2 hr at 80°C. cDNA fragments prepared from the 3' noncoding regions of pLU1001 (calpain I, RsaI/DdeI digest 494 of bp), pLM28 (calpain II, RsaI digest 639 of bp; Emori et al., 1986) and coding region pCI413 (calpstatin, EcoRI digest 1300 of bp; Emori et al., 1987). A random primer labeling kit (United States Biochemical; 70200) was used for ^{32}P-labeled cDNA fragment (Appendix 8). The baked membrane was prehybridized in Stark's buffer (.75 M NaCl, 75 mM sodium citrate, pH 7.0; 25 mM KPO_4, pH 7.0; 5X Denhardt's solution; 50% (v/v) of deionized formamide and .02% (w/v) of salmon sperm DNA; Maniatis et al., 1982) at 42°C for overnight, then hybridized in Stark's buffer containing 20% dextran sulfate and ^{32}P-labelled cDNA (500,000 cpm/ml) at 42°C for 48 hours. The membrane was washed with

0.1X SSC, 0.1%SDS at RT for 15 minutes two times and at 51°C for 3 times in the same buffer. This membrane was exposed to a Kodak X-film with two intensfying screens for 1-2 days. Quantitation of mRNA was done by scanning densitometry (Bio-Rad). A Hoefer data analysis system (GS350H) was used to integrate densitometric scans.

STATISTICAL ANALYSIS. Differences among different experimental groups were assessed using one-way ANOVA (Analysis of variance; Steel and Torrie, 1980). Individual treatment differences were examined with a Tukey multiple range test. In all cases, data were examined for equal variance and for a normal distribution prior to statistical analysis. A level of significance of 5% was adopted for all comparisons.

RESULTS

EXPERIMENT 1. EFFECTS OF AGE AND CASTRATION IN SHEEP

Slaughter weights of newborn lambs, weaned wethers, weaned rams, market weight wethers and market weight rams are shown in Table 4. No differences ($p > .05$) in rams versus wether body weights were detected at weaning; however, rams attained significantly greater body weight ($p < .05$) at 89 days post-weaning. No differences in gluteobiceps femoris weights were detected in rams versus wethers at weaning; however, at 89 days post-weaning, ram gluteobiceps femoris were larger ($p < .05$) than wethers gluteobiceps femoris. Calculation of proportion of gluteobiceps femoris versus body weight showed significant differences ($p < .05$) between wethers and rams at 89 days post-weaning (Table 4).

Concentrations of RNA in muscle taken from weaned and market weight lambs were less ($p < .05$) than newborn lambs; however, no differences were detected between rams and wethers (Table 5). Measurement of protein concentration yielded similar results. Protein concentration was increased ($p < .05$) in weaned and market weight animals compared to newborn lambs, but there were no differences ($p > .05$) between rams and wethers (Table 5). Ribosomal capacity (mg RNA/g protein) in newborn animals was higher ($p < .05$) than other animal groups; however, no differences were detected between rams and wethers at weaning or market weights (Table 5).

Effects of age and castration on calpain and calpastatin activities are shown in Table 6. Calpain I activity in neonates was higher compared to weaned and market weight lambs ($p < .05$), although there were no significant differences ($p > .05$) between weaned

and market weight animals or wethers and rams in either age group (Table 6). Similar results were observed for activities of calpastatin. Newborn animals have the highest activity of calpastatin but this was reduced as animals approached maturity. Castration did not affect calpastatin activity ($p > .05$; Table 6).

EXPERIMENT 2. EFFECTS OF AGE AND GESTATION IN RABBIT

Effects of age and gestation on body weight, biceps femoris muscle weight and muscle proportion in rabbits are shown in Table 7. No differences ($p > .05$) in non-gestational animals versus gestational animal body weight, biceps femoris muscle weight and proportion of biceps femoris muscle were detected; however, biceps femoris muscle weight increased as animals aged although its proportion did not change ($p > .05$) after 2 months (Table 7).

Muscle NMH concentration has been used as an index of myofibrillar protein degradation (Goodman, 1987). Muscle NMH was measured rabbits of various ages (Table 7). Concentration of NMH in muscle declined when animals approached maturity; however, no differences in non-gestational versus gestational animals were detected (Table 7).

No differences ($p > .05$) in muscle RNA concentration were detected between non-gestational and gestational rabbits; however, RNA concentration declined significantly ($p < .05$) with increasing age (Table 8). The most rapid loss of RNA occurred in early life (ie. between birth and weaning; Table 8). Muscle protein concentration in newborn animals was the lowest of all animal groups, although there were no differences ($p > .05$) among other animal groups (Table 8). Ribosomal capacity (RNA/g protein) declined

significantly ($p < .05$) as animals aged; however, no differences were detected between non-gestational and gestational rabbits (Table 8).

Activities of calpains and calpastatin are shown in Table 9 and Figure 1. Activities of muscle calpain I, calpain II and calpastatin declined ($p < .05$) in the first month of life (Figure 1). After one month of age, no differences in activities of these proteins were detected (Figure 1). Furthermore, no differences ($p > .05$) in activities of muscle calpains or calpastatin were detected in gestational versus non-gestational rabbits (Table 9).

Effects of age on muscle steady-state mRNA encoding calpain I concentration was analysed by Northern blot method and is shown in Figure 2. Calpain I mRNA concentration (calpain mRNA/total RNA) did not differ among groups although it tended to decrease with age ($p > .05$; Figure 5). Effects of age on muscle calpain II mRNA concentration are shown in Figure 3. Newborn, weaned (1 month), market weight (2 months) animals expressed almost the same level ($p > .05$) of calpain II mRNA; however, adults had less calpain II mRNA ($p < .05$) compared to the other three groups (Figure 6). Two mRNA species encoding calpastatin were detected (Figure 4). These correspond to mRNA species arising from the same gene (Emori et al; 1987) and are refered to as calpastatin mRNA bands I and II. In newborn and weaned rabbits, band I was not evident. However, in adult rabbits, band II appeared and total calpastatin mRNA increased ($p < .05$; Figure 7). Calpastatin band II mRNA significantly decreased ($p < .05$) as animal aged (Figure 4; Figure 7).

Effects of age on muscle calpain I, calpain II and calpastatin mRNA, expressed as densitometer units per g tissue protein, are shown in Figure 10. These results provide a

more accurate estimate of mRNA concentration whereas mRNA expressed on a total RNA basis provides insight into specific regulation of mRNA. In newborn rabbits, concentrations of mRNA (densitometer unit/g protein) encoding calpain I, calpain II and calpastatin presented the highest levels ($p < .05$) compared to weaned, market weight and adult rabbits. However, no differences ($p > .05$) in concentrations of mRNA (densitometer unit/g protein) encoding these three proteins were detected among weaned, market weight and adult animals.

No differences between non-gestational adults versus gestational adult in calpain I mRNA concentration were detected (Figure 8; Figure 9). However, calpain II mRNA concentration was increased significantly by pregnancy ($p < .05$; Figure 8; Figure 9). Pregnancy did not affect expression of calpastatin band I mRNA; however, pregnancy reduced ($p < .05$) calpastatin band II mRNA (Figure 4; Figure 9).

DISCUSSION

EFFECTS OF AGE ON SKELETAL MUSCLE PROTEIN DEGRADATION

It is well known that rates of growth and muscle protein degradation decline as animal age (Mulvaney et al., 1985; Goldspink et al., 1985; Kang et al., 1985; Oddy et al., 1986; Muramatsu et al., 1988). Due to high rates of protein degradation and high moisture content of young animals, muscle protein concentration is lower in young animals than in adults. In sheep and rabbit muscle, in early stages of growth, total protein concentration was the lowest compared to other stages of life. RNA concentration also declined with age. The RNA:protein ratio, a measure of the capacity for protein synthesis, also decreased with age. It has been reported that in adult animals the reduction of protein synthesis was due to loss of both RNA capacity and RNA efficiency (Kang et al., 1985). Our results provide support for this conclusion.

Growth rate is, in part, determined by the efficiency of protein deposition. Furthermore, the overall efficiency of muscle growth is largely influenced by the protein degradation rate. What regulates degradation of muscle proteins is not fully known. Presumably, proteolytic systems are important factors in the regulation of muscle protein degradation. Our study was intended to clarify the role that calpains and calpastatin play in the age-dependent attenuation of protein degradation. NMH concentration in muscle has been considered as an index of myofibrillar protein degradation in several species animals (Goodman, 1987). NMH concentration in rabbit biceps femoris muscle decreased as animals approached maturity. This implies that the rate of myofibrillar protein degradation in muscle decreased with age.

