

AN ABSTRACT OF THE THESIS OF

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Skeletal muscle constitutes a large portion of the body, responsible for 40% of body weight. Like other proteins, muscle proteins are continuously synthesized and degraded. Because muscle is a major tissue, the regulation of proteolysis in muscle is important to overall energy homeostasis and to body growth. In order to identify the hormonal factors which regulate proteolysis, I examined the effects of glucocorticoids, phorbol esters, thyroid hormone and insulin-like growth factor-I (IGF-I) on protein degradation in cultured muscle cells. IGF-I inhibited proteolysis, whereas glucocorticoids and phorbol ester stimulated the rate of protein degradation. Thyroid hormone was without effect on proteolysis. Studies with glucocorticoid antagonist, RU38486, showed that the catabolic effects of glucocorticoids involve intracellular glucocorticoid receptors.

To study the mechanisms by which these endocrine factors regulate proteolysis, I examined the effects of IGF-I and glucocorticoids on protease gene expression using Northern blot analysis. IGF-I treatment decreased cathepsin D and proteasome mRNA but increased m-calpain mRNA. Cathepsin B mRNA was not changed by IGF-I treatment. These results suggested the possible roles of cathepsin D and proteasome in IGF-I-dependent inhibition of proteolysis. A catabolic factor, dexamethasone, cause a 3-fold increase in cathepsin B mRNA. It also slightly increased m-calpain mRNA in L8 myotubes but did not affect cathepsin D and proteasome mRNA. These results indicate that induction of cathepsin B and m-calpain may be involved in glucocorticoid-stimulated proteolysis.

To gain understanding of the means by which phorbol esters control proteolysis in muscle cells, PKC isoforms in muscle cells were examined by immunoblot analysis. Using six isoform (α , β , γ , δ , ϵ and ζ)-specific polyclonal antibodies, PKC α , δ and ζ were detected in rat skeletal muscle, L8 myoblasts and myotubes. PKC β , γ and ϵ isozymes were not detected. These studies showed the presence of calcium- , diacylglycerol- and phospholipid-dependent PKC (α), diacylglycerol- and phospholipid-dependent PKC (δ) and phospholipid-dependent PKC (ζ) in muscle cells.

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ENDOCRINE CONTROL OF PROTEOLYSIS IN CULTURED MUSCLE CELLS

CHAPTER I

INTRODUCTION

Skeletal muscle is a major tissue of the body, responsible for forty percent of total body weight in normal adult animals. Skeletal muscle proteins, as in other cells, are subjected to continuous turnover and the overall rate of protein degradation is precisely controlled. The regulation of proteolysis in muscle is important in overall energy homeostasis, in the control of muscle size and body growth and also in adaptation of the organism to a variety of stressful conditions. Because skeletal muscle constitutes the major protein reservoir in the body, the hydrolysis of muscle proteins is an important first step in amino acid mobilization. Consequently, negative protein balance in muscle, leading to a net loss of soluble and myofibrillar proteins, is characteristic of physiological states where gluconeogenesis from body protein stores arises, such as in starvation and diabetes. Muscle protein degradation is regulated by a number of hormones and growth factors that are also critical to energy homeostasis, including insulin, cortisol, thyroid hormone and insulin-like growth factors. However, the mechanisms underlying the regulation are not known. Muscle protein turnover has been measured in vivo (Sjolin et al., 1990; Smith et al., 1990), in perfused muscle (Jefferson et al., 1977; Rannels et al., 1978), in incubated

muscle (Low and Goldberg, 1973; McGrath and Goldspink, 1982), in cultured myoblasts (Ballard and Francis, 1983; Palmer et al., 1990) and in cultured myotubes (Gulve and Dice, 1989; Gulve et al., 1991; Roeder et al. 1988). Skeletal muscle cells can be grown in vitro. Myoblasts in culture can differentiate into multinucleated myotubes, which express muscle-specific contractile proteins and enzymes such as myosin, α -actin, tropomyosin and creatine kinase. Thus, cultured muscle cells provide a unique model system for studying muscle protein turnover under a variety of culture conditions. In this study, the regulation of total protein degradation and proteinase gene expression by insulin-like growth factor I (IGF-1), thyroid hormone, glucocorticoid hormone and 12-O-tetradecanoyl phorbol-13-acetate (TPA) were determined in cultured L8 rat skeletal myotubes.

Insulin-like growth factor-I

Insulin-like growth factor-I (IGF-I), which is also known as somatomedin-C, is a peptide hormone. IGF-I consists of a single polypeptide of 70 amino acids with molecular weight of 7.6 kDa (Florini et al., 1991). Like other growth factors, IGF-I is synthesized and secreted by several cell types. The synthesis of IGF-I is controlled by growth hormone. The binding of circulating growth hormone with specific growth hormone receptors on cells induces the synthesis and secretion of IGF-I. IGF-I stimulates a variety of actions in different cells that mediate the growth rate of tissues. IGF-I has anabolic actions on skeletal muscle cells. IGF-I stimulates amino acid

uptake, protein synthesis, glucose uptake and cell proliferation (Florini et al., 1991). IGF-I also inhibits protein degradation in muscle. IGF-I has shown to regulate muscle protein turnover in vivo and in vitro. Injection of IGF-I to normal (Hizuka et al., 1986), diabetic (Scheiwiller et al., 1986), hypophysectomized (Schoenle et al., 1985), nitrogen restricted (Tomas et al., 1990) and synthetic glucocorticoid dexamethasone-treated (Tomas et al., 1992) rats leads either to an increased weight gain or to a reversal of the weight loss. IGF-I stimulates protein synthesis in isolated rat skeletal muscle (Uthne et al., 1978). Many studies with cultured muscle cells also show that IGF-1 stimulates protein synthesis and inhibits protein degradation in rat L6 myoblasts (Ballard et al., 1986) and L6 (Roeder et al., 1988) and L8 (Gulve et al., 1989) myotubes.

Thyroid hormone

Thyroxine (T_4) and triiodothyronine (T_3) are thyroid hormones secreted by the thyroid gland. T_3 is also formed in the peripheral tissues by deiodination of T_4 . Thyroid hormone is essential for the regulation of various metabolic processes and the energy consumption of the animal. The physiological effects of thyroid hormone are initiated by the binding of triiodothyronine (T_3) to DNA-binding proteins called T_3 receptors (Brent et al., 1991). The hormone-receptor complex then binds to specific genes of target tissues and either positively or negatively regulates gene transcription. Thyroid hormone has been known to regulate growth hormone gene expression in cultured rat

pituitary cell lines (Evans et al, 1982), to stimulate malic enzyme (Dozin et al, 1986) and to stimulate α -myosin heavy chain gene expression in heart (Everett et al, 1983). In addition to inducing gene expression, the thyroid hormones also inhibit the expression of certain genes, such as thyrotropin and β -myosin heavy chain gene (Gustafson et al., 1985). Thyroid hormones also regulate protein turnover in skeletal muscle. Hypophysectomy or thyroidectomy, which abolish thyroid hormone production, decrease protein degradation in skeletal muscles (Flaim et al., 1978; Tischler, 1981). Physiological levels of thyroid hormone induce muscle growth and increase both protein synthesis and breakdown. They increase protein synthesis more than they promote protein breakdown. High levels of T_3 cause muscle weakness by increasing protein degradation more than protein synthesis (Kettelhut et al, 1988). Hyperthyroidism usually causes severe muscle wasting (Goldberg et al, 1980).

Glucocorticoids

Glucocorticoids (cortisol and corticosterone) are steroid hormones which are secreted by the adrenal glands. Glucocorticoids are essential for living cells. They have a regulatory role in tissue differentiation during development (Baxter and Rousseau, 1979). In the adult animal, they control metabolism in response to stress (Rousseau, 1984). The receptor protein for glucocorticoid hormones is a member of a receptor family for steroid hormones, thyroid hormones and retinoic acid and 9-cis retinol (Funder,

1993). Unlike membrane receptors, these steroid hormone receptors are intracellular and act largely by regulation of DNA transcription (Carlstedt-Duke and Gustafsson, 1990). The receptor monomer is a 90,000 Da protein. This protein possesses three functional domains (Burnstein and Cidlowski, 1989). They are DNA binding (zinc finger), ligand binding (signaling) and 'modulating' domains. The-ligand binding domain (Muller and Renkawitz, 1991) is located in amino acid residue 518-795 at C-terminal end of rat glucocorticoid receptor. The DNA binding domain is located between residue 414-517. The modulating domain, also called a 'composite-specificity' domain (Pearce and Yamamoto, 1993), is located at N-termial (105-440). Physiological effects of glucocorticoid-receptor binding in living cells are to increase or to decrease amounts of gene products. These target genes include enzymes (tyrosine aminotransferase), housekeeping proteins (metallothionein), structural proteins (fibronectin, collagen), membrane hormone receptors (insulin receptor), secretory proteins (casein) and hormones (somatotropin) (Rousseau, 1984). The activity or concentration of those gene products changes as a result of changes in their mRNA concentration.

Like other tissues, skeletal muscle also responds to glucocorticoid hormones. It is well known that these hormones are catabolic to skeletal muscle. The overproduction of glucocorticoids, which can be seen in Cushing's disease or mimicked by pharmacological doses of glucocorticoids, causes growth retardation (Ohtsuka et al., 1992) and muscle wasting (Smith

et al, 1990). Glucocorticoids induce muscle atrophy by several ways. They reduce amino acid uptake by muscle (Kostyo and Redmond, 1966), decrease DNA (Goldberg and Goldspink, 1975) and RNA (Rannels et al, 1978) and inhibit total and myofibrillar protein synthesis (McGrath and Goldspink, 1982). A decline in protein synthesis results from an decrease in RNA content (Odedra et al, 1983), peptide chain initiation (Rannels et al, 1978) and RNA polymerase activity (Goodlad and Clark, 1991). Unlike the effects of glucocorticoids on protein synthesis, there is some disagreement on the effects of these hormones on protein degradation. Past studies have demonstrated that protein degradation is increased (Tomas et al, 1979), unchanged (Rannels and Jefferson, 1980), or decreased (McGrath and Goldspink, 1982). These conflict results may be explained by differences in the doses of hormone given method of administration and the techniques used to measure protein degradation. However, more recent in vivo (Kayali et al, 1987; Ohtsuka et al, 1992; Smith et al., 1990; Tomas et al, 1992) and in vitro (Roeder et al, 1986; Roeder and Gunn, 1987) studies indicated that glucocorticoids increase proteolysis of muscle cells. The mechanism by which glucocorticoids enhance muscle protein degradation, however, is not known.

Proteolytic systems in skeletal muscle

Lysosomal proteases. Like other cells, skeletal muscle cells contain lysosomal cathepsins. These proteases are generally small (20-40 kDa)

glycoproteins, optimally active at acidic pH and unstable at neutral or alkaline pH. Cathepsins B, H, and L are cysteine proteases and cathepsin D is an aspartic protease. Among these, cathepsin B and D are major proteolytic enzymes in muscle (Goll et al, 1989). Muscle cathepsin B has been purified from several species (Hirao et al, 1984; Okitani et al, 1988) including rat, rabbit, monkey and human. The enzyme is usually assayed with synthetic peptide, benzyloxycarbonyl-Arg-Arg-methylcoumarin as substrate. Cathepsin B shows both endopeptidase and exopeptidase activities, depending on substrate. Rabbit muscle cathepsin B can fragment and disturb the lateral arrangement of myofibrils (Matsuishi et al, 1992). Cathepsin B partly disrupts the Z-line and M-line, and causes disarrangement of filaments in the I-band. Among muscle proteins, myosin heavy chain, actin and troponin T are degraded by the enzyme. Cathepsin D, an aspartic proteinase, is also present at high concentration in lysosomes. It is a glycoprotein that resolves into several forms of similar molecular weight and different isoelectric points upon purification (Huang et al., 1980). Cathepsin D has limited activity against native proteins but considerable activity against denatured proteins at pH 3.5-5. Skeletal muscle cathepsin D degrades myosin, α -actinin, tropomyosin and troponin (Matsumoto et al, 1983) in vitro.

Proteasome. Proteasome, also called multicatalytic proteases, is high a molecular weight (700 kDa, 20 S) protein and consist of approximately 15 distinct subunits of 21-31 kDa (Tanaka et al, 1992). They have been isolated from a wide range of eukaryotic cells and tissues (Rivett, 1993).

Proteasomes appear to be located in cytosol and nuclei.

Purified proteasomes have three endoproteolytic activities which are chymotrypsin-like peptidase, trypsin-like peptidase and peptidylglutamyl-peptidase activities (Gottesman and Maurizi, 1992). Various studies indicated that cleavage of different classes of peptides is dependent on different active site within the proteasome (Orlowski, 1990). Proteasomes have proteolytic activity against a number of denatured or oxidized proteins, but this activity is usually latent in the purified proteasome and can be activated by various structural perturbators such as sodium dodecyl sulfate (SDS) or polylysine (Rivett, 1989). Recent studies indicated that proteasome may associate with the ubiquitinating system in cells to produce a 26S proteinase, which is also referred to as ubiquitin-conjugate-degrading enzyme (UCDEN) (Eytan et al., 1989; Driscoll and Goldberg, 1990; Kanayama et al., 1992).

The physiological function of proteasomes is not clear. However, their abundance in cells and association with 26S proteinase suggested that proteasomes play a role in both ubiquitin-dependent and ubiquitin-independent pathways of non-lysosomal proteolysis (Goll, 1991). Proteasomes purified from ovine skeletal muscle can degrade myosin light chain and troponin-C (Koohmaraie, 1992). However, they cause no detectable morphological changes in myofibril structure (Koohmaraie, 1992).

Calpains. Calpains (Calcium activated neutral protease, E.C. 3.4.22.17) are cysteine proteinases optimally active at neutral pH and require Ca^{++} for activity (Suzuki et al., 1987). Two isozymes with different Ca^{++} sensitivities

exist in the cell. They are called μ -calpain (calpain I) and m-calpain (calpain II). They require μ M and mM levels of calcium for activity, respectively. Calpains are heterodimers composed of large (80 kDa) and small (30 kDa) subunits. The large subunit has proteolytic activity and is unique to both isoforms while the small subunit is common to both calpains. Analysis of cDNA sequence data (Emori et al, 1986; Aoki et al., 1986) indicated that the calpain I and calpain II large subunits have essentially the same domain structures and show 50 % sequence homology. The 80 kDa large subunit contains four domains (I, II, III, IV). Domain I corresponds to a propeptide domain and domain II is a cysteine protease. Domain II has a high sequence homology to other cysteine proteinases such as papain and cathepsins B, L and H. Domain IV is a calmodulin-like Ca^{++} -binding domain with four EF hand-like structures. The 30 kDa small subunit contains two domains designated as IV' and V. Domain IV' resembles the calmodulin-like Ca^{++} -binding domain of the large subunit. Domain V is a glycine-rich hydrophobic region and plays an important role in the binding of calpain to phospholipids or membrane. Sequence comparison of the large subunits of human, rabbit and chicken indicate that the same isozyme from different species has sequence homologies higher than 95%, whereas the sequences of different isoforms from the same animal show much lower homologies of 50-60%. Thus, human and rabbit μ -calpain large subunits have 95% sequence homology, but human μ - and m-calpain have only 63% homology (Sorimachi and Suzuki, 1992). The sequences of the 30 kDa subunit (human, rabbit,

rat, bovine and porcine) are almost identical. Another member of calpain family was discovered recently (Sorimachi et al, 1989). This protein is called p94 and is highly homologous to large subunits of μ - and m-calpains except for two insertions of about 50 residues in domains II and III. p94s from human and rat are highly homologous (94%). p94 shows 50% homology with μ - and m-calpains. p94 is exclusively expressed in skeletal muscle. Northern blot analysis shows that the amount of p94 mRNA is ten times higher than the amount for μ - and m-calpains (Sorimachi et al., 1989). However, p94 proteins have not yet been detected in skeletal muscle.

The calpain substrates (Johnson, 1990) thus far identified by in vitro studies can be classified into four groups; 1) contractile and cytoskeletal proteins, like troponin I, C-protein, tropomyosin, titin, nebulin, tubulin, vimentin, spectrin, talin, vinculin and desmin, 2) enzymes, including protein kinase C, phosphorylase b kinase, pyruvate kinase, tyrosine kinase, and 3) receptor proteins, including the estrogen, progesterone, and EGF receptors. Calpain has very limited proteolytic activity and hydrolyzes substrates to large fragments. Although calpains have a variety of substrates in vitro, the physiological substrates and functions in vivo have not been elucidated.

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CHAPTER II

EFFECTS OF SERUM, GLUCOCORTICIDS, THYROID HORMONE AND INSULIN-LIKE GROWTH FACTOR-I ON PROTEIN DEGRADATION IN RAT L8 SKELETAL MYOTUBE CULTURE

ABSTRACT

We examined the effects of horse and fetal bovine sera, synthetic glucocorticoid (dexamethasone), triiodothyronine (T_3) and insulin-like growth factor-I (IGF-I) on proteolysis in rat L8 skeletal myotube cultures. Protein degradation was measured as release of radioactive trichloroacetic acid-soluble materials from intracellular proteins pre-labelled with 3H -tyrosine. Horse serum and fetal bovine serum inhibited ($P < 0.05$) protein degradation. IGF-I at 200 ng/ml inhibited protein degradation by 14% ($P < 0.01$) over a 6 hour measurement period.

Dexamethasone ($1 \mu M$), which is catabolic to muscle at high concentrations, stimulated protein degradation by 9.1% ($P < 0.01$). Dexamethasone-stimulated protein degradation was entirely blocked by adding a glucocorticoid antagonist, RU38486 (mifepristone; $P < 0.01$). Triiodothyronine ($1 \mu M$), which is also catabolic in vivo, retarded protein degradation in myotubes ($P > 0.05$).

This study indicates that IGF-I can inhibit protein degradation at physiological concentrations. Studies with RU38486 showed that the catabolic effects of dexamethasone involve intracellular glucocorticoid

receptors. The lack of response to T_3 suggests the T_3 may act on muscle indirectly, perhaps via altering concentrations of growth factors or other hormones.

INTRODUCTION

Skeletal muscle constitutes a large portion of the body, responsible for 40% of body weight. Like other proteins, muscle proteins are continuously degraded. Because muscle is a major tissue, the control of proteolysis in muscle is important to overall energy homeostasis and to body growth. Hormonal factors have been shown to be a major regulators of these process (Goldberg et al, 1980). Abnormal levels of certain hormone, such as in hyperthyroidism, Cushings's disease, and dwarfism are associated with poor muscle growth or atrophy (Kettlehut et al, 1988). Various hormones and growth factors, insulin, insulin-like growth factor-I, glucocorticoids, thyroid hormones, have been implicated in muscle protein turnover. Among these, insulin and insulin-like growth factor-I (IGF-I), which are anabolic, increase protein synthesis and decrease protein degradation (Harper et al., 1987; Gulve and Dice, 1989). However, glucocorticoids and thyroid hormones are catabolic to muscle.

Skeletal muscle cells can be cultured in vitro. Myoblasts (Wakelam, 1985; Richler and Yaffe, 1970) can be induced to differentiate into multinucleated myotubes. Myotubes (Nadel-Ginard, 1978) express muscle-specific proteins such as myosin, actin, tropomyosin, and creatin kinases etc. Thus, cultured muscle cells provide a unique model system for studying the endocrine control of proteolysis. The purpose of this study was to investigate the effects of various hormonal factors on proteolysis of cultured L8 (Yaffe and Saxel, 1977) myotubes.

MATERIALS AND METHODS

Materials

L-[ring-3,5-³H] Tyrosine (40-60 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Fetal bovine (FBS) and horse sera (HS) were from Hyclone (Logan, UT). Dulbecco's modified Eagle's medium, penicillin/streptomycin solution and trypsin solution were from GIBCO (Grand Island, NY). Human recombinant IGF-I was from Boehringer Mannheim (Indianapolis, IN). Bio-Safe II scintillation cocktail was from Research Products International Co. (Mount Prospect, IL). Trichloroacetic acid was from Mallinckrodt (Paris, KY). Cytosine- β -D-arabinofuranoside (Ara-C), 3, 3', 5-triiodo-L-thyronine, dexamethasone, 12-O-tetradecanoyl phorbol-13-acetate (TPA), dimethyl sulfoxide and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). RU38486 (Mifepristone) was generously given by Dr. Gaillard-Kelly, Roussel-Uclaf (Paris, France). Culture dishes and plates were from Corning (Corning, New York).

Cell Culture

L8 rat skeletal muscle cells were obtained from American Type Culture Collection (Rockville, MD). L8 myoblasts were established in 1969 by serial passaging of myoblasts isolated from primary skeletal muscle cell cultures prepared from neonatal Wistar rats. Cells, stored in liquid nitrogen, were thawed and maintained by repeated subculturing at low density on 10 cm

culture dishes. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 100 units of penicillin/ml, 100 μ g of streptomycin/ml, 44 mM NaHCO₃, 110 μ g of sodium pyruvate/ml in humidified atmosphere of 5% CO₂ and 95% air at 37 °C. The cells were removed with 0.25% trypsin in Ca²⁺- and Mg²⁺-free Ham's balanced salt solution (HBSS) and transferred to 12-well culture plates before experiments. Cells (2.5x10⁴ cells/cm²) were grown in presence of 10% FCS until they reached confluency. At this time the medium was replaced with DMEM containing 2% FCS for induction of differentiation. Approximately 3 days later, when myotubes formation is observed, the cells were treated with 10 μ M cytosine arabinoside for 48 hours to remove any remaining dividing myoblasts. Microscopic examination was used to monitor differentiation.

Measurement of protein degradation

Rates of protein degradation (Roeder et al, 1988; Gulve and Dice, 1989) were determined by measuring the release of trichloroacetic acid (TCA)-soluble radioactivity into the medium at various incubation time after labeling proteins with [³H] tyrosine. After complete differentiation, cells were labeled with 1.0 μ Ci of [³H] tyrosine/ml for 2 days in DMEM containing 10 % FCS. Cells were washed once with HBSS and then placed in DMEM containing 10% FCS and 2 mM non-radioactive tyrosine for 2 hours at 37 °C to allow degradation of short-lived proteins. The cells were then rinsed twice with HBSS and transferred to non-radioactive experimental medium containing 2

mM tyrosine. At the end of the experiment, culture medium was transferred to a microcentrifuge tube containing 100 μ l of bovine serum albumin (10 mg/ml). Trichloroacetic acid (TCA) was added to a final concentration of 10% (w/v). After incubation at 4 °C for at least 1 hour, samples were centrifuged for 5 minutes. The precipitates were then dissolved with Solvable tissue solubilizer (New England Nuclear). Cell monolayers were washed with ice-cold phosphate buffered saline (PBS) and solubilized with 0.5 M NaOH containing 0.1% Triton X-100. Radioactivities in the cell monolayer, TCA soluble (medium amino acids) and insoluble (medium protein) were measured using a Beckmann LS6000 SE scintillation counter. Protein degradation was expressed as the percentage protein degraded over either a 6 hour or a 24 hour period and was equal to 100 times the radioactivity in the medium amino acids divided by the radioactivity in the medium amino acids plus medium protein plus cell protein.

Statistical analysis

Values are presented as means \pm SE. Mean values were compared by Student's t-test or analysis of variance with Fisher's least-significant difference method for comparing groups.

RESULTS

Effects of serum on protein degradation

Degradation of long-lived proteins was measured over 96 hours in the media containing 10% horse serum. A semi-log plot of radioactivity remaining in cells versus time was linear (Figure 2.1.). Supplementation of DMEM with 10% horse serum resulted in a decrease of 19.7% in the rate of protein degradation during a 24 hour measurement period ($P < 0.01$) (Figure 2.2). Fetal bovine serum also inhibited (Figure 2.3.) protein degradation (8.1%, $P < 0.05$) as compared to control cultures incubated in DMEM. The ability of horse serum to inhibit proteolysis was more potent than that of fetal bovine serum. Effects of Insulin-like growth factor-I on protein degradation

Figure 2.4. shows the effect of 200 ng IGF-I/ml media on protein degradation in L8 myotubes. Treatment of L8 myotubes with human recombinant IGF-I reduced protein degradation with a 14 % (Figure 2.4; $P < 0.01$) inhibition occurring at 6 hours of incubation and 26.4 % inhibition ($P < 0.001$) being reached at 24 hours of incubation. The percentage inhibition increased as the measurement period is increased.

Effects of Thyroid hormone on protein degradation

The dose response of L8 myotubes to triiodo-thyronine (T_3) is shown in Figure 2.5. T_3 (0.1 μ M) did not affect protein degradation. At higher

concentrations, T_3 retarded ($P < 0.05$) the rate of protein degradation (Figure 2.5.). The addition of 1 μM and 10 μM T_3 in the medium decreased ($P < 0.05$) proteolysis by 6.0% and 7.7% respectively.

Effects of glucocorticoids on protein degradation

Glucocorticoids have profound effects on muscle protein metabolism and their excess is known to enhance muscle protein degradation in vivo. Figure 2.6. shows the effects of various concentrations of synthetic glucocorticoid dexamethasone on proteolysis. The addition of 1 μM dexamethasone to L8 myotube cultures during a 6 hour measurement period produced a slight stimulus ($P < 0.01$) in protein degradation. The maximal responses occurred between 10 and 1000 nM dexamethasone. Dexamethasone at 100 nM stimulated protein degradation (14.1 %; $P < 0.01$) as compared to control cells incubated in DMEM. Extension of the measurement period from 6 to 24 hours did not increase the effect (Figure 2.7.). When the measurement period was 24 hours, 1 μM dexamethasone gave rise to a smaller stimulus of protein breakdown (8.0 %; $P < 0.01$).

To further investigate the stimulation of proteolysis by dexamethasone, we examined a effects of RU38486 on glucocorticoid-stimulated protein degradation. RU38486 is a novel anti-glucocorticoid agent that effectively blocks glucocorticoid receptors without exhibiting agonist activity, even at high concentrations. Addition of RU38486 to the media containing 1 μM dexamethasone blocked the dexamethasone induced stimulation of proteolysis. A complete block was observed between 1 μM

and 10 μ M RU38486. RU38486 alone, even at high concentration (10 μ M) did not affect ($P > 0.05$) proteolysis (Figure 2.8.).

Effects of TPA on protein degradation

The tumor-promoting phorbol ester, 12-O-tetradecanoyl phorbol-13-acetate (TPA), which is an activator of protein kinase C (PKC), has been implicated in the alteration of protein synthesis and degradation in cultured muscle cells (West and Holtzer, 1982). The activities of protein kinase C, the target protein of TPA, have a close relationship with muscle development (Moraczewski et al., 1990), growth and hypertrophy (Richter and Nielsen, 1991). In order to study the effects of TPA on proteolysis, L8 myotubes were treated with 600 ng/ml TPA for either 24 h or 48 h. Addition of TPA into the experimental media produced a slight stimulation ($P < 0.01$) of proteolysis (Figure 2.9.). Protein degradation was increased ($P < 0.01$) by 5.83% and 4.31% during 24 h and 48 h incubations, respectively.

DISCUSSION

Regulation of proteolysis by hormones has been studied by several *in vivo* (Mays et al, 1991) and *in vitro*. *In vitro* techniques include incubated muscle, perfused muscle and cultured muscle cells (Kettelhut et al., 1988; Gulve and Dice, 1989). The efficient reutilization of labeled amino acids and coexistence of other hormones *in vivo* make it difficult to obtain meaningful results from *in vivo* studies. Alternatively, an incubated small muscle strip can be used for estimation of protein degradation by measuring the amount of tyrosine or phenylalanine releases in the presence of protein synthesis inhibitor, cycloheximide (Angeras and Hasselgren, 1987; Furuno et al., 1990; Hasselgren et al., 1990). However, even muscle strips develop an anoxic core because of inefficient diffusion of oxygen into muscle (Maltin and Harris, 1985). Normally, these incubated muscles are in catabolic state (Goldspink et al., 1983). Viability of such preparations is doubtful. The other *in vitro* technique used to measure protein degradation is a perfused muscle method (Kayali et al., 1987; Smith et al., 1990). Perfused muscles are more viable than incubated muscle; however, this method requires more elaborate equipment and greater surgical skill. Also, amino acids from skin, connective tissue, and bone that remain attached in the preparation contribute to the results. Recently, cultured muscle cells have been used to study direct effect of hormones on protein degradation (Roeder, et al., 1986; Roeder et al., 1988; Gulve and Dice., 1989). Major advantages of cultured myotubes are the accurate measurement of protein degradation and the

absence of contaminating non-muscle tissues or cells in preparations. In this study, we examined the effect of various hormones on protein degradation in L8 myotubes.

Horse and fetal bovine sera inhibited proteolysis of L8 myotubes. However, horse serum was more effective in inhibiting proteolysis than fetal bovine serum. A possible explanation for this difference in inhibition of protein degradation is likely due to the difference in the content of IGF-I, which is a potent inhibitor to proteolysis, in horse versus fetal calf serum. A higher concentration of IGF-I is present in adult serum than in fetal serum (Hall and Sara, 1983; Baxter, 1988). Proteolysis in L8 myotubes was effectively inhibited by a physiological concentration (200ng/ml) of IGF-I (Asakawa et al., 1992). These results are comparable with the findings by others in various mammalian cells, such as porcine (Hembree et al., 1991), ovine (Harper et al., 1987; Roe et al., 1989), L6 (Roeder et al., 1988) and L8 (Gulve and Dice, 1989) muscle cells.

Thyroid hormone has been known to participate in a regulation of muscle protein turnover (Kettelhut, 1988). Physiological levels of triiodothyronine (T_3) or thyroxine (T_4) induced normal muscle growth and increased both protein synthesis and protein degradation (Brown et al., 1981). Higher levels of these hormones elevated the rates of protein degradation further but did not increase protein synthesis rates beyond those observed with normal growth-promoting levels (Griffin and Goldberg, 1977). Thus, the muscle weakness and wasting observed in hyperthyroidism (Carter

et al., 1980; Brown and Millward, 1983) and thyrotoxicosis (Morrison et al., 1988) seems to result from increased protein degradation. In this study, L8 myotubes were treated with T_3 to assess the direct influence of the hormones on muscle protein turnover. Unlike the findings in vivo, treatment of T_3 did not increase the rate of proteolysis, even at very high concentrations (10 μ M). Similar findings have been observed in L6 myoblasts (Ballard and Francis, 1983). The lack of any proteolytic response to T_3 does not reflect an insensitivity of rat myotubes to thyroid hormones. High affinity T_3 receptors were identified in differentiated L6 myotubes (Koenig and Smith, 1985). Induction of Na^+, K^+ -ATPases and Ca^{++} -ATPases by thyroid hormone were observed in rat primary (Brodie and Sampson, 1988) and L6 muscle cell (Muller et al., 1991) cultures respectively. Our results with T_3 suggest that T_3 may act on muscle indirectly, probably through altering concentrations of other growth factors (Davis et al., 1987; Westermarck et al., 1988; Ikeda et al., 1988) or other hormones (Rousseau et al., 1987; Hayshi et al., 1986).

Administration of glucocorticoids to animal induced catabolic responses in muscle. Protein synthesis (Rannels et al., 1978; McGrath and Goldspink, 1982) was decreased and protein degradation (Kayali et al., 1987; Ohtsuka et al., 1992) was increased. Like in vivo studies, the addition of dexamethasone to L8 myotubes stimulated proteolysis. Similar stimulation of protein degradation was observed in L6 myoblast (Ballard and Francis, 1982) and myotubes (Roeder et al., 1986). A direct effect of

dexamethasone on protein degradation was confirmed by the finding that RU38486 blocked dexamethasone-stimulated proteolysis. RU38486 is a antiglucocorticoid that fully antagonizes acute and chronic hormonal effects in vivo and in vitro without showing agonist activity even in large doses (Mougilewsky and Philibert, 1984; Ulmann et al., 1990). These results provide evidence that the binding of glucocorticoid to its intracellular receptors are involved in glucocorticoid-induced muscle atrophy. However, events which follow binding of glucocorticoid to its receptor which bring about changes in proteolysis have not been identified.

Phorbol ester has been known to disassemble myofibrils of differentiated avian muscle cells by degrading myofibrillar proteins but not cytoskeletal proteins (Croop et al., 1980; Lin et al., 1987; Lin et al., 1989). In our study, TPA slightly increased ($P < 0.01$) the rates of protein degradation in L8 myotubes. These effects of TPA on L8 myotubes parallel the actions of this phorbol ester in other differentiated muscle cells (West and Holtzer, 1982; Lin et al., 1987; Lin et al., 1989). TPA, an analogue of diacylglycerol (DAG), binds to and alters protein kinase C (PKC) activity. Long term TPA exposure caused down-regulation of protein kinase C (Kishimoto et al., 1989). In our studies PKC α , δ and ζ were found in muscle. PKCs, except PKC- ζ , disappeared during a 6 hour incubation with TPA. Thus, during most of our protein degradation measurement period, PKCs are absent in L8 myotubes. Goodman (1987) reported that degradation of myofibrillar but not total proteins was inhibited by short-term

exposure (2 hour) to TPA. However, the physiological relevance of this finding is not clear. The study of the involvement of PKC in the regulation of protein turnover is at a very early stage and much work needs to be done.

In conclusion, the present study has shown that protein degradation in rat L8 myotubes was inhibited by physiological concentrations of insulin-like growth factor-I as well as by horse and fetal bovine serum. Protein degradation was stimulated by dexamethasone and TPA. T_3 , known to regulate proteolysis in vivo, did not alter rates of protein breakdown in vitro. L8 myotube culture provides a valuable model for investigating the mechanism by which these hormones directly regulate protein turnover in skeletal muscle.

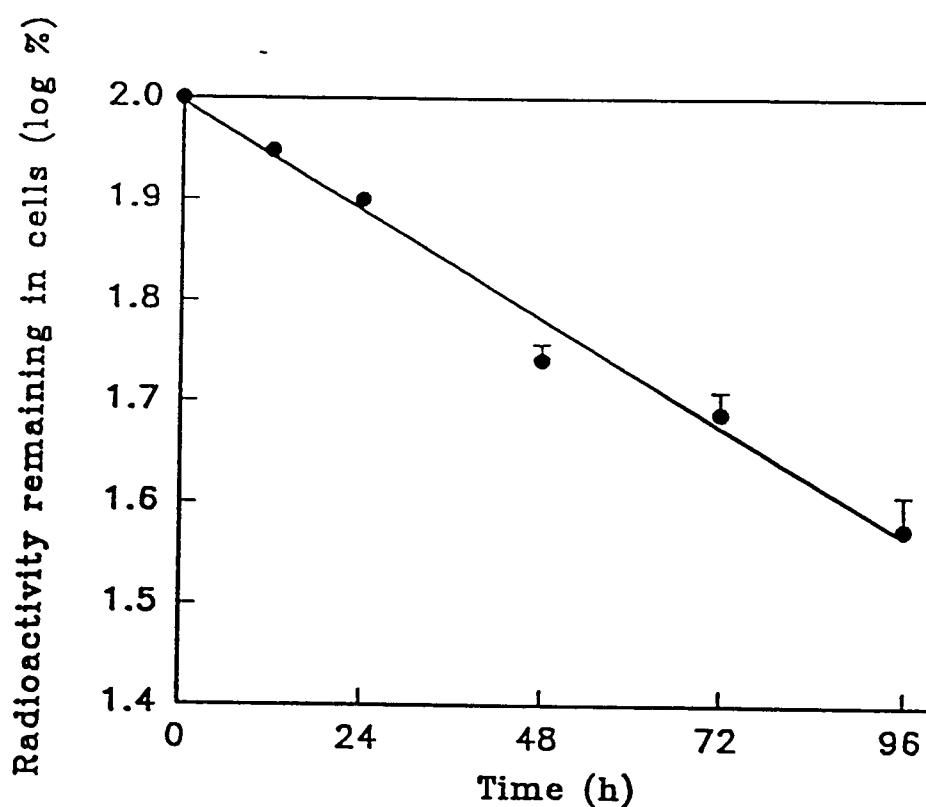


Figure 2.1. Degradation of long-lived proteins in the presence of horse serum. L8 myotubes were labeled with [^3H] tyrosine for 2 days as described in 'materials and methods'. Degradation was assessed during 96 hour incubation with DMEM supplemented with 2.0 mM unlabeled tyrosine and 10% horse serum. Medium was changed after 48 hours. Each data point represents the average of three cultures. Error bar indicates \pm SD; no error bar indicates that error lies within the point. Best line through the points was calculated using linear regression.

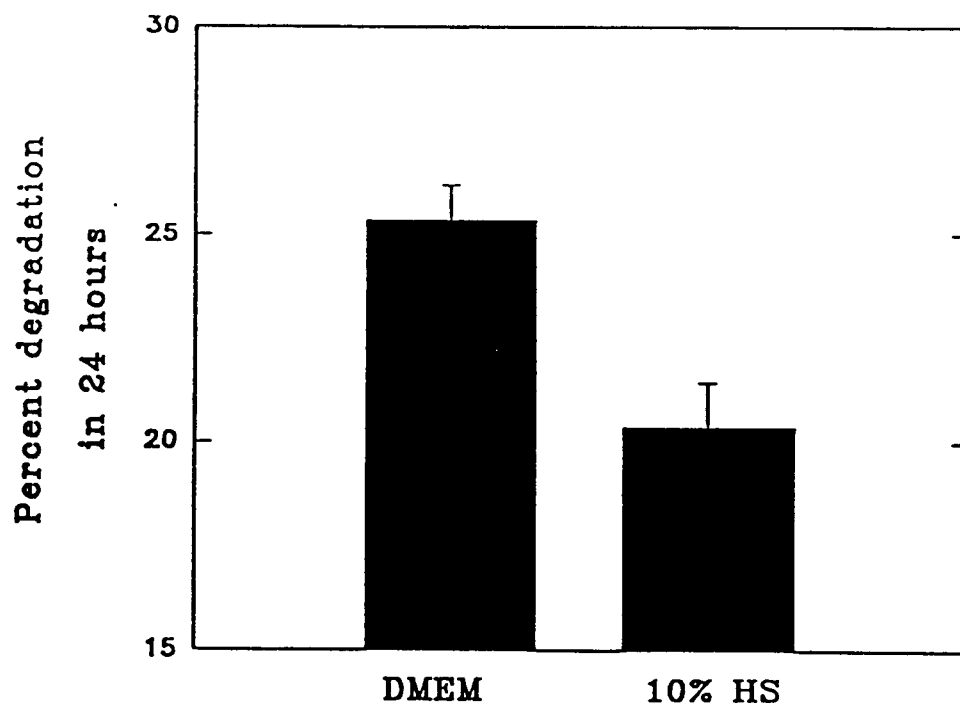


Figure 2.2. Effects of horse serum on protein degradation in cultured L8 myotubes. L8 myotubes were labeled with 1 $\mu\text{Ci/ml}$ [^3H] tyrosine for 2 days, rinsed and chased for 2 hours in DMEM + 2 mM tyrosine + 10% FCS as described in 'materials and methods'. Cells were rinsed and incubated for 24 hours in degradation medium. Degradation medium consisted of DMEM + 2mM tyrosine supplemented with or without 10% horse serum (HS). Protein degradation is expressed as the percentage (mean + SD) of labeled protein broken down in 24 hours ($n=3$). Error bar indicates + SD; * $P<0.01$ vs. DMEM.

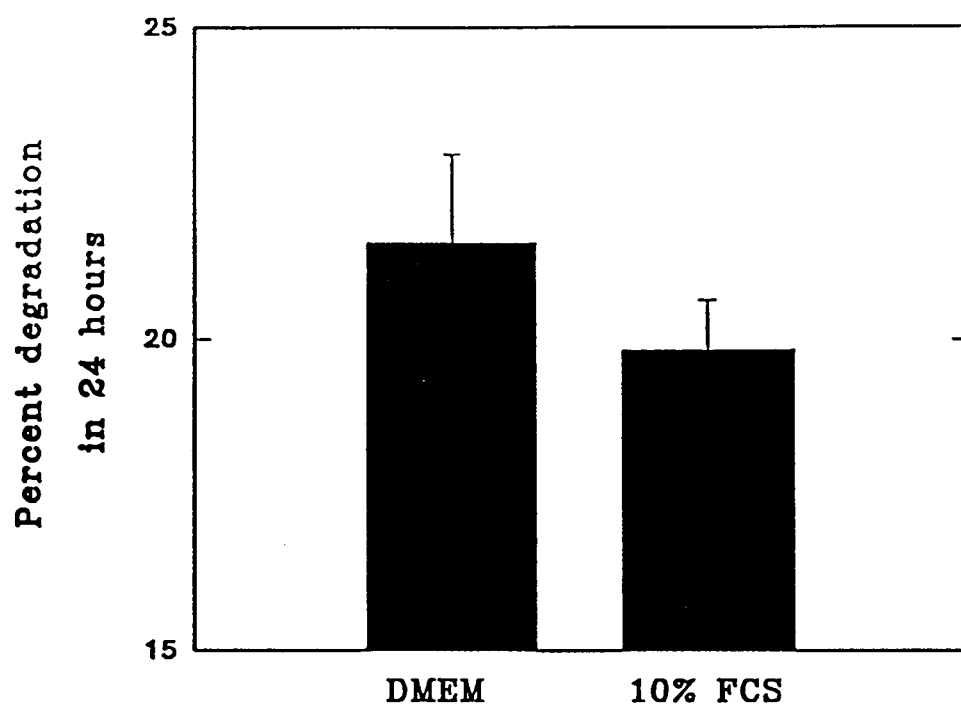


Figure 2.3. Effects of fetal calf serum on protein degradation in cultured L8 myotubes. L8 myotubes were labeled with $1 \mu\text{Ci/ml}$ [^3H] tyrosine for 2 days, rinsed and chased for 2 hours in DMEM + 2 mM tyrosine + 10% FCS as described in 'materials and methods'. Cells were rinsed and incubated for 24 hours in degradation medium. Degradation medium consisted of DMEM + 2mM tyrosine supplemented with or without 10% fetal calf serum (FCS). Protein degradation is expressed as the percentage (mean + SD) of labeled protein broken down in 24 hours ($n = 6$). Error bar indicates + SD; * $P < 0.05$ vs. DMEM.