Although it has been reported that aging was associated with reduced activities of muscle lysosomal proteases in rats (Goldspink et al.,1985) and chicks (Sanderson et al., 1989), it is unlikely that the lysosome represents the site at which changes in myofibrillar proteolysis are initiated.

Calpains have been postulated to initiate myofibrillar protein degradation by release of thick and thin filaments from the surface of myofibrils (Allen, 1986; Goll et al., 1989). Calpains are ubiquitous non-lysosomal proteases, possessing an absolute requirement for calcium. Calpastatin, an endogenous protease inhibitor, co-exists with calpains in the cytosol and specifically inhibits both calpain I and calpain II. It is believed that calpastatin is an important factor in the regulation of calpain activities (Pontremoli et al., 1985; Suzuki et al., 1988). Other investigators have demonstrated that age-related changes in calpain and calpastatin activities. Varnum et al (1989) reported that rat lens calpain II activity declined and calpastatin activity remained unchanged during aging. Blomgren et al (1989) reported that the activities of rat thymus calpain and calpastatin declined with age.

In our studies, calpain and calpastatin activities declined rapidly in sheep and rabbits prior to weaning; however, following weaning futher losses in activities of these proteins were not detected. This implies that calpains are involved in the age-dependent attenuation of protein degradation. Interestingly, in our study, calpastatin declined in parallel with calpains as animal aged. In 1988, Nakamura et al. found no differences in calpastatin activity in muscle taken from 6 and 12 months of age. Their results support our data in that there is no change in calpastatin activity after weaning. These data imply

that age-dependent attenuation of myofibrillar protein degrdation is not mediated by increase expression of calpastatin.

Chicken calpain II heavy chain was the first calpain cloned and sequenced (Ohno et al., 1984). Differential expression of calpain II in tissues was attributed to tissue-specific expression of an uncharacterized trans-element which negatively influences calpain II transcription by interacting with the negative cis-element (Hata et al., 1989). Reductions in activities of calpains and calpastatin in our rabbit study were directly associated with reduced RNA concentration (ribosomal capacity) and reduced concentrations of muscle calpain and calpastatin mRNA (expressed as desitometer units per g tissue protein; Table 5,8 and figure 10). The reduction in mRNA appeared to be associated with an overall loss of RNA from skeletal muscle because mRNA/g protein was greatly reduced whereas mRNA/total RNA for both calpains and calpastatin did not change greatly. Hence, age-related regulation specifically directed towards calpains and calpastatin was not detected. The loss of calpain II mRNA/total RNA in adults requires regulation specifically directed toward this gene; however, it does not account for neonatal attenuation of calpain II activity and may be related to increased expression of the trans-element which was proposal by Hata et al. (1989).

Three species of calpastatin mRNA have been detected in rabbit tissue (I, 38Kb; II, 30Kb; III, 25Kb; Emori et al., 1987). These arise from the same gene and differ only in lengths of their 3'non-coding regions (Emori et al., 1987). In our study, newborn and weaned rabbits expressed only the intermediate length of calpastatin mRNA (II). The longest calpastatin mRNA (I) was also expressed in market weight and adult animals;

however, the shortest mRNA (III) was not detected. In addition, it is worth noting that comparision of calpastatin activity in Figure 1 and concentration of calpastatin mRNA in Figure 7, middle length mRNA maybe the major form of mRNA which is translated because this declines with age whereas calpastatin band I increases with age. The reason why and mechanisms by which muscle cells express different forms of calpastatin mRNA at different ages is worthy of additional study.

EFFECTS OF CASTRATION ON SKELETAL MUSCLE PROTEIN DEGRADATION

In most species, the rate of gain of muscle and of total body protein is faster in the intact male compared to either the female or castrated male, even under conditions of controlled food intake (Fletcher et al., 1986). The involvement of androgens, particularly testosterone, in the accretion of carcass protein has, therefore, received considerable attention. The administration of testosterone to female and castrated animals has been applied to commercial species such as cattle and sheep (Burgess et al., 1960; Singh et al., 1985). The mechanism by which testosterone increases protein accretion in muscle is not entirely clear. One of the many mechanisms proposed is reduction of protein degradation. Lobley et al. (1987) reported that testosterone infusions into wethers reduced protein synthesis. Based on this, they suggested that an even larger reduction of protein degradation accounted for anabolic effects of androgens in sheep. Furthermore, in 1989, Hager and Kalkoff reported that testosterone infusions reduced muscle protein degradation in perfused female rat hindlimb. In our study, body weight of castrated animals was lower than ram body weight ($p < .05$) in market weight lambs (Table 4).

However, castration had no effect on activities of calpain I and calpain II or their inhibitor. These results imply that protein degradation was unaffected by castration or if degradation was affected, calpains and calpastatin were not involved in this change. Although our data indicate that castration does not affect activity of total extractable muscle calpains, these observations can not rule out possible regulation of calpains at other metabolic levels such as calpain compartmentation and interaction of calpains and calpastatin.

EFFECTS OF GESTATION ON MUSCLE PROTEIN DEGRADATION

It has been reported that skeletal muscle protein mass undergoes dramatic changes during pregnancy (Mayel-Afshar et al., 1983b). The rate of synthesis of skeletal muscle protein increases during the first half of pregnancy, which is associated with general anabolism of maternal tissues. Rate of muscle protein degradation increases during the second half of pregnancy in order to provide amino acids for fetal and placental protein synthesis (Mayel-Afshar and Grimble, 1982). Calpains have been suggested to have a role in the disassembly of myofibrils by degrading specific proteins during the initial stage of myofibrillar protein degradation. From our data, neither calpains nor calpastatin appeared to be involved in the change of muscle protein mass. Similar results have shown in the study of Mayel-Afshar et al. (1983b). In their study, calpain has been demonstrated to not be involved in the changes in muscle protein mass associated with pregnancy. Muscle NMH concentration did not change in 20-day pregnant animal compared to non-pregnant controls. These data imply that in 20-day pregnant rabbits,

which is the early stage of catabolic phase in pregnancy, enhancement of myofibrillar protein degradation had not occured. However, at the mRNA level, we found that steady-state mRNA of calpain II increased and calpastatin band II mRNA decreased in pregnant rabbits. Presumbly, in the late catabolic stage, increased calpain II activity and decreased calpastatin activity may facilitate proteolysis.

Table 1. Composition of sheep diet.[1]

Ingredient	I.F.No.[2]	% as Fed
Corn	4-02-935	50.00
Soybean meal	5-20-637	10.00
Orchardgrass hay	1-03-428	21.85
Alfalfa hay	1-00-071	15.00
Molasses	4-04-695	2.50
Calcium carbonate	6-01-069	0.65
Calculated feed content		
Metabolizable energy (Mcal/kg)		2800
Crude protein (% DM)		14.6
Calcium (% M)		0.58
Phosphorus (% DM)		0.43
Vitamin A (mg/kg cartene)		8.1
Vitamin D (IU/kg)		212
Vitamin E (mg/kg)		54.6

[1] The diet was pelleted and made available to lambs following weaning on an ad libitum basis.

[2] International Feed Number.

Table 2. Composition of rabbit diets.

Ingredient	% Dry Matter
Alfalfa meal	38.00
Soybean meal	10.00
Wheat mill run	19.00
Ground corn	25.00
Molasses	2.75
Dicalcium phosphate	0.25
Limestone	0.12
Sodium chloride	0.50
Vitamin-mineral premix[1]	0.25
Calculated composition:	
Crude protein (%)	16.00
Digestible energy (Mcal/kg)	2850.00

[1] Vitamin and mineral premix : carotene, 2 ppm; vitamin A, 15 IU/g; vitamin D, 4.41 IU/g; α-tocopherol, 35 ppm; thiamine, 14 IU/g.

Table 3. Conditions used for separation and quantitation of NMH by high performance liquid chromatography.

Time (min)	Flow (ml/min)	Percent Eluent 1	Percent Eluent 2	Gradient Curve*
Initial	1.00	100	0	-
13.50	1.00	97	3	11
24.00	1.00	94	6	8
30.00	1.00	91	9	5
50.00	1.00	66	34	6
62.50	1.00	0	100	6
67.00	1.00	100	0	6

* Gradients refer to program gradient profiles available on Water's Model 680 automatic gradient controller.

Table 4. Performance of ram and wether lambs[1].