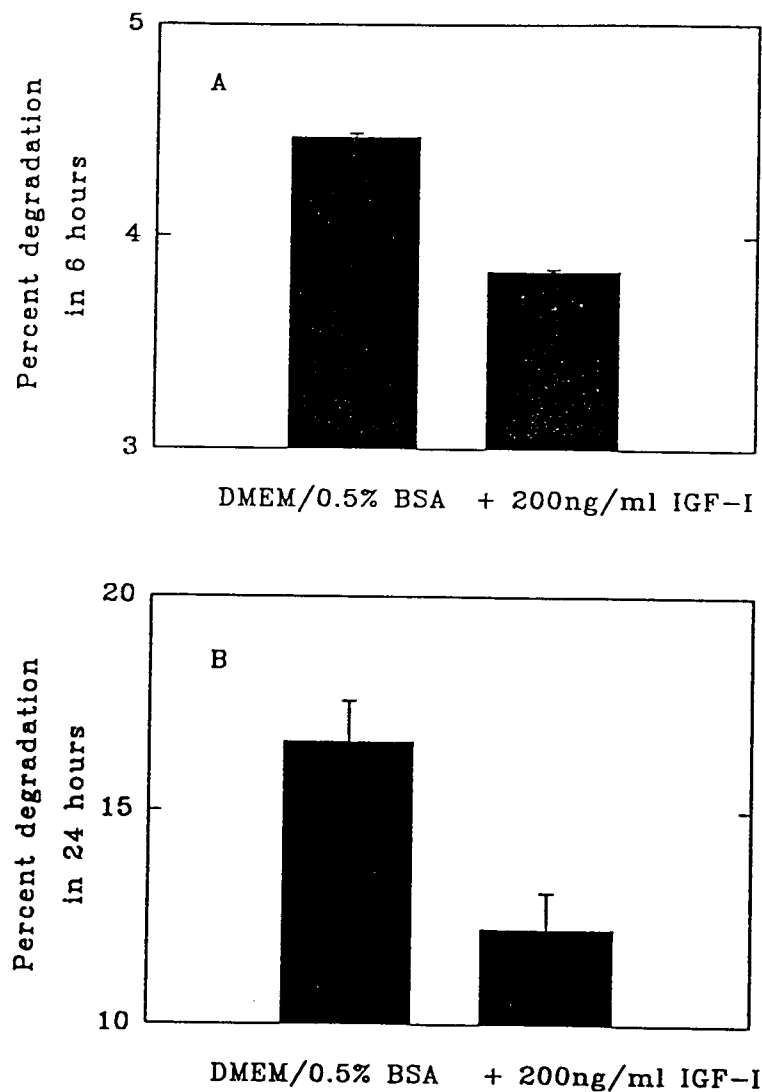


Figure 2.4. Effects of IGF-I on protein degradation in cultured L8 myotubes. L8 myotubes were labeled with 1 μ Ci/ml [3 H] tyrosine for 2 days, rinsed and chased for 2 hours in DMEM + 2 mM tyrosine + 10% FCS as described in 'materials and methods'. Cells were rinsed and incubated for 6 hours (A) or 24 hours (B) in degradation medium. Degradation medium consisted of DMEM + 2mM tyrosine + 0.5 mg BSA/ml with or without 200 ng/ml human recombinant IGF-I. Protein degradation is expressed as the percentage of labeled protein broken down in 6 or 24 hours. Each bar represents the mean + SEM of 3 experiments (6 replications in each experiments); error bar indicates + SEM. *P<0.01, **P<0.001 denotes differences from control.

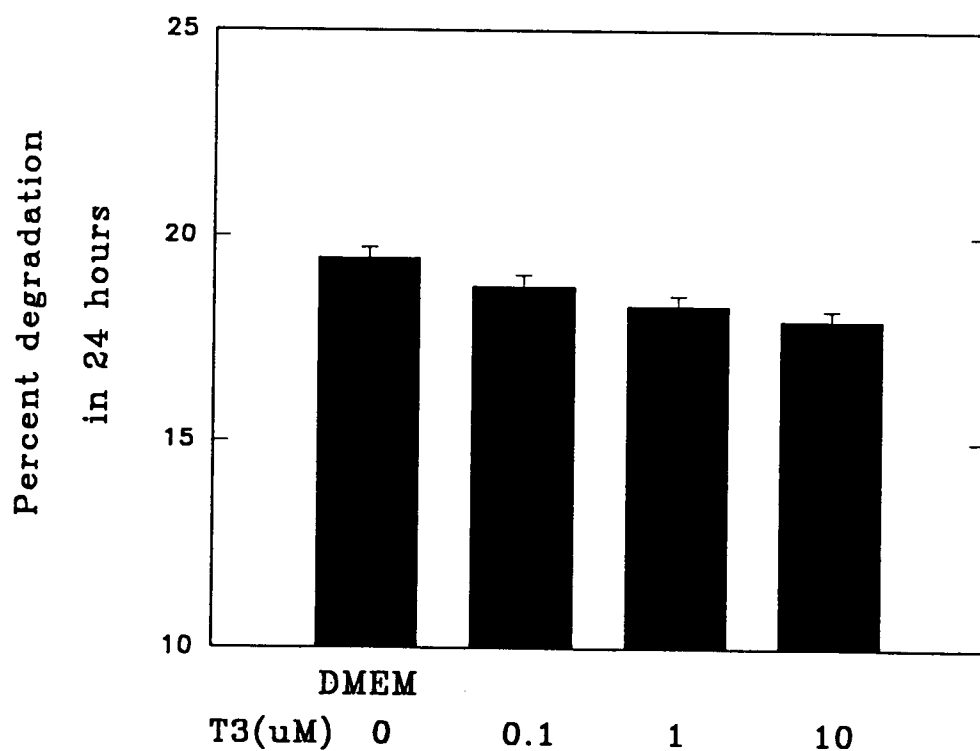


Figure 2.5. Effects of triiodothyronine (T₃) on protein degradation in cultured L8 myotubes. L8 myotubes were labeled with 1 μ Ci/ml [³H] tyrosine for 2 days, rinsed and chased for 2 hours in DMEM + 2 mM tyrosine + 10% FCS as described in 'materials and methods'. Cells were rinsed and incubated for 24 hours in degradation medium, which consisted of DMEM + 2mM non-radioactive tyrosine with addition as indicated. Protein degradation is expressed as the percentage of labeled protein broken down in 24 hours. Each bar represents the mean + SEM of 8 experiments (6 replications in each experiments); error bar indicates + SEM. *P<0.05 denotes differences from control.

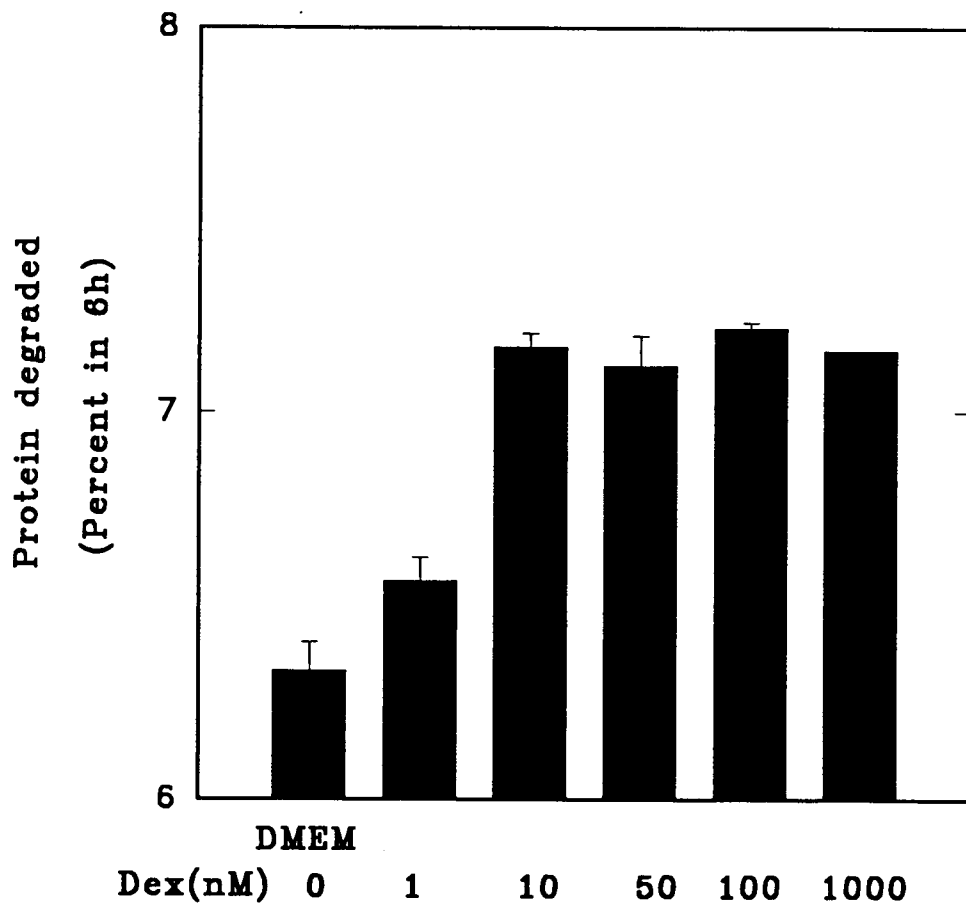


Figure 2.6. Dose response of protein degradation to dexamethasone. L8 myotubes were labeled with 1 $\mu\text{Ci/ml}$ [^3H] tyrosine for 2 days, rinsed and chased for 2 hours in DMEM + 2 mM tyrosine + 10% FCS as described in 'materials and methods'. Cells were rinsed and incubated for 6 hours in degradation medium, which consisted of DMEM + 2mM non-radioactive tyrosine with addition as indicated. Protein degradation is expressed as the percentage of labeled protein broken down in 6 hours. Each bar represents the mean + SEM of 2 experiments (6 replications in each experiments); error bar indicates + SEM. * $P < 0.01$ denotes differences from control.

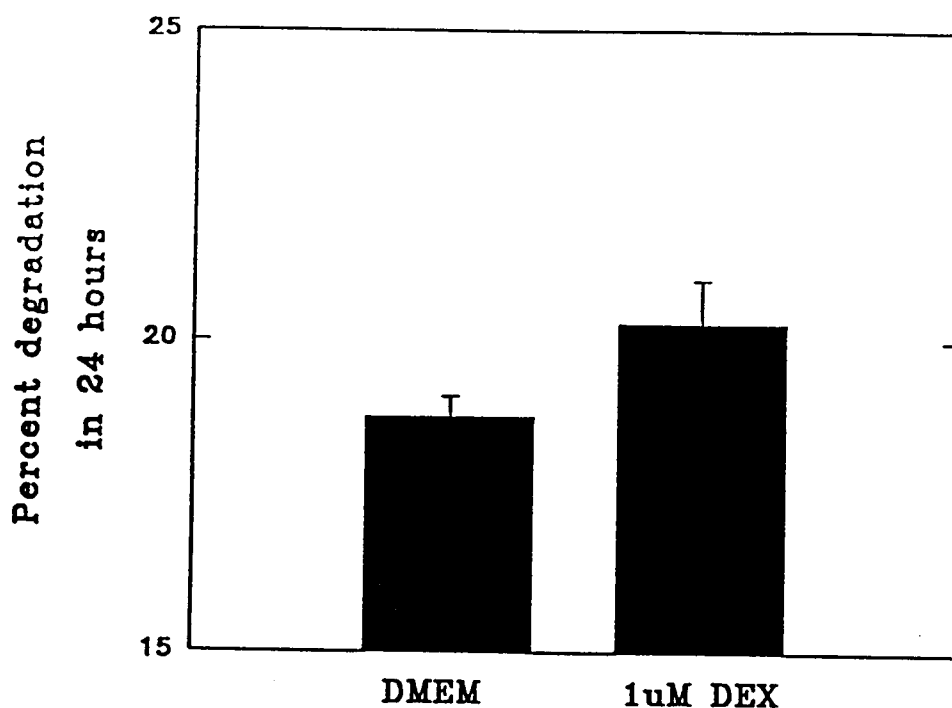


Figure 2.7. Effects of dexamethasone on proteolysis in cultured L8 myotubes. L8 myotubes were labeled with 1 $\mu\text{Ci/ml}$ [^3H] tyrosine for 2 days, rinsed and chased for 2 hours in DMEM + 2 mM tyrosine + 10% FCS as described in 'materials and methods'. Cells were rinsed and incubated for 24 hours in degradation medium. Degradation medium consisted of DMEM + 2mM non-radioactive tyrosine with or without 1 μM dexamethasone. Protein degradation is expressed as the percentage of labeled protein broken down in 24 hours. Each bar represents the mean + SEM of 5 experiments (6 replications in each experiments); error bar indicates + SEM. * $P < 0.01$ denotes differences from control.

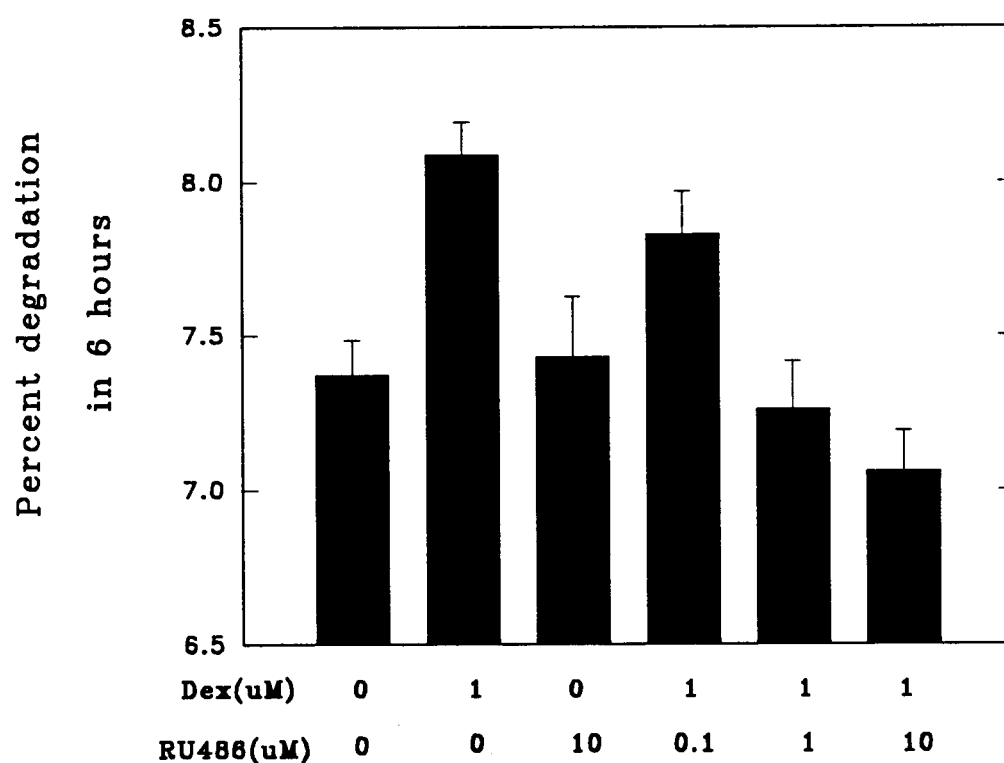


Figure 2.8. Dose dependent inhibition of dexamethasone-stimulated proteolysis by RU38486. L8 myotubes were labeled with 1 μ Ci/ml [3 H] tyrosine for 2 days, rinsed and chased for 2 hours in DMEM + 2 mM tyrosine + 10% FCS as described in 'materials and methods'. Cells were rinsed and incubated for 6 hours in degradation medium, which consisted of DMEM + 2mM non-radioactive tyrosine with addition as indicated. Protein degradation is expressed as the percentage of labeled protein broken down in 6 hours. Each bar represents the mean + SEM (n = 6). Error bar indicates + SEM. *P<0.01 denotes differences from control.

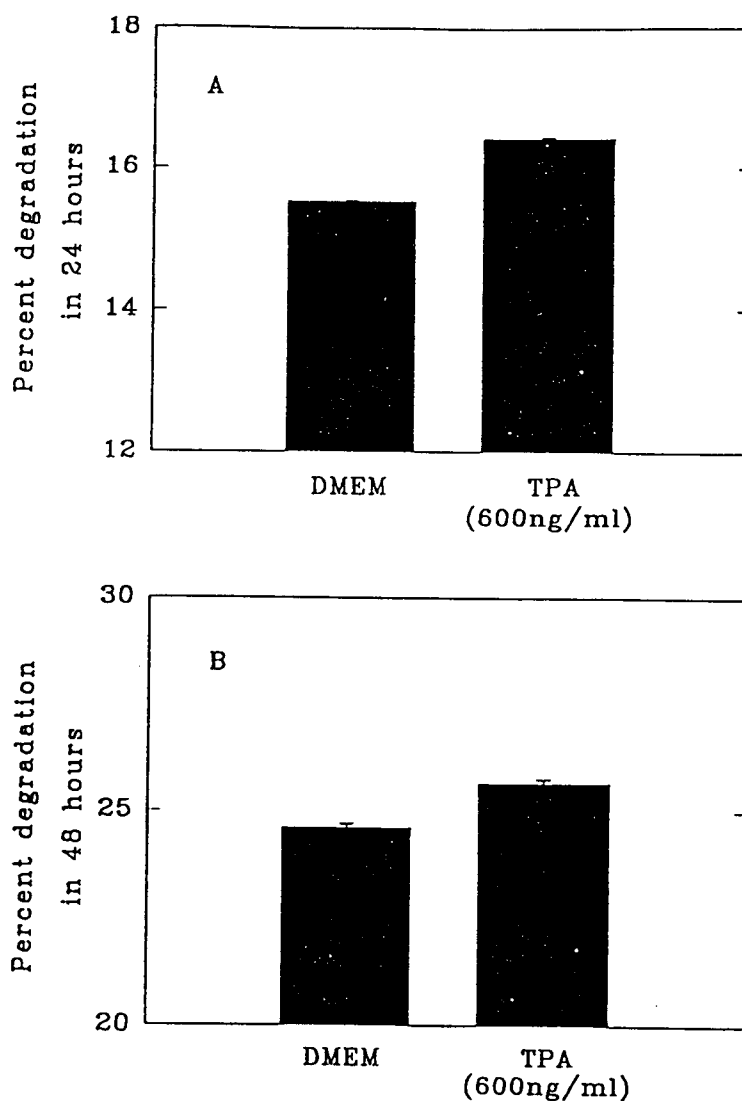


Figure 2.9. Effects of TPA on protein degradation in cultured L8 myotubes. L8 myotubes were labeled with 1 $\mu\text{Ci/ml}$ [^3H] tyrosine for 2 days, rinsed and chased for 2 hours in DMEM + 2 mM tyrosine + 10% FCS as described in 'materials and methods'. Cells were rinsed and incubated for 24 hours (A) or 48 hours (B) in degradation medium. Degradation medium consisted of DMEM + 2mM non-radioactive tyrosine with or without 600 ng/ml TPA. Protein degradation is expressed as the percentage of labeled protein broken down in 24 hours or 48 hours. Each bar represents the mean + SEM of 2 experiments (6 replications in each experiments). Error bar indicates + SEM. no error bar indicates that error lies within the point. * $P < 0.01$ denotes differences from control.