	Animal Group				
	Newborn lambs	Weaned (92 days)		Market weight (89 days post-weaning)	
		Wethers	Rams	Wethers	Rams
n	10	6	6	6	6
Body weight (kg)	2.9±0.3	23.3±0.9	22.2±0.5	55.4±1.3[a]	60.2±2.4[b]
Gluteobiceps femoris (g)	16.4±0.7	176±5	171±5	460±13[a]	554±19[b]
Muscle proportion (gluteobiceps femoris/BW)	0.57%	0.75%	0.77%	0.83%	0.92%

[1] Values are expressed as means±SEM. Means within the same row under the heading of market weight with differing superscripts differ significantly ($p < .05$).

Table 5. Effects of age and castration on muscle RNA, protein concentration and ribosomal capacity in lamb skeletal muscle[1].

	Animal Group				
	Newborn lambs	Weaned (92 days)		Market weight (89 days post-weaning)	
		Wethers	Rams	Wethers	Rams
Muscle RNA (mg/g)	5.94±0.44[a]	2.17±0.15[b]	2.03±0.10[b]	1.85±0.15[b]	2.03±0.12[b]
Muscle protein (mg/g)	86.1±2.9[a]	107±4[b]	114±4[b]	121±3[b]	106±6[b]
Ribosomal capacity (mg RNA/g protein)	70.2±6.6[a]	20.3±1.4[b]	18.1±0.5[b]	13.4±1.3[b]	19.4±2.0[b]

[1] Values are expressed as means±SEM. Values in the same row which do not share a common superscript differ significantly ($p < .05$).

Table 6. Effects of age and castration on activities of calpain I, calpain II and calpastatin in lamb skeletal muscle[1].

	Animal Group				
	Newborn lambs	Weaned (92 days)		Market weight (89 days post-weaning)	
		Wethers	Rams	Wethers	Rams
calpain I (U/g protein)	58.2 ± 2.4^{a}	40.7 ± 3.0^{b}	42.5 ± 1.7^{b}	40.7 ± 2.3^{b}	43.6 ± 2.3^{b}
calpain II (U/g protein)	113.4 ± 4.1^{a}	75.8 ± 3.9^{b}	68.9 ± 4.0^{b}	78.2 ± 4.1^{b}	79.6 ± 3.2^{b}
calpastatin (U/g protein)	1131 ± 36^{a}	752 ± 32^{b}	625 ± 40^{b}	615 ± 37^{b}	721 ± 51^{b}

[1] Values are expressed as means$\pm$SEM. Values in the same row which do not share a same superscript differ significantly ($p < .05$).

Table 7. Effects of age and gestation on body weight, muscle weight, muscle proportion and muscle NMH in rabbit[1].

	Age (months)				
	Newborn	1	2	5	
				non-gestational	gestational
n	6	6	6	6	4
Body weight (g)	57.6±1.2	1053±96	1583±65	4217±108	4050±263
Biceps femoris (g)	N/A[2]	4.64±0.33[a]	9.90±0.51[b]	26.8±1.8[c]	25.1±0.76[c]
Muscle proportion (biceps femoris)	N/A	0.44%	0.63%	0.64%	0.62%
muscle NMH (μmole/g tissue)	3.1±0.1[a]	2.8±0.3[ab]	2.2±0.2[bc]	2.1±0.2[bc]	1.8±0.3[c]

[1] Values are expressed as means±SEM. Values in the same row which do not share a common supercript differ significantly ($p < .05$).

[2] Not assayed.

Table 8. Effects of age and gestation on total RNA, protein concentration and ribosomal capacity in rabbit muscle[1].

	Age (months)				
	Newborn	1	2	5	
				non-gestational	gestational
Muscle protein (mg/g tissue)	58.1 ± 5.1^{a}	127 ± 4^{bc}	108 ± 7^{b}	143 ± 10^{bc}	127 ± 13^{c}
Muscle RNA (mg/g tissue)	5.83 ± 0.29^{a}	2.70 ± 0.21^{b}	1.81 ± 0.15^{c}	0.98 ± 0.09^{d}	0.74 ± 0.10^{d}
Ribosomal capacity (mg RNA/g protein)	91.3 ± 2.9^{a}	21.1 ± 1.0^{b}	16.9 ± 1.5^{b}	7.3 ± 0.7^{c}	5.4 ± 1.2^{c}

[1] Values are expressed as means$\pm$SEM. Values in the same row which do not share a common supercript differ significantly ($p < .05$).

Table 9. Effects of gestation on activities of rabbit muscle calpain I, calpain II and calpastatin[1].

	non-gestation	gestation
n	6	4
calpain I (U/g protein)	8.3±0.6	8.3±0.87
calpain II (U/g protein)	12.3±0.6	13.6±1.7
Calpastatin (U/g protein)	438±39	508±58

[1] Values are expressed as means±SEM.

Figure 1. Effects of age on activities of calpain I, calpain II and calpastatin in rabbit biceps femoris muscle.

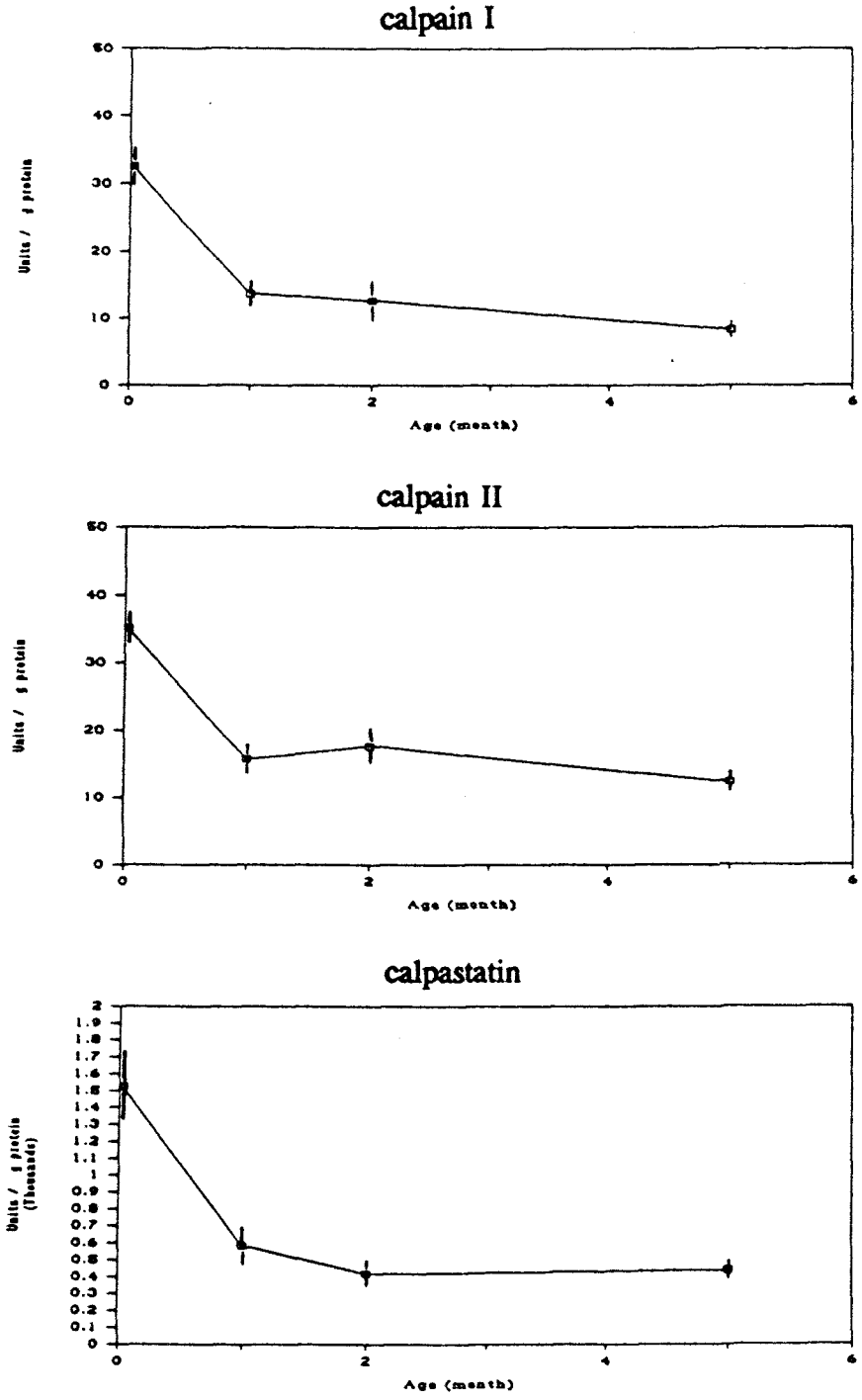


Figure 2. Effect of age on calpain I steady-state mRNA concentration in rabbit biceps femoris muscle.

Lanes 1-3: newborn rabbit; Lanes 4-6: weaned (1 month); Lanes 7-9: market weight (2 months); Lanes 9-12: adult (5 months).

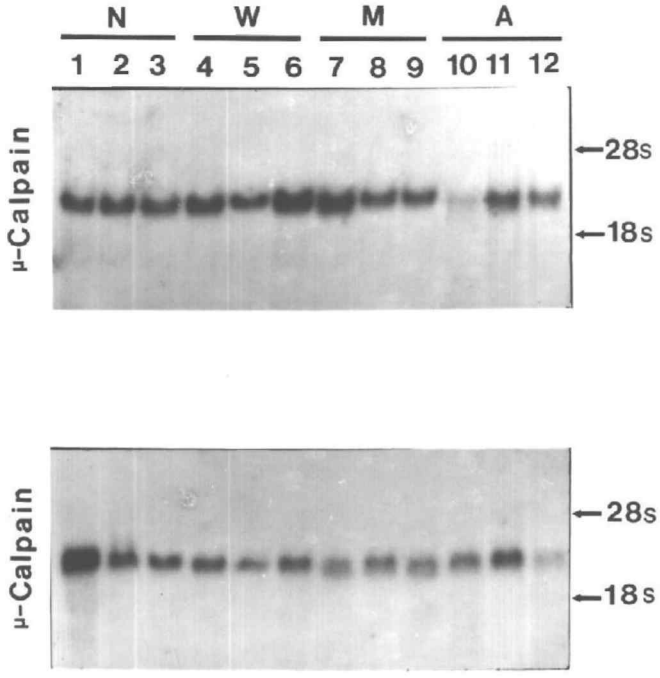


Figure 3. Effect of age on calpain II steady-state mRNA concentration in rabbit biceps femoris muscle.

Lanes 1-3: newborn rabbit; Lanes 4-6: weaned (1 month); Lanes 7-9: market weight (2 months); Lanes 9-12: adult (5 months).

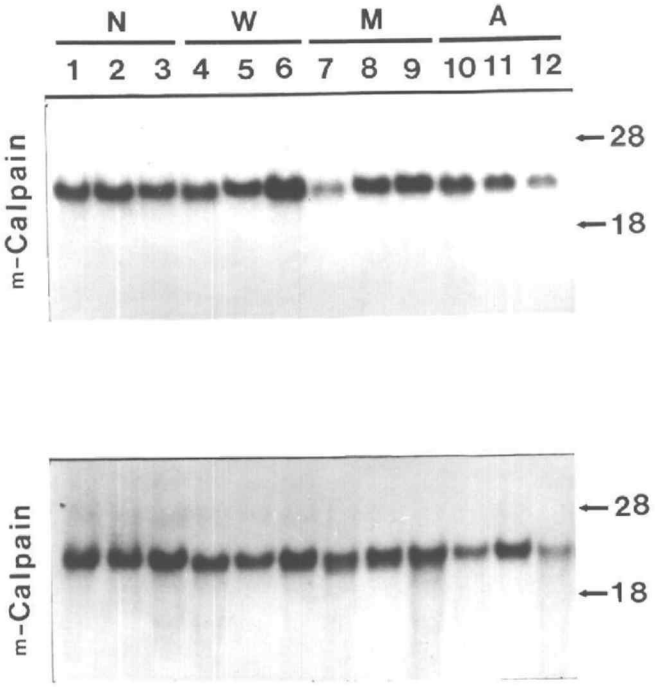


Figure 4. Effect of age on calpastatin steady-state mRNA concentration in rabbit biceps femoris muscle.

Lanes 1-3: newborn rabbit; Lanes 4-6: weaned (1 month); Lanes 7-9: market weight (2 months); Lanes 9-12: adult (5 months).

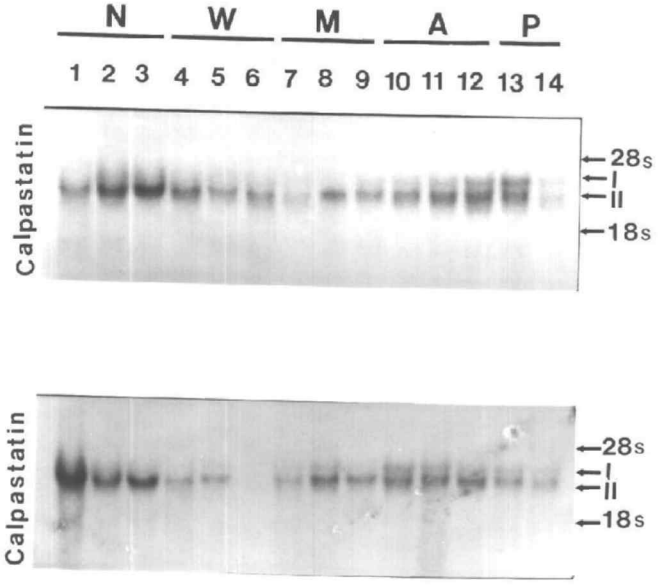


Figure 5. Scanning densitometry of Northern blot shown in Figure 2.

NB: newborn; W: weaned; M: market weight; A: adult.

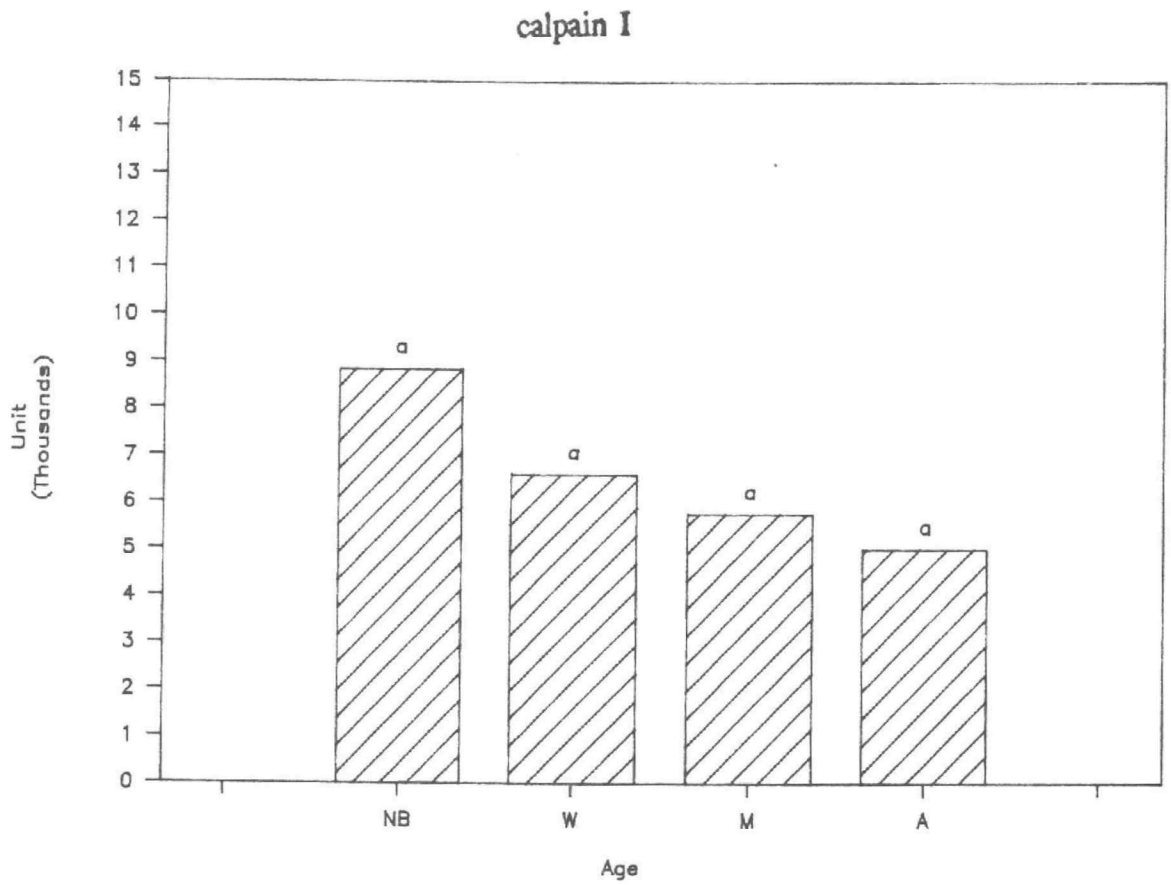


Figure 6. Scanning densitometry of Northern blot shown in Figure 3.