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CHAPTER III

EFFECTS OF INSULIN-LIKE GROWTH FACTOR-I (IGF-I) ON PROTEASE GENE EXPRESSION IN L8 MYOTUBES

ABSTRACT

Insulin-like growth factor-I (IGF-I) has pleiotypic actions on cellular metabolism in various tissues. IGF-I decreases protein degradation in muscle, but the mechanism by which IGF-I inhibits proteolysis is not known. To study the regulation of proteolysis by IGF-I, we evaluated effects of IGF-I on protease mRNA concentration by Northern blot analysis. L8 myoblasts were grown in 10 cm culture dishes, and allowed to differentiated into multinucleated myotubes. The fully differentiated L8 myotubes were then washed once with Hank's balanced salt solution (HBSS) and were incubated for 6 h or 24 h in DMEM containing 0.1% BSA in the presence or absence of 200 ng/ml human recombinant IGF-I. After appropriate incubation, cells were washed with ice-cold phosphate buffered saline (PBS) and lysed with guanidine isothyonate buffer for total RNA extraction. The isolated total RNA was subjected to 1.2% agarose gel containing 2.2 M formaldehyde, transferred to nylon membraned and hybridized with ³²P-labeled cDNA probes. IGF-I had no effect ($P > 0.05$) on cathepsin B gene expression but slightly increased ($P < 0.05$) m-calpain mRNA. Cathepsin D and proteasome mRNA were reduced ($P < 0.05$) by IGF-I treatment. The changes in cathepsin D and proteasome mRNA levels are parallel to IGF-I-dependent alterations in

proteolysis. These observations suggest that effects of IGF-I on protein degradation, at least in part, may be mediated by the down-regulation of cathepsin D and proteasome mRNA.

INTRODUCTION

Insulin-like growth factor-I (IGF-I), also called as somatomedin C, is a peptide hormone (Froesch et al., 1985; Baxter, 1987). IGF-I, synthesized and secreted mainly from liver, mediates the growth-promoting actions of growth hormone (GH). IGF-I shows pleiotypic anabolic effects on skeletal muscle cells as it does on many other types of cells (Florini, 1987). These include stimulation of amino acid uptake, protein synthesis, glucose uptake, DNA synthesis, RNA synthesis, cell proliferation and cell differentiation. It is also well established that IGF-I inhibits protein degradation in muscle (Harper et al., 1987; Roeder et al., 1988; Asaka et al., 1992; Tomas et al., 1992). Although, the effects of IGF-I on protein muscle protein turnover have been extensively characterized (Kettelhut et al., 1988; Tomas et al., 1991), the molecular and cellular mechanisms by which IGF-I inhibits muscle protein degradation remain unknown.

Several proteolytic systems have been found in skeletal muscle (Goll et al., 1989). As in other cells, lysosomal proteases, like cathepsins B, L, H and D, are present in muscle. Among these, cathepsin B and D have been regarded as the major proteolytic activity in the lysosome (Goldspink, 1991). Muscle also possesses calcium-dependent neutral proteases (calpains). Circumstantial evidences indicated that these neutral proteases may play an important role in initiating myofibrillar protein degradation (Goll et al., 1992). Recently, muscle cells have been shown to contain the ATP-dependent proteases (the proteasome or multicatalytic proteases). However, their role

in muscle protein degradation has not been identified.

In vitro culture of differentiated muscle cell offers an excellent tool to investigate the specific effects of hormones or growth factors on protein degradation in muscle without interference by the changes in the concentration of other hormones. In the present study, we examined the effects of IGF-I on gene expression of major proteolytic systems (cathepsin B, cathepsin D, proteasome and m-calpain) of skeletal muscle cells using L8 myotube culture.

MATERIALS AND METHODS

Materials

[α - ^{32}P] dCTP (6,000 Ci/mmol) was from New England Nuclear (Boston, MA). S & S Nytran⁺ (0.45 μm) membrane was from Schleicher & Schuell (Keene, NH). Denhardt's solution (100 x) was from 5 prime-3 prime Inc. (Boulder, CO). QIAEX agarose gel extraction kit was from QIAGEN Inc. (Chatsworth, CA). Random-primed DNA labeling kit was from USB (Cleveland, OH). Formamide and formaldehyde were from Mallinckrodt (Paris, KY). Safe II scintillation cocktail was from Research Products International Co. (Mount Prospect, IL). Fetal bovine (FBS) and horse (HS) sera were from Hyclone (Logan, UT). Dulbecco's modified Eagle's medium, penicillin/streptomycin solution and trypsin solution were from GIBCO (Grand Island, NY). Human recombinant IGF-I and Quick Spin G-50 Sephadex column were from Boehringer Mannheim (Indianapolis, IN). Cytosine- β -D-arabinofuranoside (Ara-C), dextran sulfate, sonicated, denatured salmon testes DNA, ethidium bromide and bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO). Culture dishes were from Corning (Corning, New York).

Cell Culture

L8 rat skeletal muscle cells were obtained from American Type Culture Collection (Rockville, MD). Cells, stored in liquid nitrogen, were thawed and maintained by repeated subculturing at low density on 10 cm culture dishes.

Cells were grown in Dulbecco's-modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 100 units of penicillin/ml, 100 μ g of streptomycin/ml, 44 mM NaHCO₃, 110 μ g of sodium pyruvate/ml in humidified atmosphere of 5% CO₂ and 95% air at 37 °C. The medium was changed every 2 days. Cells (2.5×10^4 cells/cm²) were grown in presence of 10% FCS until they reached confluency. At this time the medium was replaced by DMEM containing 2% FCS for induction of differentiation. Approximately 3 days later, when myotube formation was observed, the cells were treated with 10 μ M cytosine arabinoside for 48 hours to remove any remaining dividing myoblasts. Microscopic examination was used to monitor differentiation. To study the effect of IGF-1 on protease gene expression, fully-differentiated L8 myotubes were treated for 6 h or 24 h with DMEM containing 0.5 % BSA in the presence or absence of 200 ng/ml IGF-I. After the appropriate incubation period, cells were harvested for isolation of RNA.

Extraction of total RNA

Extraction of total RNA has been described previously (Chomczynski and Sacchi, 1987). Briefly, myotube cultures grown in 10-cm diameter dishes were washed three times with ice-cold phosphate buffered saline (PBS) and lysed directly on the dishes using 2 ml of Solution A (4 M guanidium isothionate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 10 mM β -mercaptoethanol). The monolayer was scraped with a rubber policeman to

ensure that all cells were released from the dishes. The lysate was transferred to a 15 ml polypropylene tube. To this, 0.2 ml of 2 M sodium acetate (pH 4.0), 2 ml of phenol and 0.4 ml of a chloroform-isoamyl alcohol mixture (49:1) were added sequentially. The lysate was mixed by inversion after each addition. After brief vortexing, the mixture was kept on ice for 15 min and was separated by centrifugation (12,000 g) for 30 min at 4°C. The upper aqueous phase was transferred to a new tube and the same volume of ice-cold isopropanol was added. The samples were mixed and stored at -20°C overnight. RNA was collected by centrifugation (12,000 g, 20 min at 4°C). The supernatant was discarded and the RNA pellet was resuspended in 0.4 ml of solution A. One volume of isopropanol was added, and the mixture was stored at -20°C overnight. The RNA pellet was recovered by centrifugation at 12,000 g for 15 min at 4°C, washed twice with 70% ethanol and dried under vacuum. The dried RNA pellet was then dissolved in diethylpyrocarbonate (DEPC)-treated water and was quantitated by spectrophotometry at a wavelength of 260 nm.

cDNA probes

pC2- α , which encodes rat liver proteasome (30.8 kDa C2 subunit; Fujiwara et al., 1989) was provided by Dr. K. Tanaka (Institute for Enzyme Research, University of Tokushima, Japan). pHcPDEco1.1, which encodes human cathepsin D, was provided by Dr. John Chirgwin (University of Texas Health Science Center; Faust et al., 1985). prCB5, which encodes rat liver

cathepsin B, was provided by Dr. S. J. Chan (Howard Hughes Medical Institute, University of Chicago; Segundo et al., 1985). pT1, which encodes chicken α -tubulin, was provided by Dr. Donald W. Cleveland (Johns Hopkins University; Valenzuela et al., 1981). Rat m-calpain cDNA probe (0.9 kb), which was subcloned in pUC19, was provided by Dr. John S. Elce (Queen's University, Canada). Plasmids were amplified and recovered using standard techniques (Maniatis et al., 1982). A cDNA probe encoding proteasome (1000 bp HindIII/PvuII fragment of pC2- α), cathepsin D (1000 bp EcoRI fragment of pHcPDEco1.1), cathepsin B (600 bp HindIII fragment of prCB5) and α -tubulin (1,500 bp HindIII fragment of pT1) and m-calpain (900 bp EcoRI fragment) were prepared as previously described (Ou and Forsberg, 1991; Ilian and Forsberg, 1992). After restriction enzyme digestion, cDNA fragments were separated by electrophoresis and were recovered by electroelution onto a dialysis membrane or by QIAEX agarose gel extraction kit. cDNA fragments (25ng) were labeled with [α - 32 P] dCTP (6000 Ci/mmol) using a random-prime kit. A labeled cDNA probe was purified using Quick Spin G-50 Sephadex column (Boehringer Mannheim).

Northern blot hybridization

RNA samples (15 ug) were denatured at 65°C for 15 min, applied to a 1.2% agarose gel containing 2.2 M formaldehyde, then electrophoresed at 30 V for 12 h. The quality and the relative abundance of RNA/lane were judged by comparing the ethidium bromide staining of the ribosomal bands. RNA

was transferred to Nytran⁺ nylon membrane and immobilized by baking at 80°C for 40 min. Membranes were incubated at 43 °C for overnight in prehybridization buffer (5 x SSPE, 0.2% SDS, 5 x Denhardt's solution, 100 ug/ml sonicated salmon testes DNA (Sigma) and 50% formamide). Following prehybridization, the membranes were hybridized for 36 hours at 43 °C in prehybridization buffer containing 10% dextran sulfate and [³²P] cDNA (1,000,000 cpm/ml). After hybridization, membranes were washed three times with 1 x SSPE and 0.1% SDS for 15 min at room temperature and were washed two times with 0.1 x SSPE and 0.1% SDS for 15 min at 50 °C. Membranes were exposed to Kodak X-Omat or Fuji RX film with intensifying screens for 6-12 hours at -80 °C. Quantitation of exposures on autoradiographic films was performed using a Bio-Rad model 1650 scanning densitometer and a Hoefer GS-350H scanning program. To remove probe from blot for rehybridization, membrane was washed in 6 x SSPE containing 50% formamide at 65 °C for 30 minutes and then rinsed in 2 x SSPE. After removal, the membrane was prehybridized and hybridized as indicated above.

RESULTS

In previous studies we determined that IGF-I reduced proteolysis in muscle cells. In this study we examined effects of IGF-I on protease gene expression. Effects of 200 ug/ml IGF-I on cathepsin D expression were examined by Northern blotting (Figure 3.1.) and scanning densitometer data are shown in Figure 3.5. Steady-state levels of cathepsin D mRNA gradually decreased during IGF-I treatment. IGF-I caused a 30% reduction of cathepsin D mRNA in 24 hours. However, mRNA encoding another lysosomal protease, cathepsin B, was not altered by IGF-I (Figure 3.2.).

Proteasome (multicatalytic protease) consists of at least 15 subunits of 21-31 kDa. We examined effects of IGF-I on the proteasome gene expression using a cDNA probe for the C2 subunit, which is the largest component of rat proteasome. Like cathepsin D, IGF-I reduced steady-state levels of proteasome gradually (Figure 3.3.). Proteasome mRNA was reduced 27% at 24 hours incubation. To explore the effects of IGF-I treatment on cytoskeletal protein as a control, the same membrane was stripped and rehybridized with the α -tubulin probe. The results are shown in the bottom panel of Figure 3.3. By contrast, the presence of IGF-I in the medium caused a 37% increase in steady-state level of α -tubulin mRNA (Figure 3.6.). We also examined the effects of IGF-I on m-calpain gene expression (Figure 3.4.). Unlike the two other proteolytic systems, m-calpain mRNA levels were up-regulated by IGF-I. IGF-I treatment caused a 30% increase ($P < 0.05$) in m-calpain mRNA (Figure 3.5.).

DISCUSSION

IGF-I has been known to suppress proteolysis in muscle cells (Gulve and Dice, 1989; Hembree et al., 1991). Our own results also indicated that IGF-I inhibits proteolysis (Chapter II). Despite these findings, the mechanisms by which IGF-I inhibits protein degradation are not known. To investigate whether regulation of protease gene expression by IGF-I is involved in this process, we examined effects of IGF-I on steady-state mRNA levels encoding major proteolytic enzymes using Northern blot analysis.

Expression of cathepsin D, which is a lysosomal aspartic protease, was reduced by IGF-I treatment. However, IGF-I had no effect on cathepsin B expression. Cathepsin D and B have been considered to constitute the major proteolytic activity in the lysosome. Goldspink and Lewis (1987) showed that changes in the activities of cathepsin B and D appeared to parallel alterations in proteolysis of muscle. Hence, a decrease in cathepsin D gene expression may be involved in the inhibition of protein degradation by IGF-I.

Proteasome mRNA level also decreased by IGF-I treatment. Proteasome, found in skeletal muscle as in other tissues, seems to degrade proteins by an ATP-dependent manner with or without ubiquitin. However, its role in muscle protein degradation has not been identified. These results suggested that its involvement in IGF-I-dependent control of protein degradation in muscle.

In contrast, m-calpain gene expression was slightly up-regulated. Calpain has implicated in myofibrillar protein degradation. However, its precise role in myofibrillar protein degradation is not clear yet. No change or an increase in m-calpain mRNA was observed in a condition where muscle proteolysis was inhibited (Parr et al., 1992; Alyan, 1991). A possible explanations for these observations is that slightly increased in m-calpain expression may be counteracted by a simultaneous increase in its specific inhibitor, calpastatin as shown by Parr et al., (1991).

In the present study, we showed that changes in cathepsin D and proteasome steady-state mRNA levels are similar in direction and degree to the changes in proteolysis. This suggests that these two proteolytic systems play a role in IGF-I-dependent control of protein degradation.

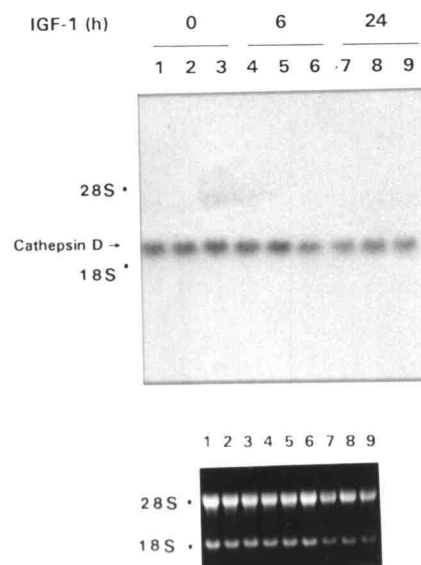


Figure 3.1. Effects of IGF-I (200 ng/ml) on cathepsin D mRNA abundance in L8 myotubes. Total RNA (15 μ g) extracted from cells cultured in triplicate for the indicated times (control; lanes 1,2,3 :6 hr; lanes 4,5,6: 24 hr; lanes 7,8,9) with 200 ng/ml IGF-I were examined by Northern blot analysis (top panel). Relative amounts of RNA loaded in each lane were visualized by staining with ethidium bromide (bottom panel).

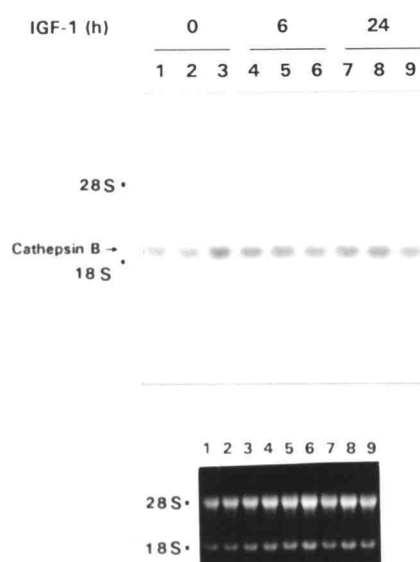


Figure 3.2. Effects of IGF-I (200 ng/ml) on cathepsin B mRNA abundance in L8 myotubes. Total RNA (15 μ g) extracted from cells cultured in triplicate for the indicated times (control; lanes 1,2,3: 6 hr; lanes 4,5,6: 24 hr; lanes 7,8,9) with 200 ng/ml IGF-I were examined by Northern blot analysis (top panel). Relative amounts of RNA loaded in each lane were visualized by staining with ethidium bromide (bottom panel).

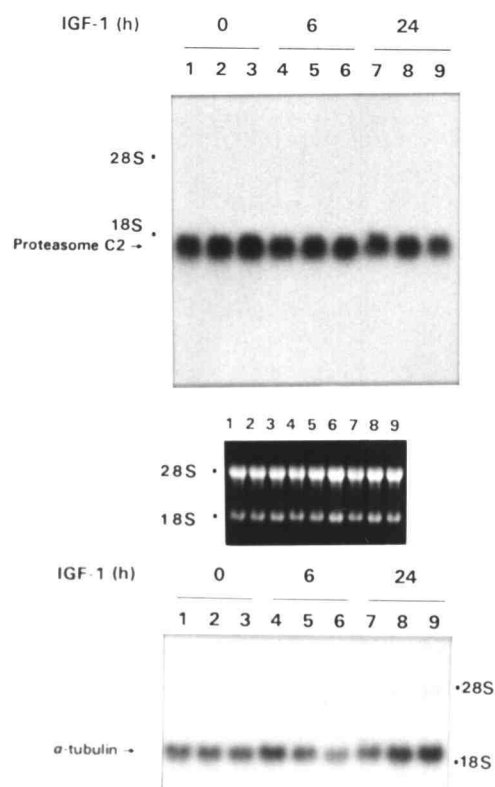


Figure 3.3. Effects of IGF-I (200 ng/ml) on proteasome C2 and α -tubulin mRNA abundance in L8 myotubes. Total RNA (15 μ g) extracted from cells cultured in triplicate for the indicated times (control; lanes 1,2,3: 6 hr; lanes 4,5,6: 24 hr; lanes 7,8,9) with 200 ng/ml IGF-I were examined by Northern blot analysis (top panel). Relative amounts of RNA loaded in each lane were visualized by staining with ethidium bromide (middle panel). After hybridization to a proteasome probe, the filter was stripped and rehybridized with a probe for α -tubulin.

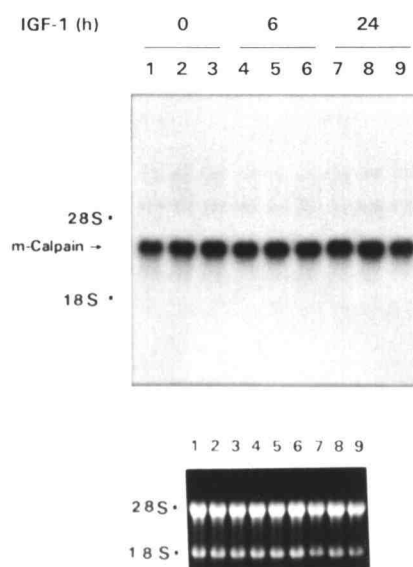


Figure 3.4. Effects of IGF-I (200 ng/ml) on m-calpain mRNA abundance in L8 myotubes. Total RNA (15 μ g) extracted from cells cultured in triplicate for the indicated times (control; lanes 1,2,3: 6 hr; lanes 4,5,6: 24 hr; lanes 7,8,9) with 200 ng/ml IGF-I were examined by Northern blot analysis (top panel). Relative amounts of RNA loaded in each lane were visualized by staining with ethidium bromide.