NB: newborn; W: weaned; M: market weight; A: adult.

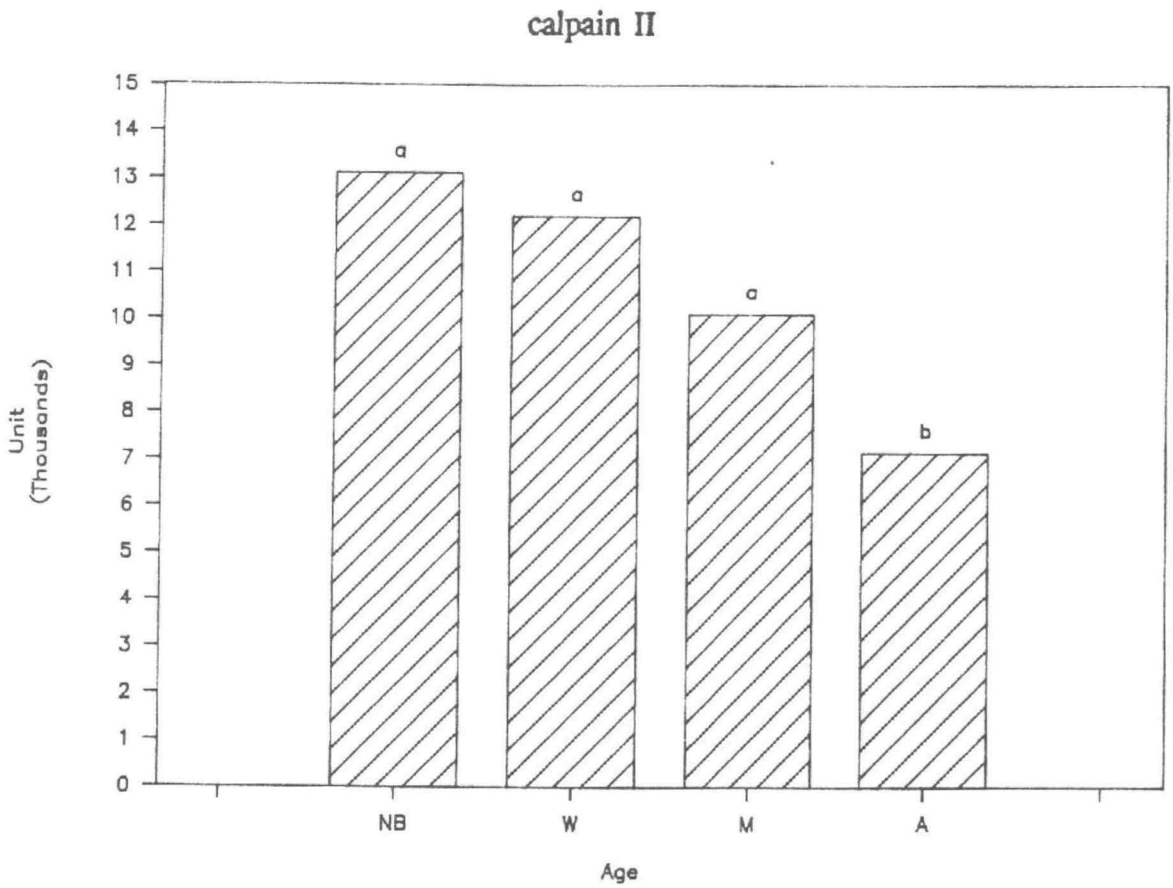


Figure 7. Scanning densitometry of Northern blot shown in Figure 4.

NB: newborn; W: weaned; M: market weight; A: adult.

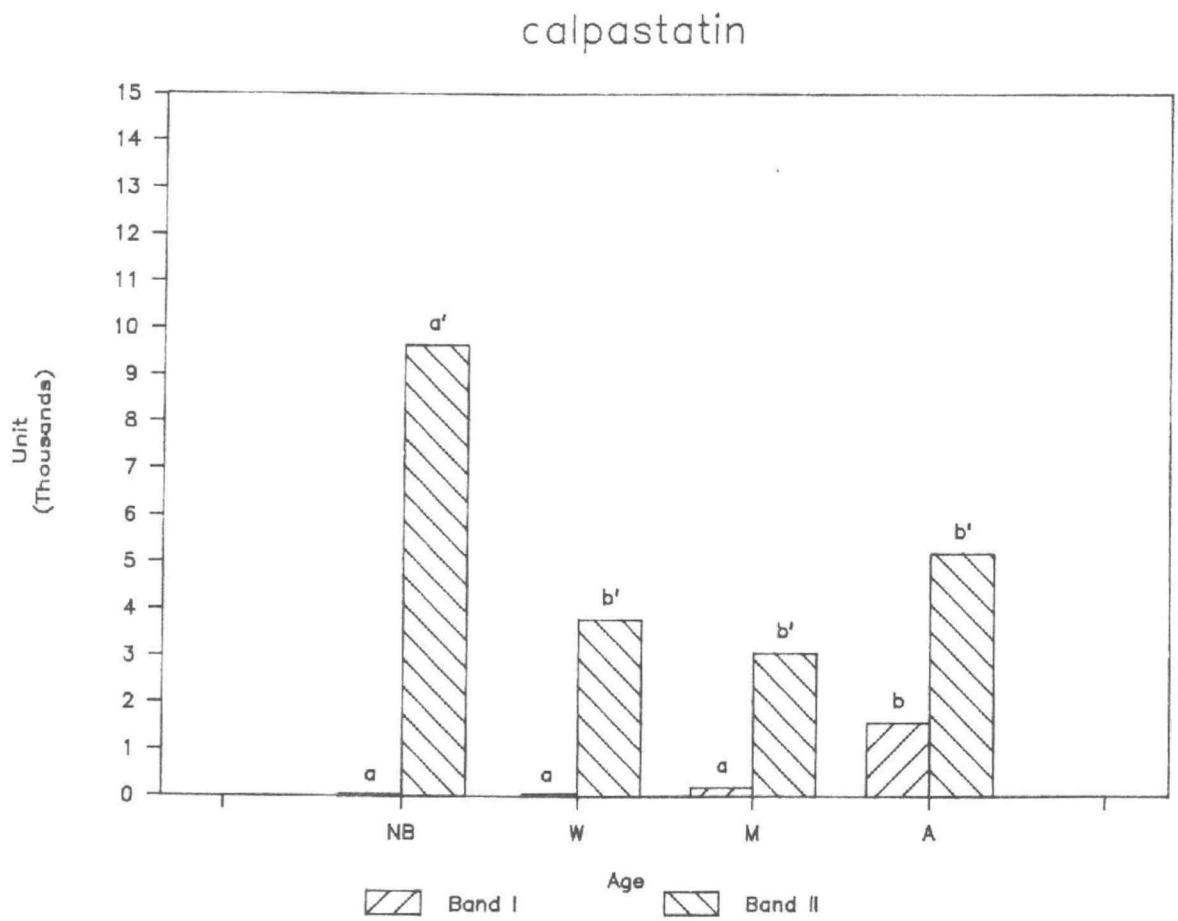


Figure 8. Effect of gestation on calpain I and calpain II steady-state mRNA concentration in rabbit biceps femoris muscle.

Lanes 1-6: adult (5 months); Lanes 7-10: gestational adult (20 day pregnant).