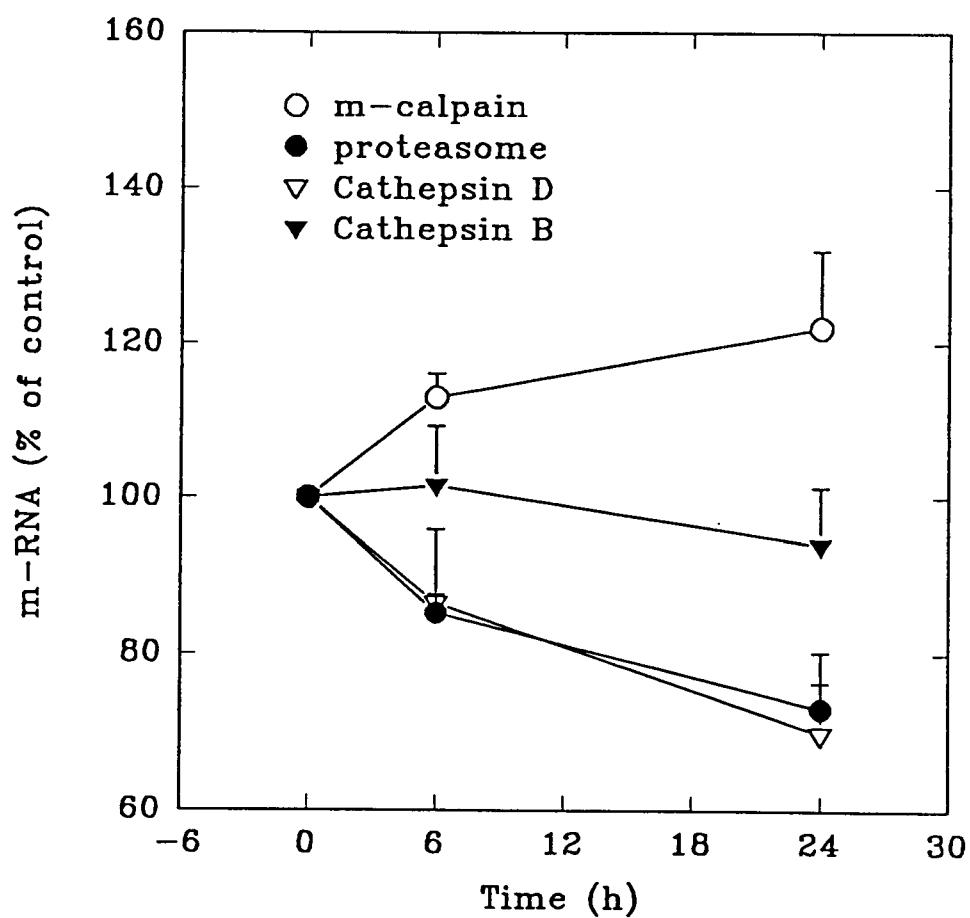


Figure 3.5. Time course of the effects of IGF-I (200 ng/ml) on the abundance of cathepsin D, cathepsin B, proteasome and m-calpain mRNA in L8 myotubes. Autoradiograms shown in Fig. 3.1., 3.2, 3.3, and 3.4 were quantitated by scanning densitometer.

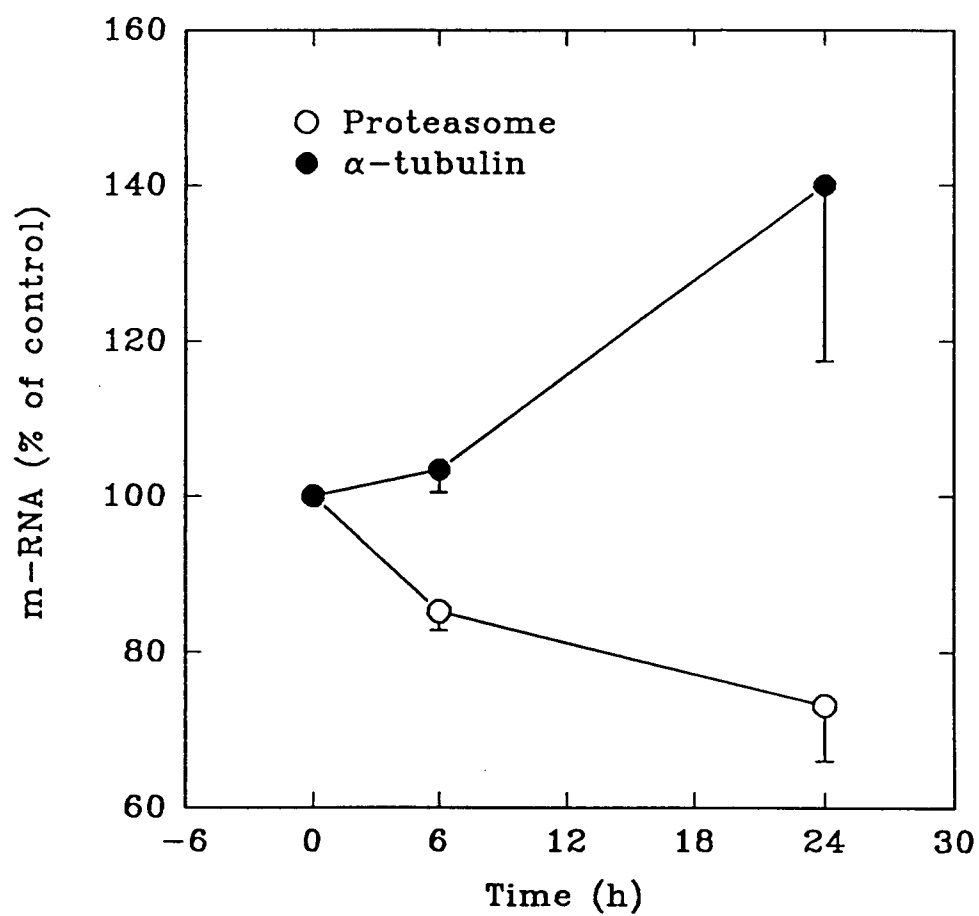


Figure 3.6. Time course of the effects of IGF-I (200 ng/ml) on the abundance of proteasome and α -tubulin mRNA in L8 myotubes. Autoradiogram shown in Fig. 3.3 was quantitated by scanning densitometer.

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CHAPTER IV

EFFECTS OF GLUCOCORTICOIDS ON PROTEASE GENE EXPRESSION IN L8 MYOTUBES

ABSTRACT

Glucocorticoids stimulate protein degradation in skeletal muscle but the molecular mechanisms are not known. We investigated the regulation of protease (cathepsin B, cathepsin D, proteasome and m-calpain) gene expression by dexamethasone in cultured L8 muscle cells. L8 myoblasts were grown in 10 cm culture dishes, and allowed to differentiate into multinucleated myotubes. The fully-differentiated cells were incubated for 6 h or 24 h in DMEM in the presence or absence of 1 μ M dexamethasone. After appropriate incubation, cells were washed with ice-cold phosphate buffered saline (PBS) and lysed with guanidine isothyonate buffer for total RNA extraction. The isolated total RNA was subjected to 1.2% agarose gel containing 2.2 M formaldehyde, transferred to nylon membranes and hybridized with 32 P-labeled cDNA probes. Northern blot analysis indicated that steady-state level of cathepsin B mRNA was marked enhanced by dexamethasone treatment. Dexamethasone caused a 3-fold increase in cathepsin B mRNA. The dexamethasone-mediated induction of cathepsin B gene expression was blocked by RU38486 (mifepristone), a glucocorticoid antagonist, suggesting the involvement of glucocorticoid receptors in the induction process. RU38486 alone did not affect cathepsin B mRNA levels.

Effects of glucocorticoids on other protease mRNAs were also studied. Expression of m-calpain was increased 50% by glucocorticoids. Like cathepsin B, this effect was abolished by addition of RU38486 to culture medium. However, concentrations of proteasome and cathepsin D mRNA were only slightly increased by dexamethasone. We also investigated the effects of glucocorticoids on α -tubulin, a cytoskeletal protein, expression. Unlike proteases, glucocorticoids down-regulated α -tubulin mRNA. Thus, glucocorticoids regulate the expression of proteases, mainly cathepsins, in cultured muscle cells through interaction with their receptors. Such regulation may be important in control of proteolysis in muscle.

INTRODUCTION

Glucocorticoids (cortisol and corticosterone) are steroid hormones which are secreted by the adrenal glands. Glucocorticoids at high concentrations cause severe atrophy or wasting of skeletal muscle (Goldberg et al., 1980; Tischler, 1981; Kettelhut et al., 1988). These hormones induce muscle atrophy by decreasing amino acid uptake (Kostyo and Redmond, 1966) and DNA (Goldberg and Goldspink, 1975), RNA (Rannels et al., 1978), protein synthesis (McGrath and Goldspink, 1982; Odedra et al., 1983; Goodlad and Clark, 1991). This atrophy process also involves stimulation of proteolysis (Kayali et al., 1987; Ohtsuka et al., 1992; Tomaz et al., 1992). However, the precise biochemical nature of glucocorticoid-mediated muscle atrophy has not been studied and the molecular mechanisms of this atrophy are not known.

Like other tissues, skeletal muscle contains multiple proteolytic systems (Goll et al., 1989), each of which could play important roles in glucocorticoid-induced atrophy. Lysosomal proteases, such as cathepsin B, L, H, and D, are present in muscle. Muscle also contains calcium-dependent proteases, μ -calpain and m-calpain (Goll et al., 1992), which have been known to play an important role in myofibrillar protein degradation. Recently, muscle has been shown to contain proteasome (multicatalytic protease), which can degrade proteins in an ATP-dependent manner.

In this study, we examined the effects of synthetic glucocorticoid (dexamethasone) on protease gene expression by Northern blot analysis and

determined whether regulation was mediated via the glucocorticoid receptor.

MATERIALS AND METHODS

Materials

[α -³²P] dCTP (6,000 Ci/mmol) was from New England Nuclear (Boston, MA). S & S Nytran⁺ (0.45 μ m) membrane was from Schleicher & Schuell (Keene, NH). Denhardt's solution (100 x) was from 5 prime-3 prime Inc. (Boulder, CO). QIAEX agarose gel extraction kit was from QIAGEN Inc. (Chatsworth, CA). Random-primed DNA labeling kit was from USB (Cleveland, OH). Formamide and formaldehyde were from Mallinckrodt (Paris, KY). Safe II scintillation cocktail was from Research Products International Co. (Mount Prospect, IL). Fetal bovine (FBS) and horse (HS) sera were from Hyclone (Logan, UT). Dulbecco's modified Eagle's medium, penicillin/streptomycin solution and trypsin solution were from GIBCO (Grand Island, NY). Quick-Spin G-50 Sephadex columns were from Boehringer Mannheim (Indianapolis, IN). RU38486 (mifepristone) was generously given by Dr. Gaillard-Kelly, Roussel-Uclaf (Paris, France). Cytosine- β -D-arabinofuranoside (Ara-C), dexamethasone, dextran sulfate, sonicated, denatured salmon testes DNA, ethidium bromide and bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO). Culture dishes were from Corning (Corning, New York).

Cell Culture

L8 rat skeletal muscle cells were obtained from American Type Culture Collection (Rockville, MD). Cells, stored in liquid nitrogen, were thawed and

maintained by repeated subculturing at low density on 10 cm culture dishes. Cells were grown in Dulbecco's-modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 100 units of penicillin/ml, 100 ug of streptomycin/ml, 44 mM NaHCO₃, 110 ug of sodium pyruvate/ml in humidified atmosphere of 5% CO₂ and 95% air at 37 °C. The medium was changed every 2 days. Cells (2.5×10^4 cells/cm²) were grown in the presence of 10% FCS until they reached confluency. At this point the medium was replaced by DMEM containing 2% FCS for induction of differentiation. Approximately 3 days later, when myotube formation was observed, the cells were treated with 10 uM cytosine arabinoside for 48 hours to remove any remaining dividing myoblasts. Microscopic examination was used to monitor differentiation. To study the effect on protease gene expression, fully differentiated L8 myotubes were treated with dexamethasone and RU38486. After the appropriate incubation period, cells were harvest for isolation of RNA.

Extraction of total RNA

Extraction of total RNA has been described previously (Chomczynski and Sacchi, 1987). Briefly, myotube cultures grown in 10-cm diameter dishes were washed three times with ice-cold phosphate buffered saline (PBS) and lysed directly on the dishes using 2 ml of Solution A (4 M guanidium isothionate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 10 mM β-mercaptoethanol). The monolayer was scraped with a rubber policeman to

ensure that all cells were released from the dishes. The lysate was transferred to a 15 ml polypropylene tube. To this, 0.2 ml of 2 M sodium acetate (pH 4.0), 2 ml of phenol and 0.4 ml of a chloroform-isoamyl alcohol mixture (49:1) were added sequentially. The lysate was mixed by inversion after each addition. After brief vortexing, the mixture was kept on ice for 15 min and was separated by centrifugation (12,000 g) for 30 min at 4°C. The upper aqueous phase was transferred to a new tube and the same volume of ice-cold isopropanol was added. The samples were mixed and stored at -20°C overnight. RNA was collected by centrifugation (12,000 g, 20 min at 4°C). The supernatant was discarded and the RNA pellet was resuspended in 0.4 ml of solution A. One volume of isopropanol was added, and the mixture was stored at -20°C overnight. The RNA pellet was recovered by centrifugation at 12,000 g for 15 min at 4°C, washed twice with 70% ethanol and dried under vacuum. The dried RNA pellet was then dissolved in diethylpyrocarbonate (DEPC)-treated water and was quantitated by spectrophotometry at a wavelength of 260 nm.

cDNA probes

pC2- α , which encodes rat liver proteasome (30.8 kDa C2 subunit; Fujiwara et al., 1989) was provided by Dr. K. Tanaka (Institute for Enzyme Research, University of Tokushima, Japan). pHcPDEco1.1, which encodes human cathepsin D, was provided by Dr. John Chirgwin (University of Texas Health Science Center; Faust et al., 1985). prCB5, which encodes rat liver

cathepsin B, was provided by Dr. S. J. Chan (Howard Hughes Medical Institute, University of Chicago; Segundo et al., 1985). pT1, which encodes chicken α -tubulin, was provided by Dr. Donald W. Cleveland (Johns Hopkins University; Valenzuela et al., 1981). Rat m-calpain cDNA probe (0.9 kb), which was subcloned in pUC19, was provided by Dr. John S. Elce (Queen's University, Canada). Plasmids were amplified and recovered using standard techniques (Maniatis et al., 1982). A cDNA probe encoding proteasome (1000 bp HindIII/PvuII fragment of pC2- α), cathepsin D (1000 bp EcoRI fragment of pHcPDEco1.1), cathepsin B (600 bp HindIII fragment of prCB5) and α -tubulin (1,500 bp HindIII fragment of pT1) and m-calpain (900 bp EcoRI fragment) were prepared as previously described (Ou and Forsberg, 1991; Ilian and Forsberg, 1992). After restriction enzyme digestion, cDNA fragments were separated by electrophoresis and were recovered by electroelution onto a dialysis membrane or by QIAEX agarose gel extraction kit. cDNA fragments (25ng) were labeled with [α - 32 P] dCTP (6000 Ci/mmol) using a random-prime kit. A labeled cDNA probe was purified using Quick Spin G-50 Sephadex column (Boehringer Mannheim).

Northern blot hybridization

RNA samples (15 ug) were denatured at 65°C for 15 min, applied to a 1.2% agarose gel containing 2.2 M formaldehyde, then electrophoresed at 30 V for 12 h. The quality and the relative abundance of RNA/lane were judged by comparing the ethidium bromide staining of the ribosomal bands. RNA

was transferred to Nytran⁺ nylon membrane and immobilized by baking at 80°C for 40 min. Membranes were incubated at 43 °C for overnight in prehybridization buffer (5 x SSPE, 0.2% SDS, 5 x Denhardt's solution, 100 ug/ml sonicated salmon testes DNA (Sigma) and 50% formamide). Following prehybridization, the membranes were hybridized for 36 hours at 43 °C in prehybridization buffer containing 10% dextran sulfate and [³²P] cDNA (1,000,000 cpm/ml). After hybridization, membranes were washed three times with 1 x SSPE and 0.1% SDS for 15 min at room temperature and were washed two times with 0.1 x SSPE and 0.1% SDS for 15 min at 50 °C. Membranes were exposed to Kodak X-Omat or Fuji RX film with intensifying screens for 6-12 hours at -80 °C. Quantitation of exposures on autoradiographic films was performed using a Bio-Rad model 1650 scanning densitometer and a Hoefer GS-350H scanning program. To remove probe from blot for rehybridization, membrane was washed in 6 x SSPE containing 50% formamide at 65 °C for 30 minutes and then rinsed in 2 x SSPE. After removal, the membrane was prehybridized and hybridized as indicated above.

RESULTS

We have shown that protein degradation in L8 myotubes can be effectively stimulated by 1 μ M dexamethasone (Figure 2.6. and 2.7.). Therefore, in the present study, L8 myotubes were treated with 1 μ M dexamethasone.

Effects of dexamethasone on cathepsin B expression were examined by Northern blotting analysis (Figure 4.1.). The time course of the effect of dexamethasone on cathepsin B mRNA is seen Figure 4.2. Cathepsin B mRNA levels were increased about 3.3-fold by dexamethasone treatment. However, dexamethasone decreased α -tubulin mRNA (Figure 4.1. bottom panel). Dexamethasone-mediated cathepsin B induction was blocked by addition of glucocorticoid antagonist, RU38486 (10 μ M) (Figure 4.3.). This level of RU38486 has been shown to effectively block the glucocorticoid-stimulated proteolysis in L8 myotubes (Figure 2.8.). RU38486 alone had no effect on cathepsin B mRNA (Figure 4.4.). Cathepsin D mRNA levels were also slightly increased by dexamethasone treatment (Figure 4.5.). As seen in Figure 4.6., cathepsin D mRNA was elevated 35% by a 6 hour incubation with dexamethasone. RU38486 treatment blocked these effects (Figure 4.7.). Steady-state levels of proteasome were not altered by dexamethasone (Figure 4.8. and 4.9.).

Dexamethasone increased the expression of m-calpain in L8 myotubes (Figure 4.10.). Steady-state m-calpain mRNA was increased 48% (Figure 4.11.) at 6 hours. Like cathepsin B, the increase produced by 1 μ M

dexamethasone was completely blocked by 10 μ M RU38486 (Figure 4.12.). However, RU38486, alone, had no effect on m-calpain gene expression (Figure 4.13.).

DISCUSSION

Glucocorticoids have been known to be a catabolic factor to muscle. Administration of glucocorticoids at high levels causes marked atrophy in skeletal muscle (Rannels et al., 1978; Kayali et al., 1987). These studies indicated that this process involved inhibition of protein synthesis and stimulation of protein degradation. However, the mechanisms underlying glucocorticoid-induced muscle atrophy are not clear. In the present study, we examined the regulation of protease gene expression by glucocorticoids to elucidate the molecular mechanisms by which glucocorticoids increase muscle protein degradation.

Cathepsin B expression was markedly increased by glucocorticoid treatment. Glucocorticoid antagonist, RU38486 (Ulmann et al., 1990), blocked the glucocorticoid-mediated induction of cathepsin B mRNA. These results indicated that intracellular glucocorticoid receptors (Burnstein and Cidlowski, 1989; Muller and Renkawitz, 1991) are involved in glucocorticoid-mediated cathepsin B induction. Although its role in muscle protein degradation is not clear, close correlations between cathepsin B activity and muscle protein degradation have been observed (Gerard et al., 1988; Furuno et al., 1990; Tawa et al., 1992). Although the changes are small compared to cathepsin B, dexamethasone also increased steady-state cathepsin D mRNA concentration. This induction was blocked by RU38486 suggesting again involvement of a glucocorticoid receptor.

Calpains (Goll et al., 1992) have been implicated in muscle protein degradation. Two isoforms with different Ca^{2+} requirements exist in muscle cells. In this study, we examined effects of glucocorticoids on gene expression of m-calpain, which require mM levels of Ca^{2+} for activity. In muscle, m-calpain is three times more abundant than μ -calpain (Kawashima et al., 1988). In the present study, we examined the regulation of m-calpain gene expression by dexamethasone to investigate the involvement of calcium dependent proteolytic system in this process. The steady-state m-calpain mRNA was increased by dexamethasone treatment. This induction was abolished by RU38486. Dexamethasone had no effect on proteasome gene expression. The role of proteasome, an ATP-dependent multicatalytic protease, in muscle protein degradation, is not clear.

In summary, this study shows that induction of cathepsin B, D, and m-calpain by dexamethasone is involved in glucocorticoid-induced atrophy of muscle and this induction process requires intracellular glucocorticoid receptors. This suggests that the lysosomal and calcium-dependent proteolytic systems may have a major role in glucocorticoid-induced muscle atrophy.

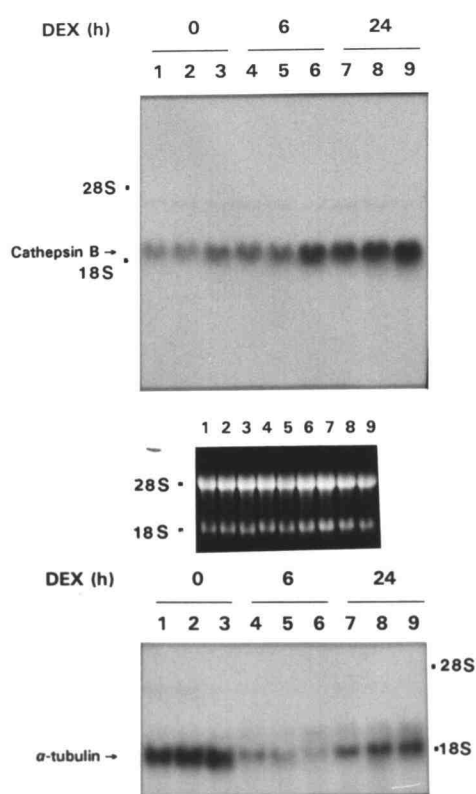


Figure 4.1. Effects of dexamethasone on cathepsin B mRNA abundance in L8 myotubes. Total RNA (20 μ g) extracted from cells cultured in triplicate for the indicated times (control; lanes 1,2,3 :6 hr; lanes 4,5,6: 24 hr; lanes 7,8,9) with 1 μ M dexamethasone was examined by Northern blot analysis (top panel). Relative amounts of RNA loaded in each lane are visualized by staining with ethidium bromide (middle panel). After hybridization to a cathepsin B probe, the filter was stripped and rehybridized with a probe for α -tubulin (bottom panel).

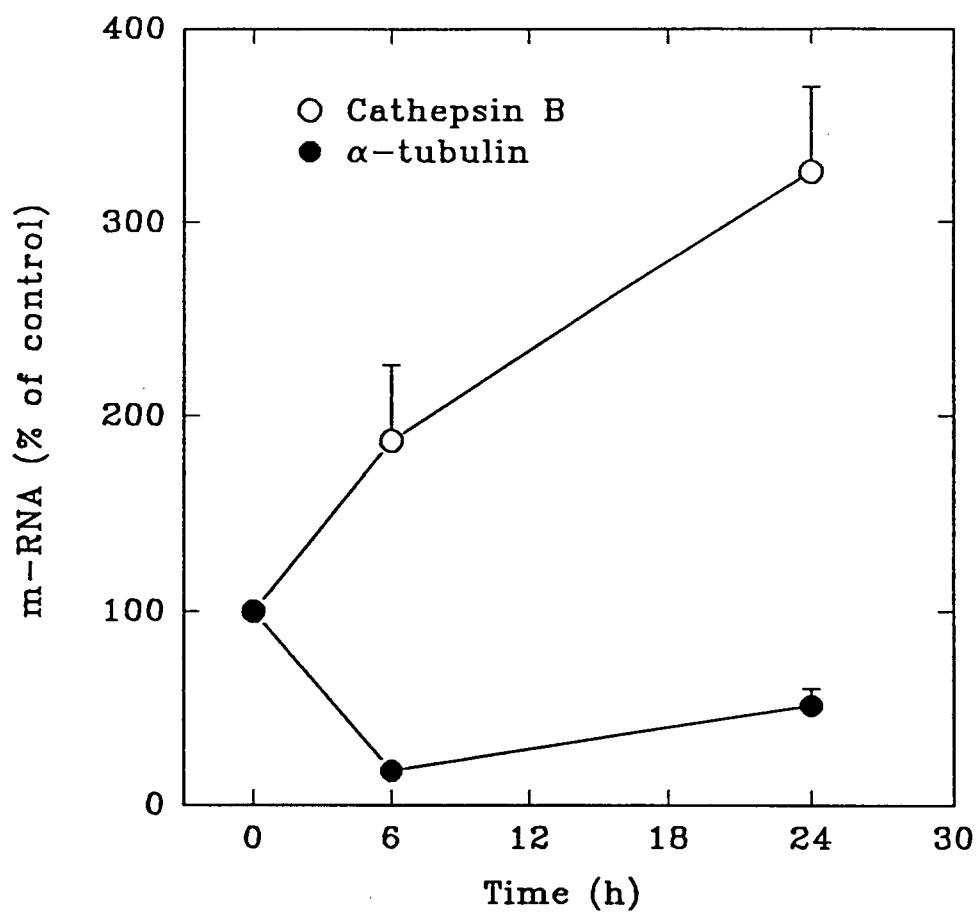


Figure 4.2. Time course showing the effects of dexamethasone on the abundance of cathepsin B and α -tubulin mRNA in L8 myotubes. Autoradiogram shown in Fig. 4.1 was quantitated by scanning densitometer.

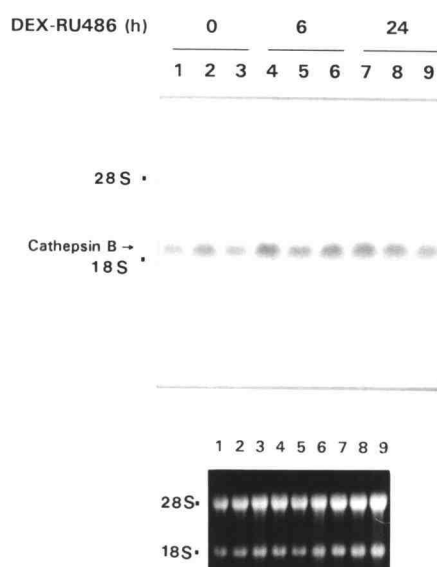


Figure 4.3. Effects of RU38486 on dexamethasone-mediated cathepsin B induction in L8 myotubes. Total RNA (20 μ g) extracted from cells cultured in triplicate for the indicated times (control; lane 1,2,3: 6 hr; lane 4,5,6: 24 hr; lane 7,8,9) with both 10 μ M RU38486 and 1 μ M dexamethasone was examined by Northern blot analysis (top panel). Relative amounts of RNA loaded in each lane are visualized by staining with ethidium bromide (bottom panel).

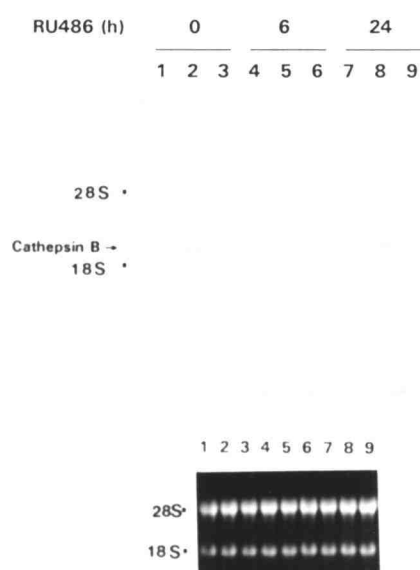


Figure 4.4. Effects of RU38486 on cathepsin B mRNA abundance in L8 myotubes. Total RNA (20 μ g) extracted from cells cultured in triplicate for the indicated times (control; lanes 1,2,3: 6 hr; lanes 4,5,6: 24 hr; lanes 7,8,9) with 10 μ M dexamethasone was examined by Northern blot analysis (top panel). Relative amounts of RNA loaded in each lane were visualized by staining with ethidium bromide (bottom panel).

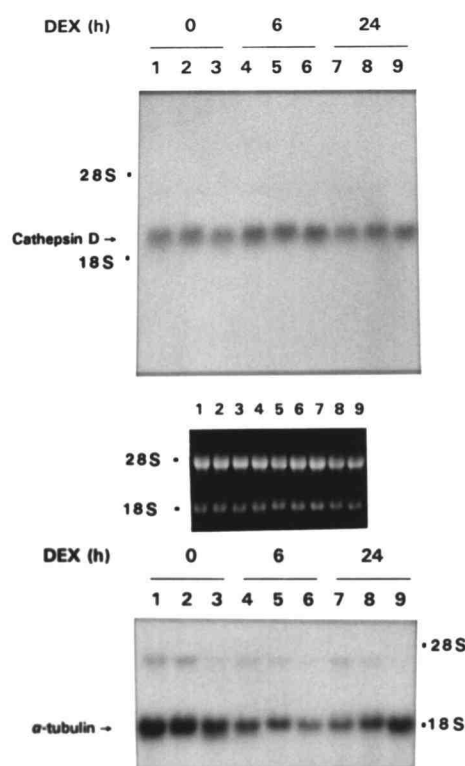


Figure 4.5. Effects of dexamethasone on cathepsin D mRNA abundance in L8 myotubes. Total RNA (20 μ g) extracted from cells cultured in triplicate for the indicated times (control; lanes 1,2,3 :6 hr; lanes 4,5,6: 24 hr; lanes 7,8,9) with 1 μ M dexamethasone was examined by Northern blot analysis (top panel). Relative amounts of RNA loaded in each lane are visualized by staining with ethidium bromide (middle panel). After hybridization to a cathepsin D probe, the filter was stripped and rehybridized with a probe for α -tubulin (bottom panel).

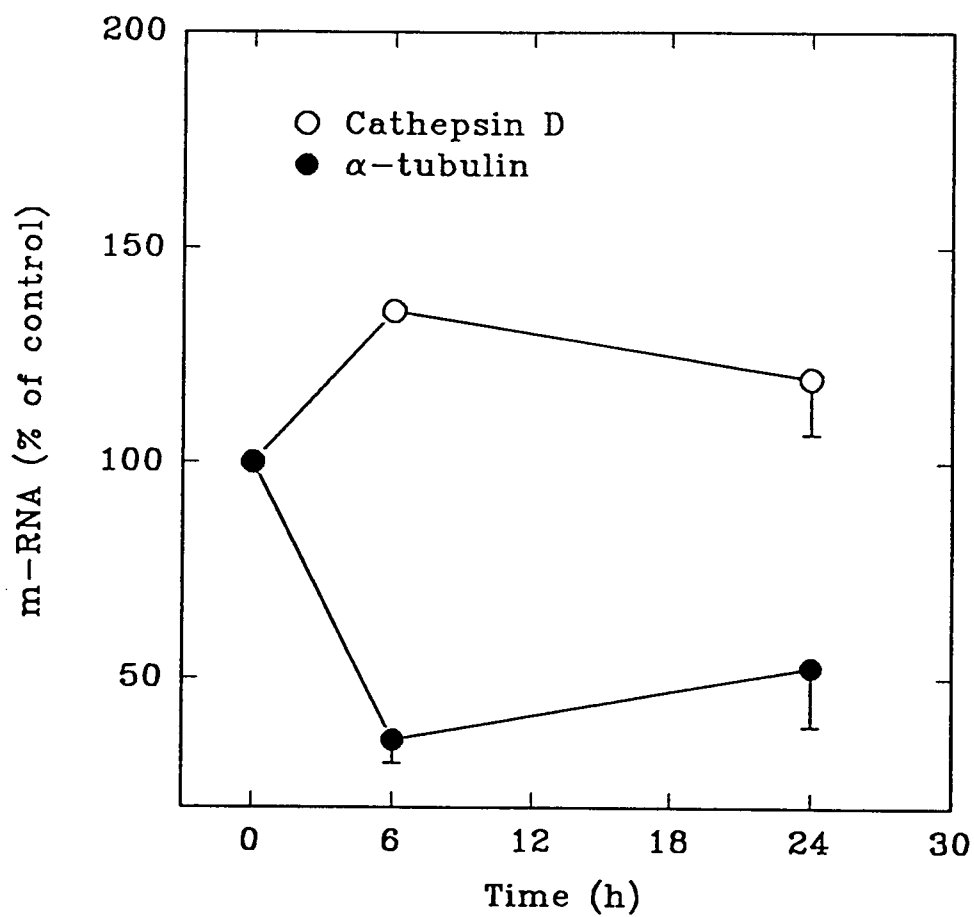


Figure 4.6. Time course showing the effects of dexamethasone on the abundance of cathepsin D and α -tubulin mRNA in L8 myotubes. Autoradiogram shown in Fig. 4.5 was quantitated by scanning densitometer.

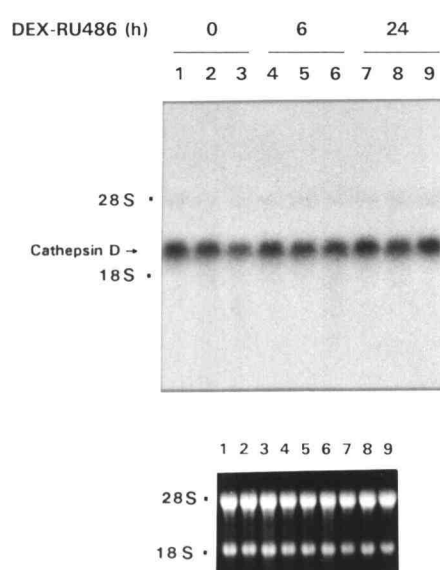


Figure 4.7. Effects of RU38486 on dexamethasone-mediated cathepsin D induction in L8 myotubes. Total RNAs (20 μ g) extracted from cells cultured in triplicate for the indicated times (control; lanes 1,2,3: 6 hr; lanes 4,5,6: 24 hr; lanes 7,8,9) with both 10 μ M RU38486 and 1 μ M dexamethasone was examined by Northern blot analysis (top panel). Relative amounts of RNA loaded in each lane were visualized by staining with ethidium bromide (bottom panel).

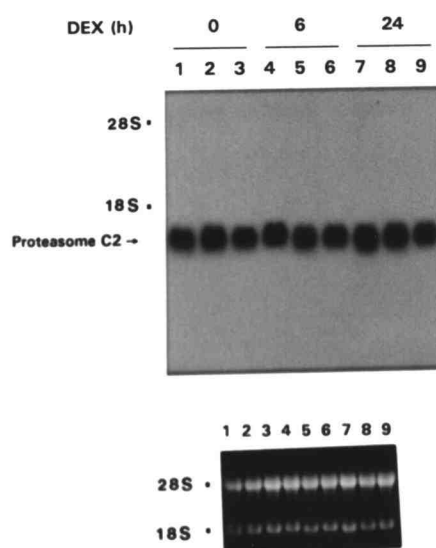


Figure 4.8. Effects of dexamethasone on proteasome mRNA abundance in L8 myotubes. Total RNA (20 μ g) extracted from cells cultured in triplicate for the indicated times (control; lanes 1,2,3: 6 hr; lanes 4,5,6: 24 hr; lanes 7,8,9) with 10 μ M dexamethasone were examined by Northern blot analysis (top panel). Relative amounts of RNA loaded in each lane were visualized by staining with ethidium bromide (bottom panel).

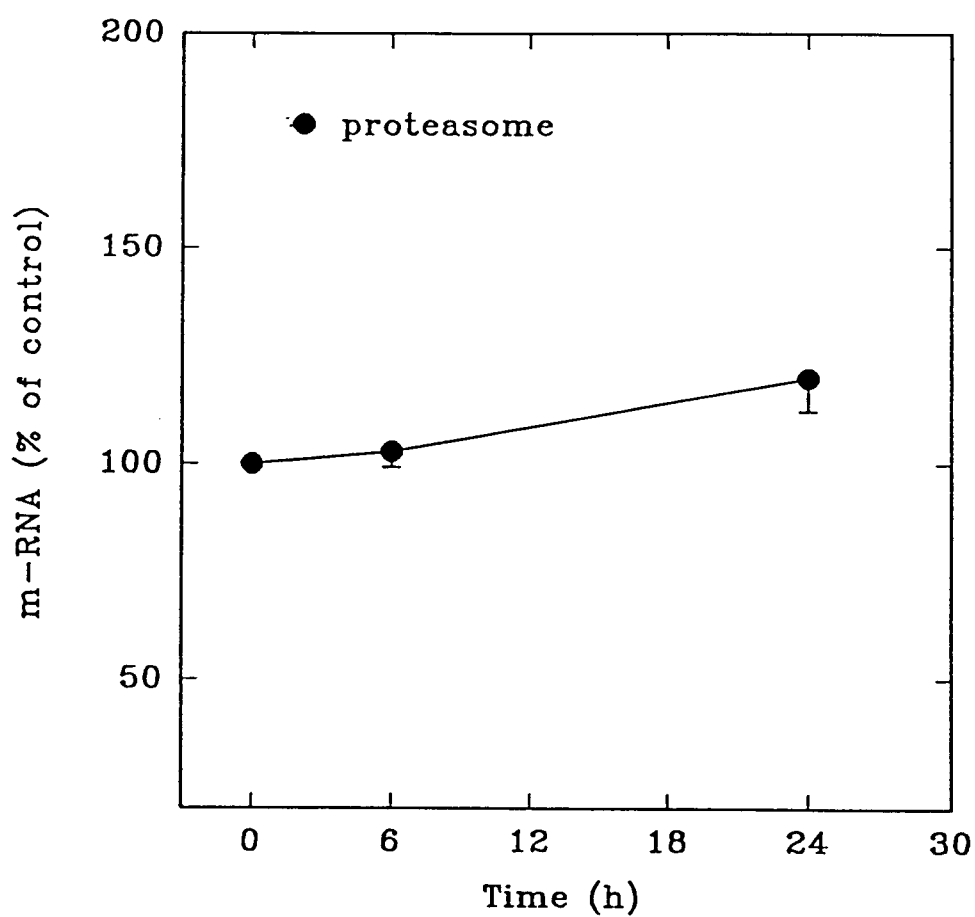


Figure 4.9. Time course showing the effects of dexamethasone on the abundance of proteasome mRNA in L8 myotubes. The autoradiogram shown in Fig. 4.8 was quantitated by scanning densitometer.

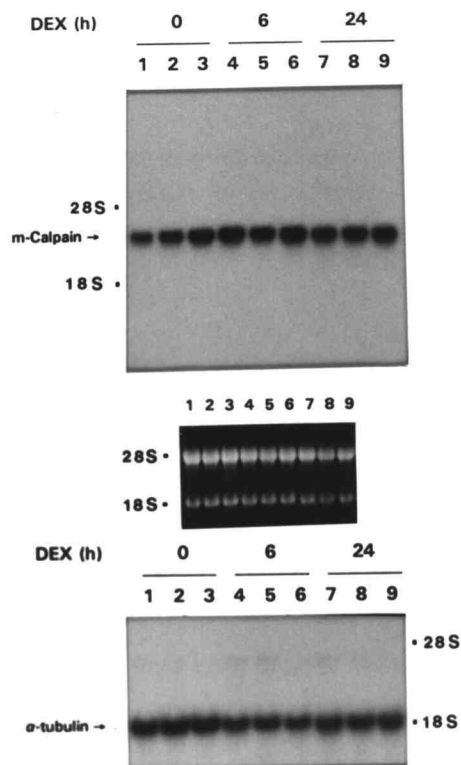


Figure 4.10. Effects of dexamethasone on m-calpain mRNA abundance in L8 myotubes. Total RNA (20 μ g) extracted from cells cultured in triplicate for the indicated times (control; lanes 1,2,3 :6 hr; lanes 4,5,6: 24 hr; lanes 7,8,9) with 1 μ M dexamethasone were examined by Northern blot analysis (top panel). Relative amounts of RNA loaded in each lane are visualized by staining with ethidium bromide (middle panel). After hybridization to a m-calpain probe, the filter was stripped and rehybridized with a probe for α -tubulin (bottom panel).

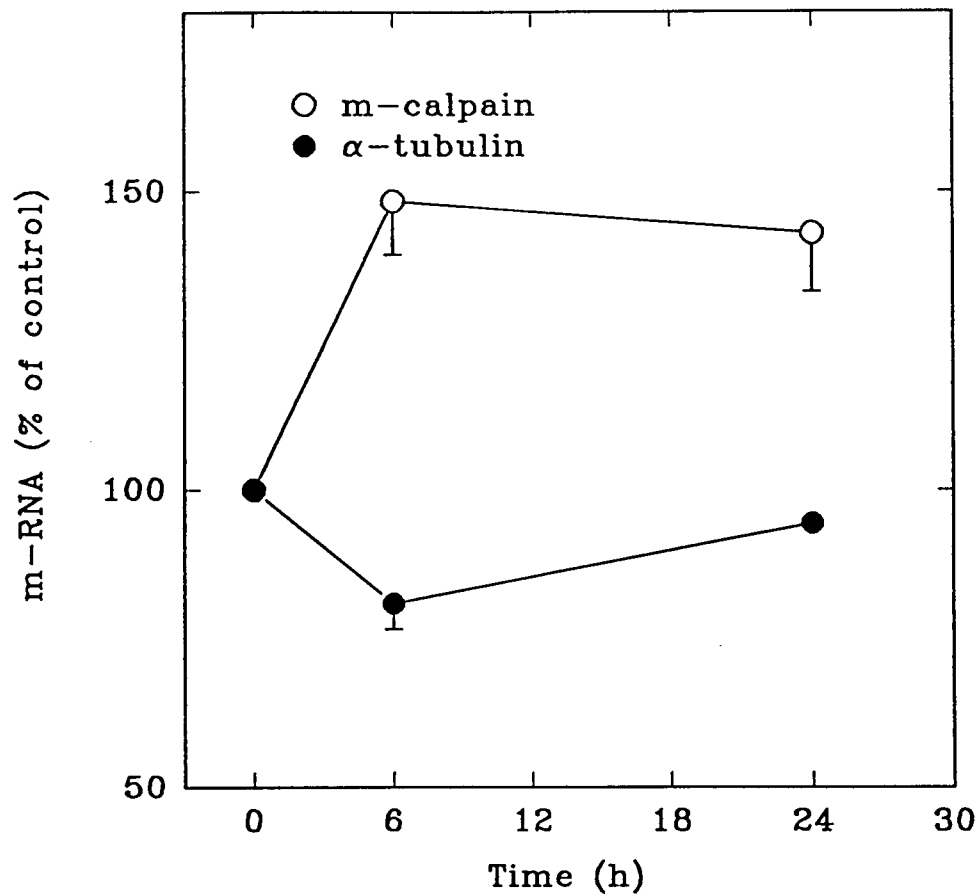


Figure 4.11. Time course showing the effects of dexamethasone on the abundance of m-calpain and α -tubulin mRNA in L8 myotubes. The autoradiogram shown in Fig. 4.10 was quantitated by scanning densitometer.