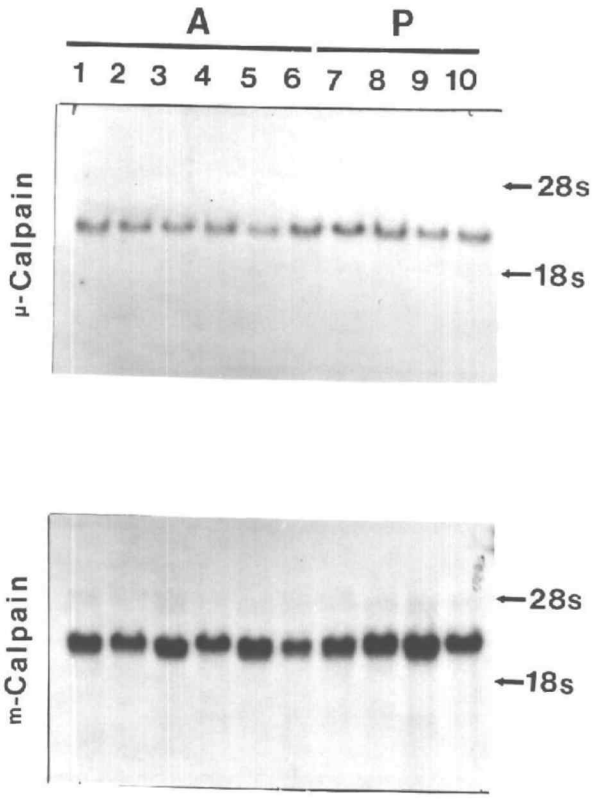


Figure 9. Scanning densitometry of Northern blot showing effects of pregnancy on mRNA concentrations encoding calpain I, calpain II and calpastatin.

A: nongestational adult; P: gestational adult.

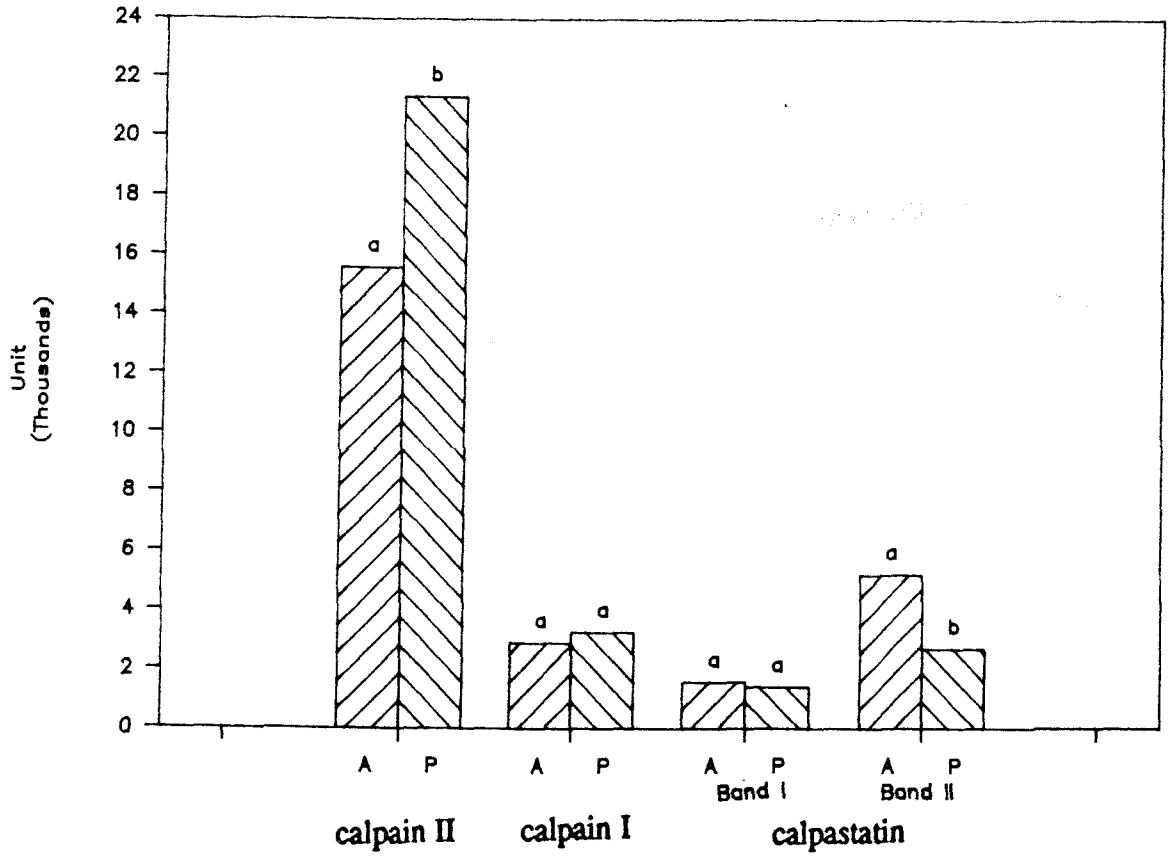
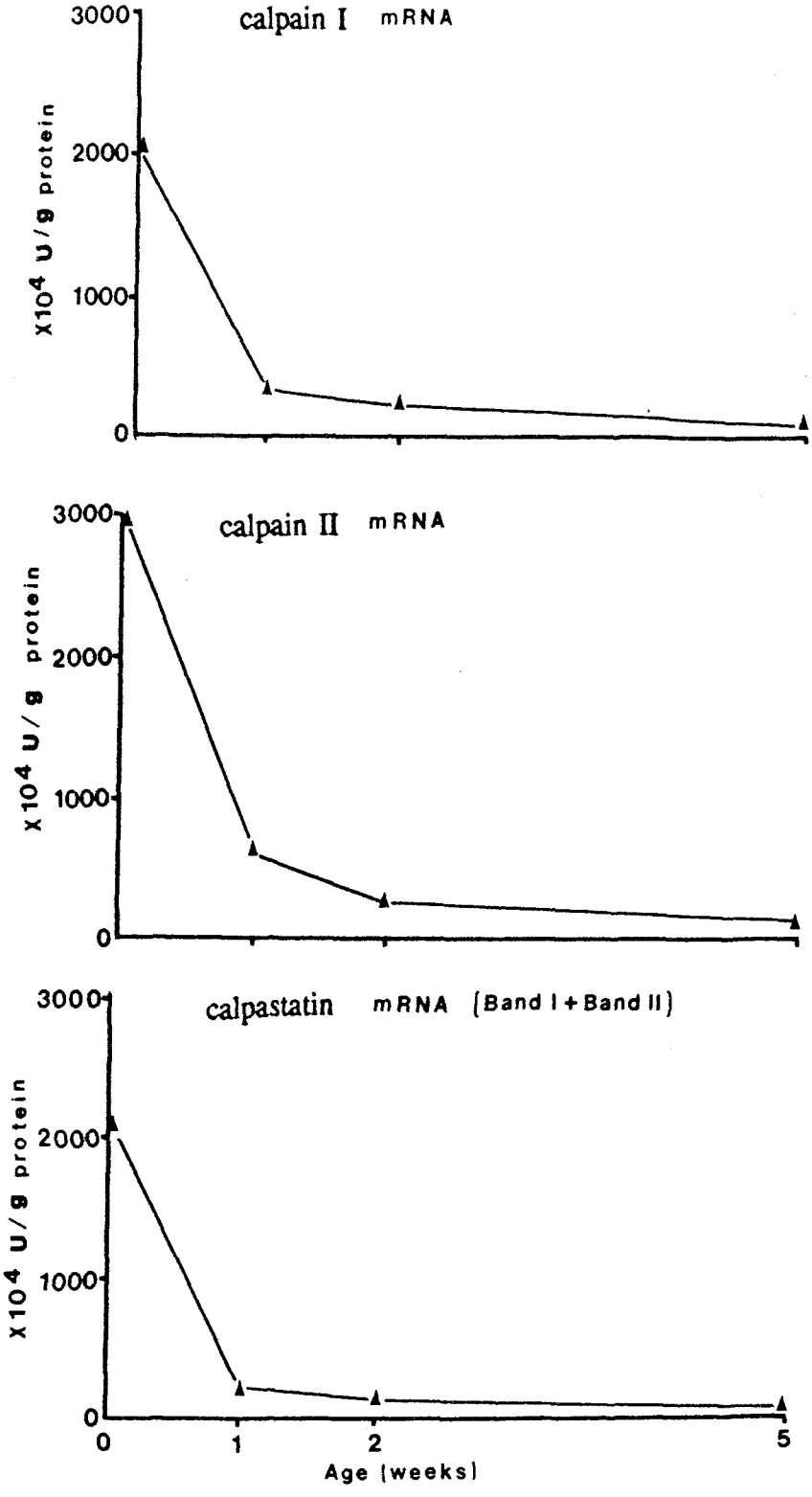


Figure 10. Effects of age on muscle calpain I, calpain II and calpastatin mRNA expressed as densitometer units per g tissue protein.



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APPENDICES

Appendix 1. Assessment of calpain activity.

1. Homogenize muscle tissue (3 g) in 5 volume of homogenization buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 10 mM 2-mercaptoethanol and 150 nM pepstatin A).

2. Centrifuge at 13000 x g at 4°C for 30 min.

3. Mix 5 ml of supernatant with 10 μl of 1 mM leupeptin, 1 ml of phenyl sepharose and .3 ml of 5 M NaCl, and shake for 5 min.

4. Add .6 ml of .1 M $CaCl_2$ and continue shaking for another 10 min.

5. Pour gel suspension into a .8 X 4 cm column (Bio-Rad).

6. Wash with 2 ml of Buffer A (20 mM Tris-HCl [pH 7.5], .1 mM $CaCl_2$, 10 mM 2-mercaptoethanol and 20 μm leupeptin) containing .25 M NaCl to remove calpain inhibitor.

7. Wash with 2 ml of Buffer A.

8. Wash with 2 ml of Buffer A without leupeptin.

9. Elute calpain II (m-calpain) with 4 ml of Buffer B (20 mM Tris-HCl [pH 7.5], 1 mM EGTA and 10 mM 2-mercaptoethanol) containing .1 M NaCl and collect this fraction for calpain II assay.

10. Wash with 2 ml of Buffer B containing .1 M NaCl.

11. Elute calpain I (μ-calpain) with 4 ml of Buffer B and collect this fraction for calpain I assay.

12. Mix 3.2 ml of calpain fraction with 1 ml of casein solution (8 mg/ml casein in solution of 100 mM Tris·HCl and 1 mM NaN_3) and 4 mM $CaCl_2$, and incubate at 25°C (RT) for 30 min.

13. Add 1 ml of ice cold 36% TCA and let stand at 4°C for several hr.

14. Centrifuge for 15 min and take 3.2 ml of supernatant into cuvet.

15. Add .8 ml of Bradford solution and incubate at room temperature for 10 min.

16. Measure absorbance (595 nm) by spectrophotometer.

Appendix 2. Assessment of calpastatin activity.

1. Homogenize muscle (0.5g) in 5 ml of homogenization buffer (50 mM Tris-Cl, [pH 7.5], 1 mM EDTA, 10 mM 2-mercaptoethanol and 150 nM pepstatin A).

2. Heat muscle homogenate at 100°C for 10 min and then cool down to RT.

3. Centrifuge at 10000xg at 4°C for 10 min to remove pellet.

4. Take .3 ml calpain II and .1 ml crude homogenate with 2 mM $CaCl_2$, mix and incubate at 25°C for 5 min.

5. Add .4 ml casein solution (8 mg/ml casein in solution of 100 mM Tris-HCl and 1 mM NaN_3) to the mixture, incubate at 25°C for another 30 min.

6. Add .2 ml 36% TCA to stop reaction and store at 4°C for 2 hr.

7. Centrifuge at 10000xg for 10 min.

8. Add .8 ml of supernatant to .2 ml Bradford solution and incubate at RT for 10 min.

9. Measure absorbance (595 nm) by spectrophotometer.

Appendix 3. Extraction of total RNA.

1. Homogenize muscle tissue (.5 g) at room temperature in 10 volume (5 ml) of Solution A (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], .5 % Sarcosyl and .36 ml of 2-mercaptoethanol), and transfer muscle homogenate to a polypropylene tube.

2. Add .5 ml of 2 M sodium acetate (pH 4.0), 5 ml of phenol and 1 ml of chloroform:isoamyl alcohol (49:1) mixture, and mix by inversion after each addition at room temperature.

3. Shake the final suspension vigorously for 10 sec and centrifuge at 10000 x g for 20 min at 4°C.

4. Transfer the aqueous phase to a new tube, add 5 ml of isopropanol, mix well and place at -20°C for at least 1 hr.

5. Centrifuge at 10000 x g for 20 min at 4°C and resuspend the RNA pellet in .5 ml of Solution A.

6. Transfer the solution to a 1.5 ml microcentrifuge tube, add 1 volume (.5 ml) of isopropanol, mix well and place at -20°C for at least 1 hr.

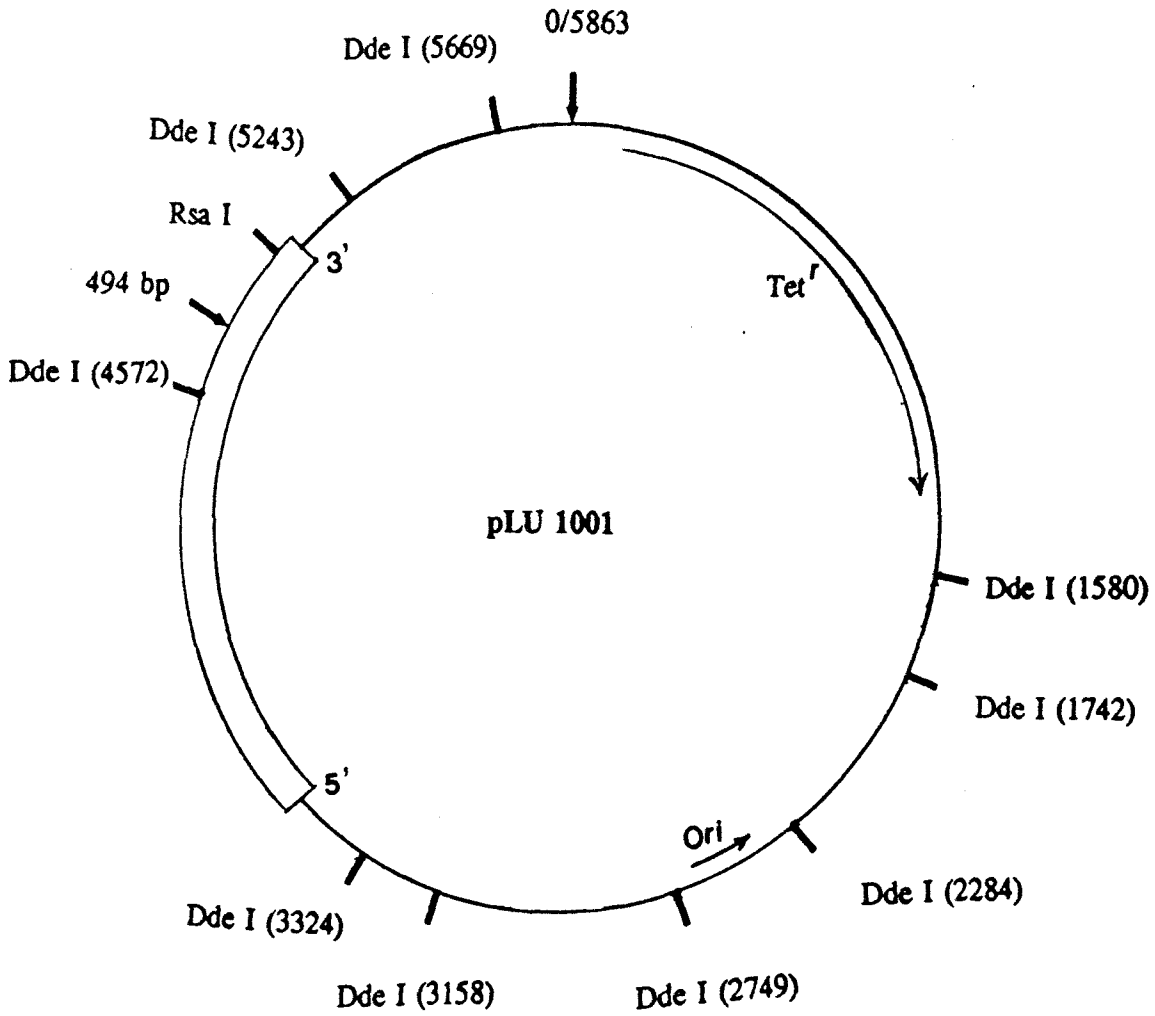
7. Centrifuge at 12000 x g for 10 min at 4°C, wash the RNA pellet with 1 ml of 75 % ethanol and dry the RNA pellet in a vacuum desicator.

8. Resuspend the RNA pellet in 50 μl of diethyl-pyrocarbonate (DEPC) treated-water and quantitate by spectrophotometer at 260 nm wavelength.

Appendix 4. Preparation of saturated phenol solution.