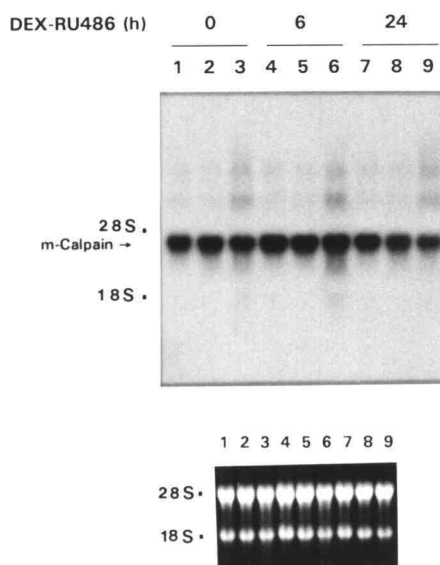


Figure 4.12. Effects of RU38486 on dexamethasone-mediated m-calpain induction in L8 myotubes. Total RNA (20 μ g) extracted from cells cultured in triplicate for the indicated times (control; lanes 1,2,3: 6 hr; lanes 4,5,6: 24 hr; lanes 7,8,9) with both 10 μ M RU38486 and 1 μ M dexamethasone was examined by Northern blot analysis (top panel). Relative amounts of RNA loaded in each lane were visualized by staining with ethidium bromide (bottom panel).

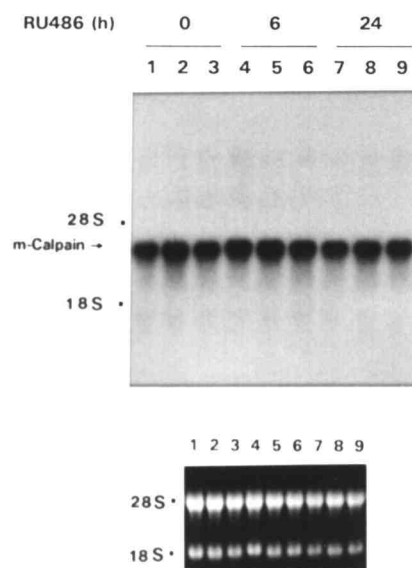


Figure 4.13. Effects of RU38486 on m-calpain mRNA abundance in L8 myotubes. Total RNA (20 μ g) extracted from cells cultured in triplicate for the indicated times (control; lanes 1,2,3: 6 hr; lanes 4,5,6: 24 hr; lanes 7,8,9) with 10 μ M RU38486 was examined by Northern blot analysis (top panel). Relative amounts of RNA loaded in each lane were visualized by staining with ethidium bromide (bottom panel).

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CHAPTER V

PROTEIN KINASE C ISOFORMS IN MUSCLE CELLS AND THEIR REGULATION BY PHORBOL ESTER

ABSTRACT

Protein kinase C (PKC) is a multi-enzyme family that encodes at least ten isoforms of phospholipid- and calcium-dependent serine-threonine kinases which play important roles in the regulation of signal transduction, cell growth and differentiation. PKC has also been implicated in control of muscle growth, m-calpain expression and muscle protein degradation. We determined protein kinase C isoforms in muscle cells (adult rat skeletal muscle, L8 myoblasts and myotubes) by immunoblot analysis. Using six isoform (α , β , γ , δ , ϵ , ζ)-specific polyclonal antibodies, PKC α , δ and ζ were detected in rat skeletal muscle, L8 myoblasts and myotubes. PKC β , γ and ϵ isozymes were not detected. L8 myoblasts (undifferentiated muscle cells) expressed the same isoforms as did myotubes and adult rat muscle. These studies showed the presence of calcium-dependent PKC (α) and calcium-independent PKC (δ) and (ζ) in muscle cells. PKC α and ζ were mainly localized in the cytosolic fraction of L8 myotubes whereas PKC δ was more abundant in the membrane fraction. Treatment of L8 myotubes with 12-O-tetradecanoyl 13-acetate (TPA) resulted in the down-regulation of PKC α and PKC δ isoforms. TPA induced down-regulation of PKC α and δ was partially blocked by calpain inhibitor (N-acetyl-leucyl-leucyl-methionine), indicating the

involvement of calpains in the process. However, PKC ζ did not translocate or down-regulate in response to TPA. In other studies we determined that TPA regulated proteolysis in muscle cells. Hence, we also studied the effects of TPA on protease gene expression by Northern blot analysis. TPA increased mRNA concentrations encoding m-calpain, proteasome and cathepsin B. Cathepsin D mRNA concentration was reduced by TPA. These results suggested that the PKC isoforms, which exist in muscle, are differentially regulated by extracellular and intracellular signals and may thereby influence muscle growth through distinct mechanisms.

INTRODUCTION

Protein kinase C (PKC), a serine/threonine kinase, is thought to play important roles in the control of a wide range of cellular processes, which include secretion, contraction, membrane receptor function, cell differentiation and tumor promotion (Nishizuka, 1984; Houslay, 1991). Protein kinase C is activated by calcium and diacylglycerol, which is generated from the receptor-mediated hydrolysis of membrane phospholipids. Molecular cloning studies revealed that the PKC gene family contains at least ten different isozymes that can be divided into three major groups (Kikkawa et al., 1989; Nishizuka, 1992; Hug and Sarre, 1993). The first group consists of four classical or conventional PKCs (cPKC): α , β I, β II and γ . The cPKC isoforms are activated by Ca^{2+} , phosphatidylserine, and diacylglycerol. The second group consists of four new PKCs (nPKC): δ , ϵ , η (L), and θ which are activated by phosphatidylserine and diacylglycerol but not by Ca^{2+} . The third group consists of two atypical PKCs (aPKC): ζ and λ . aPKCs require only phosphatidylserine for activator. Since the individual isozymes may have different cofactor requirements, subcellular distribution and substrate specificity, the profile of PKC isoforms expressed in a particular tissue is likely to be an important determinant in PKC-mediated cellular processes. Differential tissue distribution of PKC isoforms have been reported by many authors (Wetsel et al., 1992; Dlugosz et al., 1992, Chaudhyry and Casillas, 1992; Smallwood and Malawista, 1992)

Some PKCs are activated by the tumor promoting agent 12-O-tetradecanoyl phorbol-13-acetate (TPA), an analogue of diacylglycerol. Long-term exposure to TPA leads to down-regulation of PKCs (Ase et al., 1988; Huang, et al., 1989). It has been established that this TPA-induced down-regulation of PKC is due to an increased rate of proteolysis (Pontremoli, et al., 1990). Calcium-activated neutral protease (calpain) has been suggested to be the responsible protease for PKC down-regulation (Kishimoto et al., 1989; Savart et al., 1992).

In skeletal muscle, PKC has been implicated in hypertrophy (Richter and Nielsen, 1991), contraction-induced muscle metabolism (Richter et al., 1987), glucose transport (Farese et al., 1992), myoblast differentiation (David et al., 1990) and proliferation. We have determined that TPA increased muscle protein degradation. However, very little is known about the PKC isoforms and their regulation. In this study, we characterized the isoenzymes of PKC in skeletal muscle and their differential down-regulation by phorbol ester (TPA). We also investigated the effects of TPA on calpain gene expression. For comparative purpose, effects of TPA on expression of other proteases (cathepsin B and D and proteasome) were also studied.

MATERIALS AND METHODS

Materials

[α - 32 P] dCTP (6,000 Ci/mmol) was from New England Nuclear (Boston, MA). S & S Nytran⁺ (0.45 μ m) membrane was from Schleicher & Schuell (Keene, NH). Denhardt's solution (100 x) was from 5 prime-3 prime Inc. (Boulder, CO). QIAEX agarose gel extraction kit was from QIAGEN Inc. (Chatsworth, CA). Random-primed DNA labeling kit was from USB (Cleveland, OH). Formamide and formaldehyde were from Mallinckrodt (Paris, KY). Safe II scintillation cocktail was from Research Products International Co. (Mount Prospect, IL). Fetal bovine (FBS) and horse (HS) sera were from Hyclone (Logan, UT). Dulbecco's modified Eagle's medium, penicillin/streptomycin solution and trypsin solution, affinity-purified anti-PKC isozyme-specific (α , β , γ , δ , ϵ , and ζ) antibodies were from GIBCO (Grand Island, NY). Calpain inhibitor II, leupeptin and Quick Spin G-50 Sephadex column were from Boehringer Mannheim (Indianapolis, IN). Cytosine- β -D-arabinofuranoside (Ara-C), 12-O-tetradecanoyl phorbol-13-acetate (TPA), dimethyl sulfoxide, dextran sulfate, sonicated, denatured salmon testes DNA, ethidium bromide and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). Hybond-C Super and Hybond-ECL nitrocellulose membranes, horseradish peroxidase (HRP) conjugated donkey anti-rabbit IgG and ECL Western blotting analysis system were from Amersham (Arlington Heights, IL). Alkaline phosphatase (AP) conjugated goat anti-rabbit (GAR) IgG,

alkaline phosphatase conjugate substrate kit, colloidal gold total protein stain, SDS-PAGE low molecular weight standard, and Bio-Rad protein assay dye reagent were from Bio-Rad (Richmond, CA). Culture dishes were from Corning (Corning, New York).

Cell Culture

L8 rat skeletal muscle cells were obtained from American Type Culture Collection (Rockville, MD). Cells, stored in liquid nitrogen, were thawed and maintained by repeated subculturing at low density on 10 cm culture dishes. Cells were grown in Dulbecco's-modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 100 units of penicillin/ml, 100 ug of streptomycin/ml, 44 mM NaHCO₃, 110 ug of sodium pyruvate/ml in humidified atmosphere of 5% CO₂ and 95% air at 37 °C. The medium was changed every 2 days. Cells (2.5×10^4 cells/cm²) were grown in presence of 10% FCS until they reached confluency. At this point the medium was replaced by DMEM containing 2% FCS for inducing differentiation. Approximately 3 days later, when myotube formation was observed, the cells were treated with 10 uM cytosine arabinoside for 48 hours to remove any remaining dividing myoblasts. Microscopic examination was used to monitor differentiation. To study protease gene expression, fully differentiated L8 myotubes were treated with TPA. After the appropriate incubation period, cells were harvested for Western blotting or Northern blotting analysis.

Preparation of Tissue and Cell Extract for Western blotting

Male rats were killed by cervical dislocation and the brain and muscle were immediately removed and homogenized in a ice-cold Buffer A (20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 50 mM β -mercaptoethanol, 1 mM PMSF, 200 ug/ml of leupeptin, 5 mM EDTA, 2 mM EGTA, 10 mM benmidine, 0.2% Triton X-100). For protein extraction from L8 myoblasts and myotubes cells were washed with ice-cold phosphate buffered saline (PBS), scraped into ice-cold Buffer A and homogenized by sonication. The homogenates were kept for 1 h at 4 °C and centrifuged at 100,000 g for 30 min at 4 °C. The supernatant was either used immediately or frozen at -80 °C for SDS-PAGE. Protein concentration was determined by the method of Bradford (1976).

Preparation of cytosol and membrane extract form L8 myotubes

Cytosol and membrane fractions were prepared by homogenizing cells in a Buffer B (20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 50 mM β -mercaptoethanol, 1 mM PMSF, 200 ug/ml of leupeptin, 5 mM EDTA, 2 mM EGTA, 10 mM benmidine). The cells were disrupted by a Dounce homogenizer and centrifuged at 100,000 g for 20 min. Supernatants were used as cytosolic protein. Pellets were washed and extracted with Buffer B containing 1% Triton X-100. After 30 min incubation at 4 °C, the samples were centrifuged at 12,000 g for 20 min yielding the solubilized membrane fraction.

Western blot analysis

Protein samples were subjected to 10% SDS-PAGE according to the method of Laemmli (1970). The separated proteins were electrophoretically transferred to nitrocellulose membrane (Hybond-C Super or Hybond-ECL) at 4 °C for overnight using a Bio-Rad Trnasfer Blot apparatus (30 mAmp) by the method of Towbin et al. (1979). Nonspecific sites were blocked by incubation of nitrocellulose membranes with 1% BSA in TBST (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20) for 1 h at room temperature. Anti-PKC antibodies were incubated with the membranes for 2 h at room temperature. The antibody dilutions were as follows: α , 1:333; β , 1:333; γ , 1:333; δ , 1:200; ϵ , 1:333; and ζ , 1:200. After three washes (15 min) with TBST, secondary antibody (AP-conjugated goat anti-rabbit IgG, at 1:3000 dilution; or HRP-conjugated donkey anti-rabbit IgG, at 1:2000) was added and incubated for 1 h at room temperature. Following this incubation, the membrane was washed (15 min) three times with TBST. Specific binding of anti-PKC antibody onto the membrane was detected by either the alkaline phosphatase detection kit (Bio-Rad) or the ECL detection system (Amersham).

Extraction of total RNA

Extraction of total RNA has been described previously (Chomczynski and Sacchi, 1987). Briefly, myotube cultures grown in 10-cm diameter dishes were washed three times with ice-cold phosphate buffered saline (PBS) and

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

cDNA probes

pC2- α , which encodes rat liver proteasome (30.8 kDa C2 subunit; Fujiwara et al., 1989) was provided by Dr. K. Tanaka (Institute for Enzyme Research,

University of Tokushima, Japan). pHcPDEco1.1, which encodes human cathepsin D, was provided by Dr. John Chirgwin (University of Texas Health Science Center; Faust et al., 1985). prCB5, which encodes rat liver cathepsin B, was provided by Dr. S. J. Chan (Howard Hughes Medical Institute, University of Chicago; Segundo et al., 1985). pT1, which encodes chicken α -tubulin, was provided by Dr. Donald W. Cleveland (Johns Hopkins University; Valenzuela et al., 1981). Rat m-calpain cDNA probe (0.9 kb), which was subcloned in pUC19, was provided by Dr. John S. Elce (Queen's University, Canada). Plasmids were amplified and recovered using standard techniques (Maniatis et al., 1982). A cDNA probe encoding proteasome (1000 bp HindIII/PvuII fragment of pC2- α), cathepsin D (1000 bp EcoRI fragment of pHcPDEco1.1), cathepsin B (600 bp HindIII fragment of prCB5) and α -tubulin (1,500 bp HindIII fragment of pT1) and m-calpain (900 bp EcoRI fragment) were prepared as previously described (Ou and Forsberg, 1991; Ilian and Forsberg, 1992). After restriction enzyme digestion, cDNA fragments were separated by electrophoresis and were recovered by electroelution onto a dialysis membrane or by QIAEX agarose gel extraction kit. cDNA fragments (25ng) were labeled with [α - 32 P] dCTP (6000 Ci/mmol) using a random-prime kit.

Northern blot hybridization

RNA samples (15 ug) were denatured at 65°C for 15 min, applied to a 1.2% agarose gel containing 2.2 M formaldehyde, then electrophoresed at 30 V

for 12 h. The quality and the relative abundance of RNA/lane were judged by comparing the ethidium bromide staining of the ribosomal bands. RNA was transferred to Nytran⁺ nylon membrane and immobilized by baking at 80°C for 40 min. Membranes were incubated at 43 °C for overnight in prehybridization buffer (5 x SSPE, 0.2% SDS, 5 x Denhardt's solution, 100 ug/ml sonicated salmon testes DNA (Sigma) and 50% formamide). Following prehybridization, the membranes were hybridized for 36 hours at 43 °C in prehybridization buffer containing 10% dextran sulfate and [³²P] cDNA (1,000,000 cpm/ml). After hybridization, membranes were washed three times with 1 x SSPE and 0.1% SDS for 15 min at room temperature and were washed two times with 0.1 x SSPE and 0.1% SDS for 15 min at 50 °C. Membranes were exposed to Kodak X-Omat or Fuji RX film with intensifying screens for 6-12 hours at -80 °C. Quantitation of exposures on autoradiographic films was performed using a Bio-Rad model 1650 scanning densitometer and a Hoefer GS-350H scanning program. To remove probe from blot for rehybridization, membrane was washed in 6 x SSPE containing 50% formamide at 65 °C for 30 minutes and then rinsed in 2 x SSPE. After removal, the membrane was prehybridized and hybridized as indicated above.

RESULTS

PKC isoforms in muscle

We used affinity-purified isozyme-specific polyclonal antibodies for Western blotting to detect the presence of PKC α , β , γ , δ , ϵ and ζ isoforms in extracts from rat skeletal muscle, L8 myoblasts and L8 myotubes. Extracts from rat brain were used as a positive control. These results are shown in Figure 5.1. and 5.2. We detected the expression of PKC α , PKC δ and PKC ζ in all muscle cells. The apparent molecular weight of PKC α and PKC δ was 80 kDa. The PKC ζ isozyme-specific antibody recognized two proteins of approximately 70 kDa and 85 kDa in rat brain, while in muscle cells, a predominant 70 kDa protein was detected. PKC ζ content in L8 muscle cells was much higher than that detected in adult muscle.

Subcellular distribution and down-regulation of PKC isoforms by TPA

We next examined the subcellular distribution of three PKC isoform in L8 myotubes and their down-regulation by TPA (Figure 5.3., 5.4. and 5.5.). PKC α and PKC ζ resided predominantly in the cytosol, whereas 70% of PKC δ was found in the membrane fraction. The subcellular localization studies showed that PKC ζ appeared in the cytosol as a 70 kDa protein, while in the membrane 70 and 80 kDa bands were detected (Figure 5.5.). Treatment of L8 myotubes with 300 ng/ml TPA caused down-regulation of PKC α and PKC δ . PKC α was rapidly depleted in response to TPA and was not

detected in L8 myotubes treated with TPA for 6 hour. On the other hand, PKC δ was more resistant to TPA-induced down-regulation since 20% of this isoform was still detectable in the membrane fraction of cells treated for 6 hours. However, longer exposure to TPA resulted in complete depletion of PKC δ . To investigate the role of calpain in the down-regulation of PKC α and δ , we treated L8 cells with a cell penetrating calpain inhibitor (N-Acetyl-leucyl-leucyl-methioninal). TPA-induced down-regulations of PKC α and δ were partially blocked by the addition of calpain inhibitor. In calpain inhibitor-treated cells, PKC α and PKC δ were still detected. PKC ζ , which is known to not bind to TPA, was neither translocated nor down-regulated by TPA treatment (Figure 5.5.).

Effects of TPA on protease gene expression

In previous studies, we determined that TPA enhanced proteolysis. However, the mechanism underlying this regulation has not been established. In order to examine the role of protease gene expression in TPA-induced PKC and muscle protein degradation, we performed Northern blot analysis in TPA-treated L8 myotubes. Treatment of L8 myotubes with 300 ng/ml TPA increased m-calpain mRNA by 40% (Figure 5.6. and 5.10.). The steady-state mRNA level encoding proteasome was also slightly increased by 28% (Figure 5.7.). For comparative purposes we also measured the effects of TPA on gene expression of two major lysosomal proteases, cathepsin B and cathepsin D. Cathepsin B was transiently increased by 83% at 6 hours, and

then decreased to control level. However, steady-state cathepsin D mRNA was decreased by 33%.

DISCUSSION

In this study, we demonstrated the presence of calcium-dependent PKC (α) and calcium-independent PKC (δ and ζ) in L8 myoblasts, L8 myotubes and rat adult muscle. The differentiation of muscle cells did not cause any change in the expression of PKC isoforms. PKC α , δ and ζ were present in both undifferentiated and differentiated L8 muscle cells. These findings confirmed the early studies showing the presence of PKC α (Osada et al., 1992) and δ (Mizuno et al., 1991) in muscle. The presence of PKC β in muscle was reported by Hanaina et al (1992) and Nakano et al (1992). However, we were unable to detect PKC β in myoblasts, myotubes, and rat muscle. This may be due to the presence of only small amount of PKC β in skeletal muscle. The quantitative immunoblotting by Yoshida et al. (1988) indicated that PKC β in muscle was very low compared other tissues. We detected a 70 kDa single band with PKC ζ antibody in muscle cells, whereas 70 and 85 kDa doublet in rat brain. These results are consistent with previous findings showing presence of multiple PKC ζ s of different molecular weights (Farese et al., 1992; Goodnight et al., 1992; Simboli-Campbell, 1993).

In an effort to assess the roles of these multiple isoforms we examined the subcellular distribution and phorbol ester induced translocation and down-regulation of PKCs. Immunoblotting with subcellular fractions showed that PKC α and ζ isoforms are cytosolic while significant amounts of PKC δ were recovered in solubilized particulate fractions. Mizuno et al

(1991) also reported the predominant presence of mouse PKC δ in the membrane fraction. It has been well established that PKCs are translocated to the membrane fractions in response to phorbol ester, diacylglycerol and calcium (Kikkawa et al., 1989; Smallwood and Malawista, 1992; Borner et al., 1992). We also observed the TPA induced-translocation of PKC α and δ from cytosol to the membrane fraction. Exposure to TPA failed to translocate PKC ζ of L8 myotubes to the membrane fractions. In contrast, Borner et al.(1992) reported the translocation of rat fibroblast PKC ζ by phorbol ester. However, most studies indicate that PKC ζ is resistant to TPA-induced translocation or down-regulation (Ways et al., 1992; Liyanage et al., 1992; Gschwendt et al., 1992).

Chronic exposure of cells to the phorbol ester induces a down-regulation of PKC , which results in a depletion of the enzyme (Ito et al., 1988). Calcium-dependent neutral protease (calpain) has been implicated in the down-regulation of PKC (Pontremoli et al., 1986; Kishimoto et al., 1989; Kurota et al., 1991). The mechanism by which phorbol esters induce down-regulation of PKC is not understood. Savart et al.(1992) suggested that TPA binding to the regulatory domains (C1 region) may cause a conformational change of the PKC, increasing its affinity for calpain and allowing its proteolysis at physiological concentrations of calcium. A recent circular dichroism study showed that binding of TPA to PKC caused marked changes in secondary structure (Bosca and Moran, 1993). This may explain why PKC ζ , which lacks TPA binding sites, is not down-regulated by TPA.

Although PKC down-regulation has been extensively studied, the physiological role of this proteolysis is not clear yet. Two possible roles have been proposed (Kikkawa, 1989). First, proteolytic cleavage produces a PKC fragment possessing constitutive kinase activity. James and Olson (1992) showed the specific role of PKM, a catalytic fragment of PKC in muscle cells. Another possible role is that the limited proteolysis by calpain may initiate the degradation of PKC, eventually depleting the enzyme from the cell.

We also have used calpain inhibitor to investigate the involvement of muscle calpain in the down regulation process. This inhibitor has been used as selective inhibitor for both μ - and m-calpain (Saito and Nixon, 1993). Down-regulation of PKC α and PKC δ was blocked by calpain inhibitor treatments. Adachi et al. (1990) also reported a similar finding with calpastatin. These results suggests that calpain plays a role in down-regulation of PKC isoforms.

We also studied the effects of TPA on protease gene expression. TPA has been shown to activate the transcription of certain genes, such as c-jun, collagenase, and metallothionein II A. These genes commonly possess a cis-acting element, TRE (TPA responsive element), which binds with jun homodimer or jun/fos heterodimer. Interestingly, Hata et al (1989) identified the presence of TRE in the human m-calpain promotor. Recently, the same group showed the transcriptional activation of human m-calpain in HeLa cell by TPA (Hata, 1992). We also observed the TPA mediated induction of m-calpain in L8 myotubes. But, TPA treatment increased m-calpain expression

to a much lower extent compared to HeLa cells. This may be due to the presense of high concentration of serum in our media. To study the involvement of other proteases in degradation of PKC, we examined the effects of TPA on cathepsins B and D and proteasome mRNA by Northern blotting. A transient increase in cathepsin B mRNA suggested its possible role in in further degradation of PKC fragments produced by calpain. However, roles of other proteases in the down-regulation process need to be elucidated.

In conclusion, the distinct expression pattern, activator requirements, substrate specificity and response to extra- or intracellular stimuli of different PKC isoforms suggest that each isoforms are independently regulated, respond differently to hormones, growth factors, and other signals.

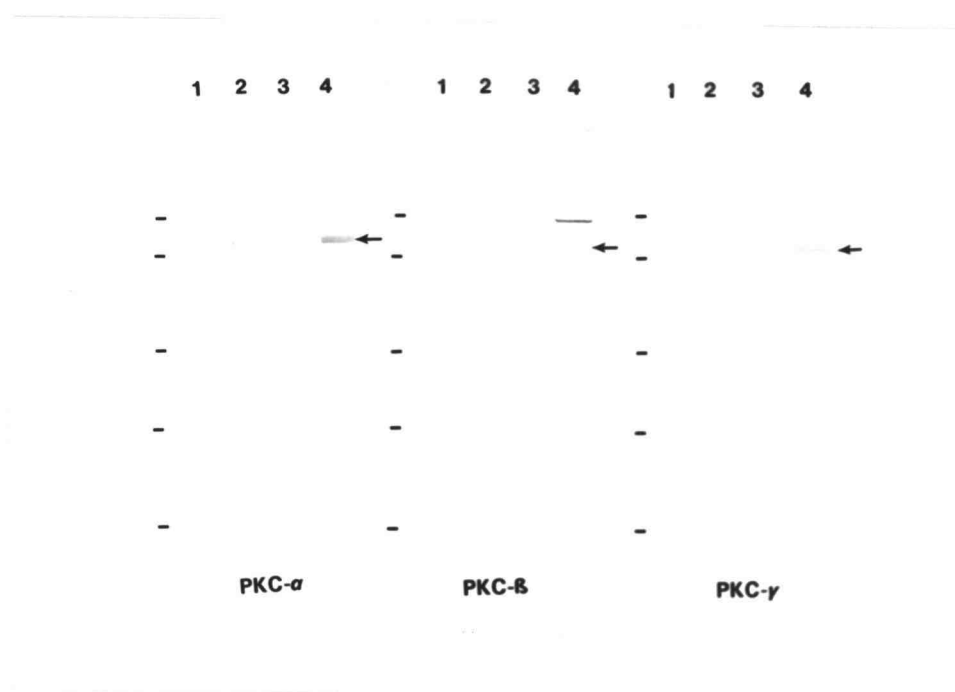


Figure 5.1. Western blot analysis of protein kinase C isozymes (α , β , and γ) isozymes in muscle. Protein samples from L8 myoblasts (lane 1), L8 myotubes (lane 2), rat skeletal muscle (lane 3) and rat brain (lane 4) were subjected to Western blot analysis as described in materials and methods. Positions of molecular weight markers are shown on the left: (from the top) 97.4, 66.2, 42.7, 31.0 and 21.5 kDa.

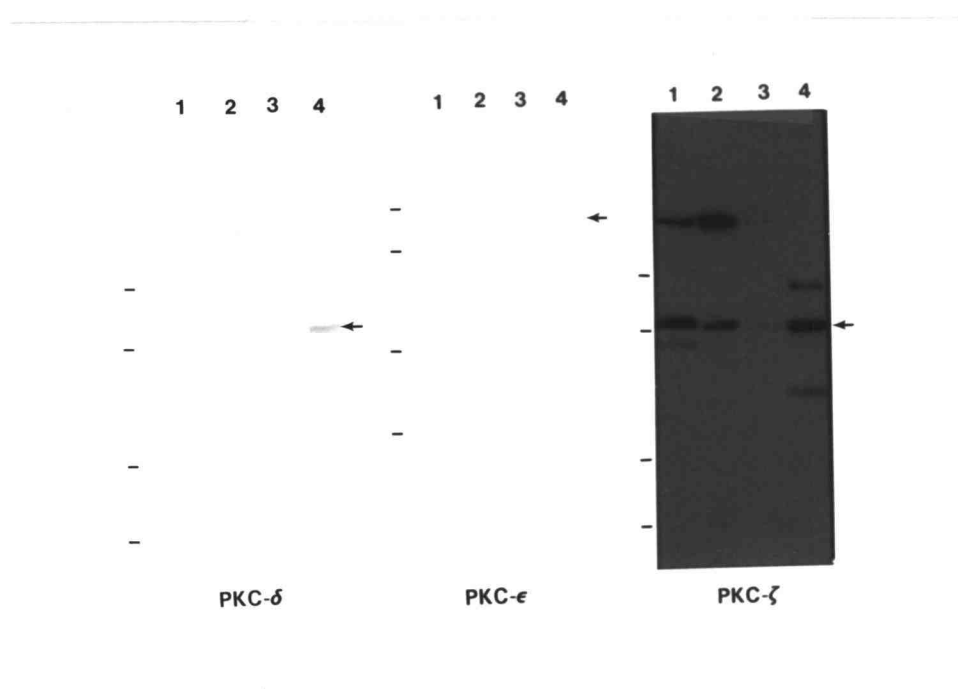


Figure 5.2. Western blot analysis of protein kinase C isozymes (δ , ϵ , and ζ) isozymes in muscle. Protein samples from L8 myoblasts (lane 1), L8 myotubes (lane 2), rat skeletal muscle (lane 3) and rat brain (lane 4) were subjected to Western blot analysis as described in materials and methods. Positions of molecular weight markers are shown on the left: (from the top) 97.4, 66.2, 42.7, 31.0 and 21.5 kDa.

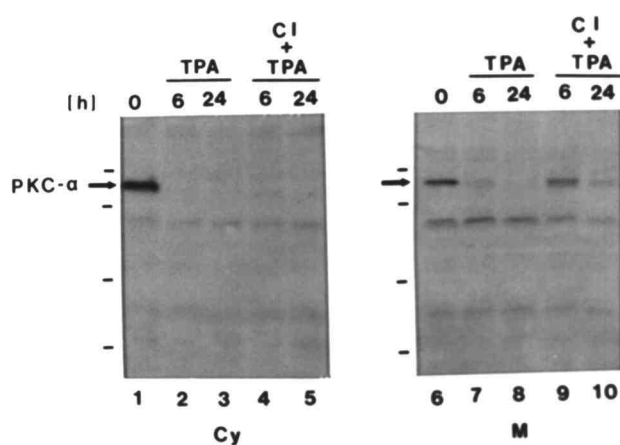


Figure 5.3. Effects of calpain inhibitor on TPA-induced down-regulation of PKC- α of L8 myotubes. L8 myotubes were treated with 300 ng/ml TPA in the absence or presence of 100 nM calpain inhibitor (N-Acetyl-leucyl-leucyl-methionial). Cytosolic (lane 1-5) and membrane (lane 6-10) fractions were prepared for Western blot analysis. Positions of molecular weight markers are shown on the left: (from the top) 97.4, 66.2, 42.7, and 31.0 kDa.

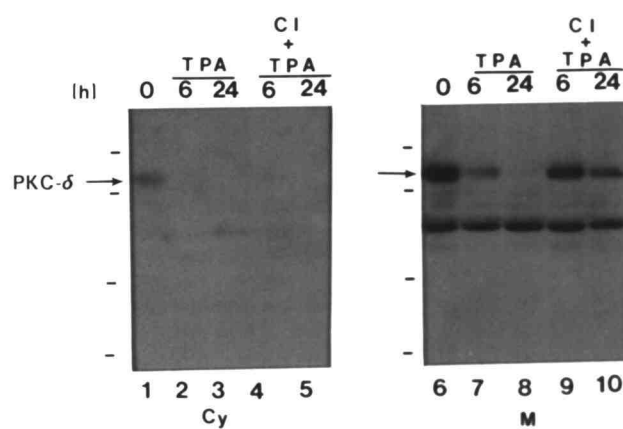


Figure 5.4. Effects of calpain inhibitor on TPA-induced down-regulation of PKC- δ of L8 myotubes. L8 myotubes were treated with 300 ng/ml TPA in the absence or presence of 100 nM calpain inhibitor (N-Acetyl-leucyl-leucyl-methionial). Cytosolic (lane 1-5) and membrane (lane 6-10) fractions were prepared for Western blot analysis. Positions of molecular weight markers are shown on the left: (from the top) 97.4, 66.2, 42.7, and 31.0 kDa.

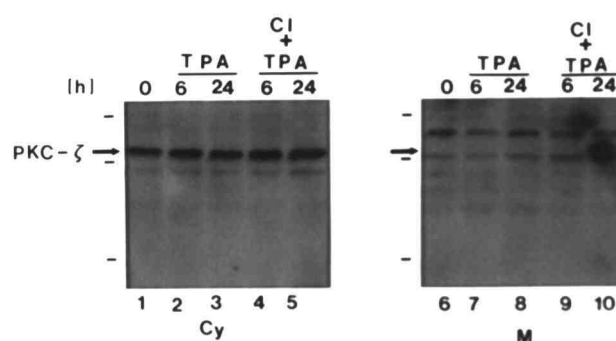


Figure 5.5. Effects of calpain inhibitor on TPA-induced down-regulation of PKC- ζ of L8 myotubes. L8 myotubes were treated with 300 ng/ml TPA in the absence or presence of 100 nM calpain inhibitor (N-Acetyl-leucyl-leucyl-methionial). Cytosolic (lane 1-5) and membrane (lane 6-10) fractions were prepared for Western blot analysis. Positions of molecular weight markers are shown on the left: (from the top) 97.4, 66.2, 42.7, and 31.0 kDa.

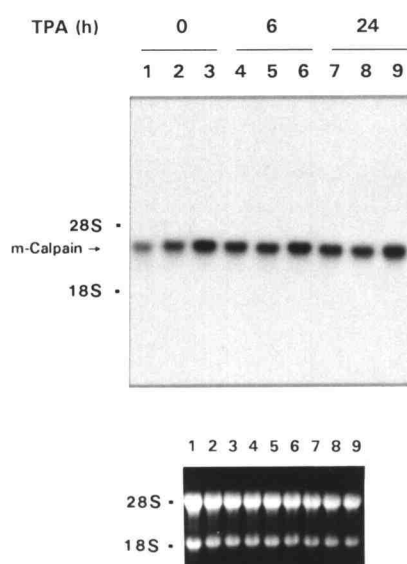


Figure 5.6. Effects of TPA on m-calpain mRNA abundance in L8 myotubes. Total RNA (20 μ g) extracted from cells cultured in triplicate for the indicated times (control; lanes 1,2,3 :6 hr; lanes 4,5,6: 24 hr; lanes 7,8,9) with 300 ng/ml TPA was examined by Northern blot analysis (top panel). Relative amounts of RNA loaded in each lane were visualized by staining with ethidium bromide (bottom panel).

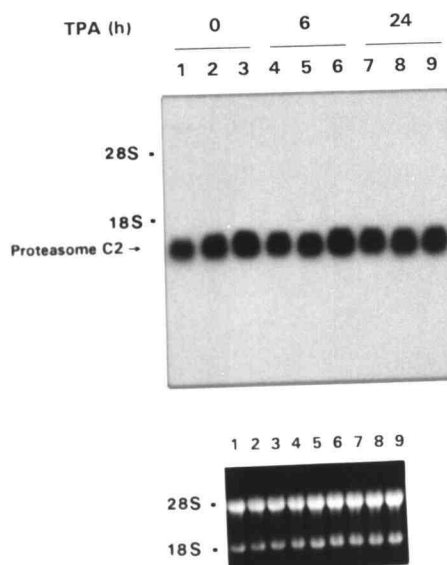


Figure 5.7. Effects of TPA on proteasome mRNA abundance in L8 myotubes. Total RNA (20 μ g) extracted from cells cultured in triplicate for the indicated times (control; lanes 1,2,3 :6 hr; lanes 4,5,6: 24 hr; lanes 7,8,9) with 300 ng/ml TPA was examined by Northern blot analysis (top panel). Relative amounts of RNA loaded in each lane were visualized by staining with ethidium bromide (bottom panel).

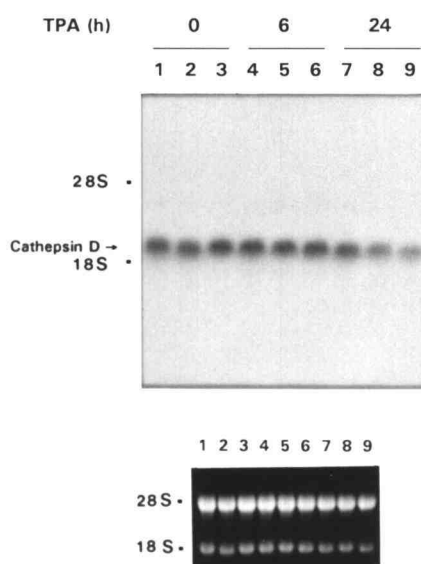


Figure 5.8. Effects of TPA on cathepsin D mRNA abundance in L8 myotubes. Total RNA (20 μ g) extracted from cells cultured in triplicate for the indicated times (control; lanes 1,2,3 :6 hr; lanes 4,5,6: 24 hr; lanes 7,8,9) with 300 ng/ml TPA was examined by Northern blot analysis (top panel). Relative amounts of RNA loaded in each lane are visualized by staining with ethidium bromide (bottom panel).

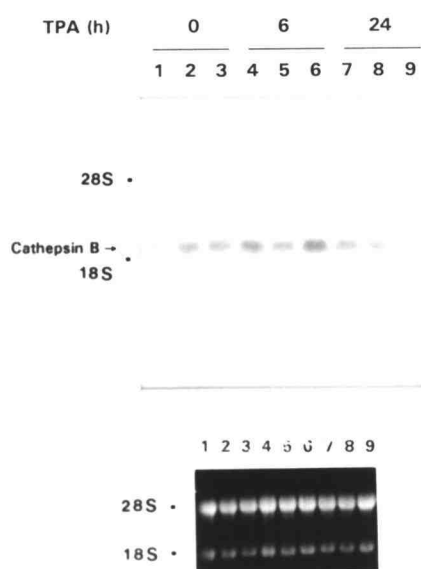


Figure 5.9. Effects of TPA on cathepsin B mRNA abundance in L8 myotubes. Total RNA (20 μ g) extracted from cells cultured in triplicate for the indicated times (control; lanes 1,2,3 :6 hr; lanes 4,5,6: 24 hr; lanes 7,8,9) with 300 ng/ml TPA was examined by Northern blot analysis (top panel). Relative amounts of RNA loaded in each lane were visualized by staining with ethidium bromide (bottom panel).

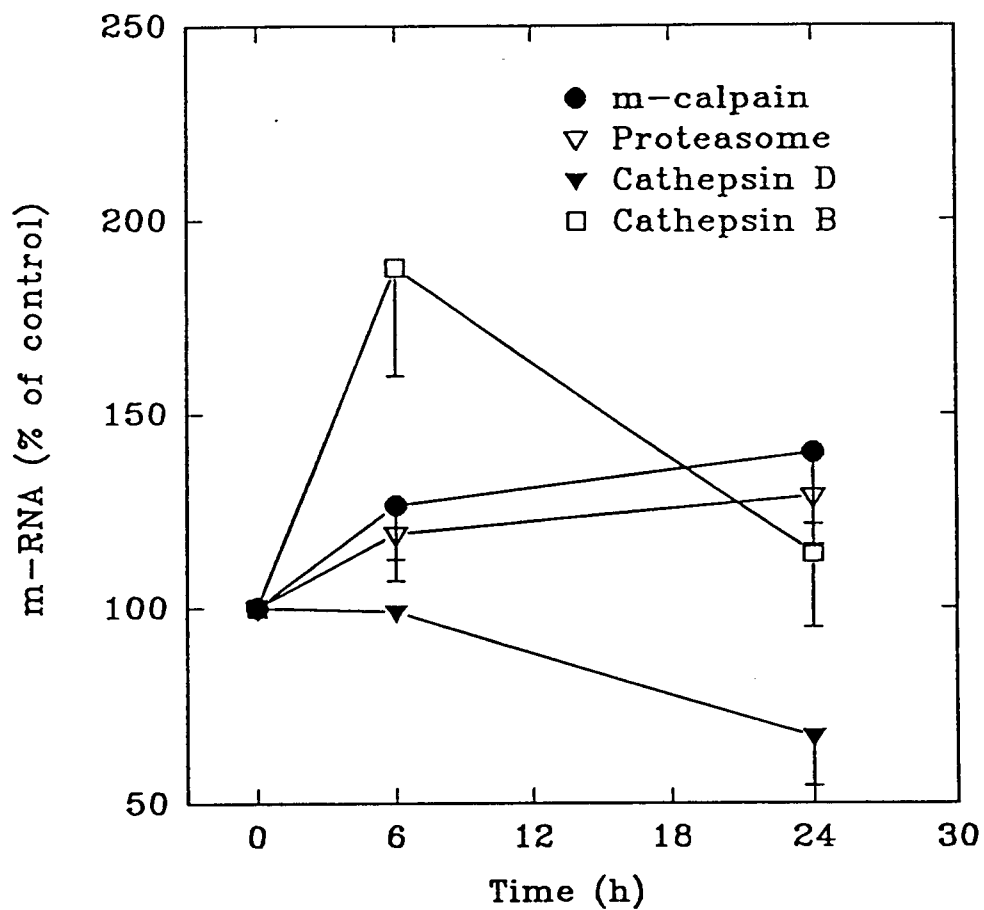


Figure 5.10. Time course showing the effects of TPA on the abundance of m-calpain, proteasome, cathepsin D and cathepsin B mRNA in L8 myotubes. Autoradiograms shown in Fig. 5.6., 5.7, 5.8, and 5.9 were quantitated by scanning densitometer.

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