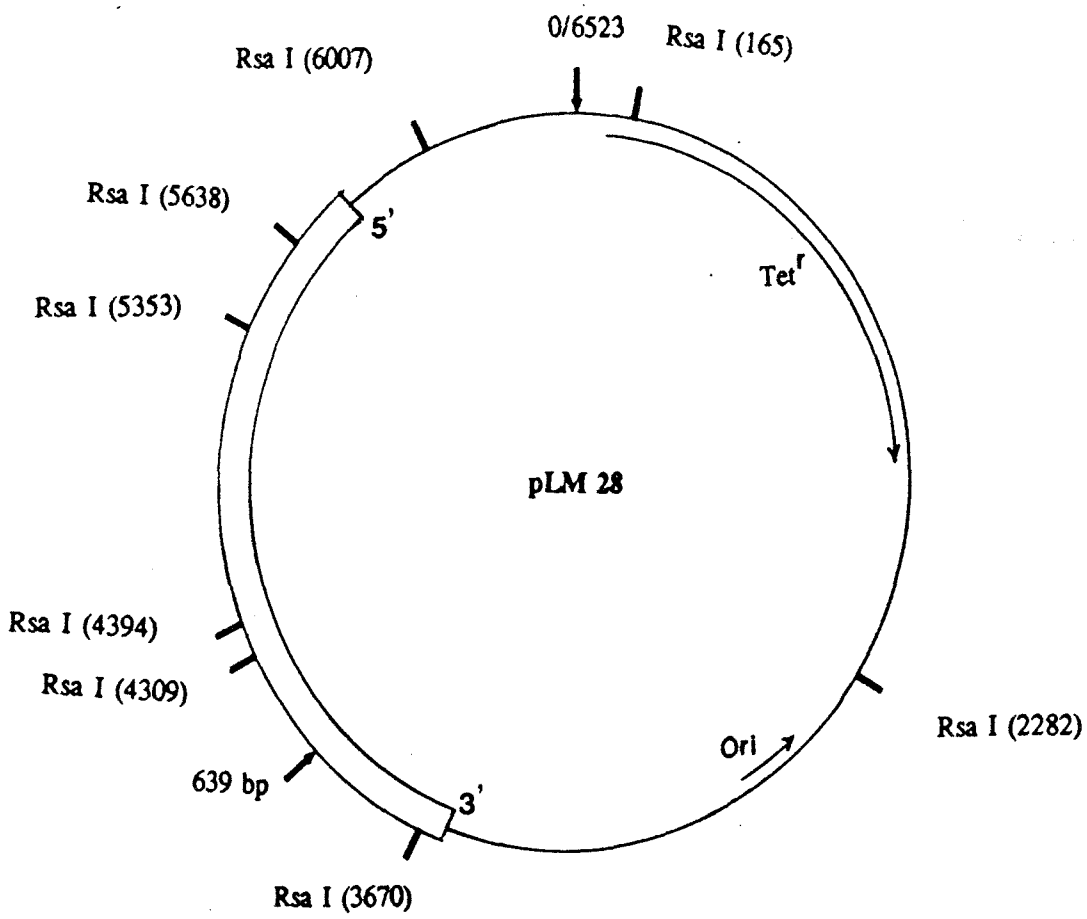
1. Add .5 g of 8-hydroxyquinoline (Sigma; H-6878) to a 2 L glass beaker.
2. Melt solid phenol in 65°C water bath.
3. Pour 500 ml of liquified phenol into beaker.
4. Add 500 ml of 50 mM Trizma base solution and stir 10 min at room temperature with low speed.
5. Stop stiring and let phases separate at room temperature.
6. Gently discard the upper phase into a suitable waste container.
7. Substitute 50 mM Trizma base solution with 50 mM Tris·Cl solution (pH 8.0) and repeat procedure 4-6 twice.
8. Add 250 ml of 50 mM Tris·Cl solution (pH 8.0) and store this saturated phenol solution in dark at 4°C.

Appendix 5. Plasmid (pLU 1001) cycle map[1].



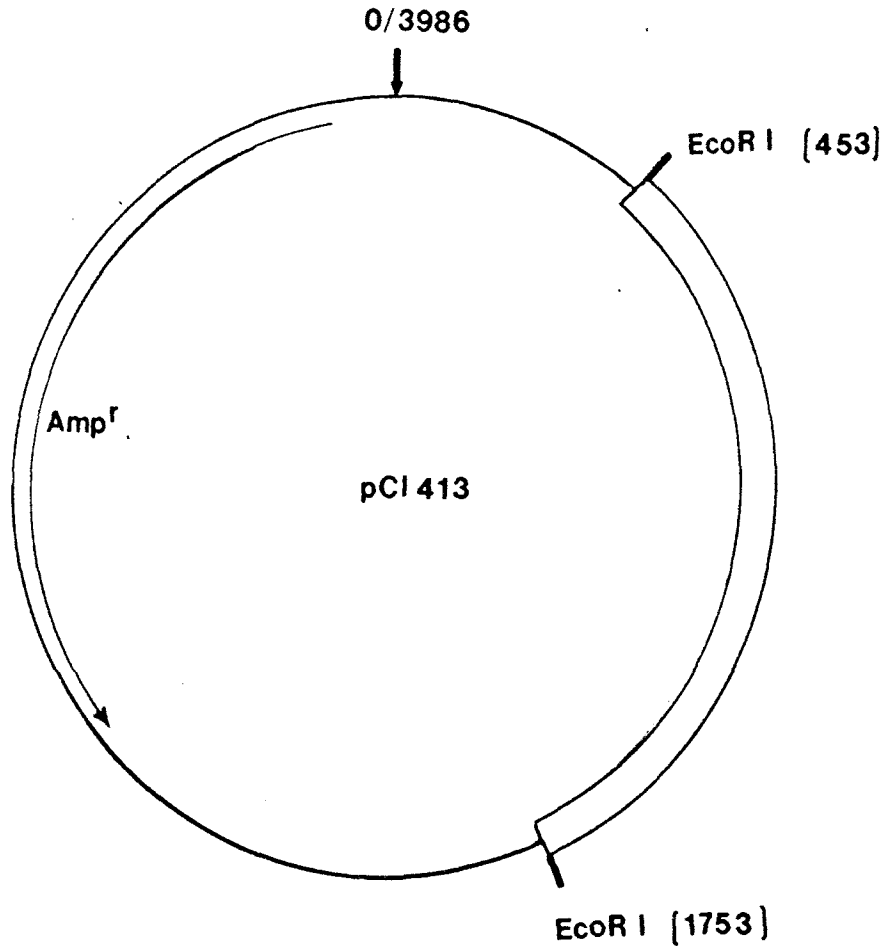
[1] pLU 1001 is constructed in the Pst I site of pBR 322.

Appendix 6. Plasmid (pLM 28) cycle map[1].



[1] pLM 28 is constructed in the Pst I site of pBR 322.

Appendix 7. Plasmid (pCI 413) cycle map[1].



[1] pCI 413 is constructed in the EcoR I site of pUC 18.

Appendix 8. Preparation of ^{32}P-labeled cDNA probe.

1. Add 100 ng cDNA fragment and 5 μl of primer to tube A, boil for 4 min and place on ice.

2. Add 4.0 μl of Klenow enzyme, 50 μCi of ^{32}P-dCTP (New England Nuclear; NEG-013H), and 7.5 μl of dATP, dGTP and dTTP (1:1:1) mixture into tube B.

3. Mix tube A and tube B, and incubate at 37°C for 4 hr.

4. Add 5.0 μl of .2 M EDTA solution to stop reaction and make volume to 100 μl by deionized distill water.

5. Extract once with 1 volume of phenol and 1 volume of chloroform and transfer the aqueous phase to a new tube.

6. Add 30 g of Bio-gel P-60 (Bio-Rad; 150-1640) to 300 ml of TE buffer and incubate overnight at room temperature to swell the renin.

7. Discard excess TE buffer and replace with fresh TE buffer.

8. Pack a column (20 cm X .5 cm) with swelled Bio-gel P-60 and put the column behind a plastic shield to protect from exposure of radioactivity.

9. Separate the ^{32}P-labeled cDNA probe from the unincorporated nucleotide by chromatography on Bio-gel P-60.

10. Apply ^{32}P-labeled cDNA probe to Bio-gel P-60 column in a volume no more than 200 μl.

11. Collect 12 fractions of 300 μl each.

12. Take 5 μl of each fraction and add 5 ml of counting solution to count the radioactivity.

13. Pool peak fractions and add .1 volume of 3 M sodium acetate and 2.5 volume of ethanol to precipitate the ^{32}P-labeled cDNA probe.

14. Rinse the cDNA pellet with 80 % ethanol and dry the cDNA pellet in a vacuum desicator.

15. Dissolve the dried ^{32}P-labeled cDNA probe in a suitable amount of TE buffer.