AN ABSTRACT OF THE THESIS OF

Jan-ying Yeh for the degree of Master of Science in Animal Science
presented on September 4, 1990

Title: Temporal Effects of Dexamethasone on Skeletal Muscle Protein Metabolism in Rabbits

Abstract approved: Redacted for Privacy Dr. Neil E. Forsberg

Glucocorticoids are growth-inhibiting steroids. They have been reported to reduce muscle growth by reducing protein synthesis. However, their actions on muscle protein degradation remain equivocal. Glucocorticoids have been reported to transiently increase muscle protein degradation, to not affect this process and to reduce muscle protein degradation. Reasons for these conflicting reports are not understood but may be related to species, glucocorticoid doses and route and duration of administration.

Dexamethasone is a synthetic glucocorticoid and is not rapidly metabolized in vivo. As an inflammatory agent, dexamethasone is more potent than natural glucocorticoids. The objective of this study was to clarify the role in molecular regulation of calpain expression as a proteolytic catalyst linked to initiation of myofibrillar-protein degradation by using glucocorticoid-dependent changes in muscle protein degradation as a model. A secondary objective of investigating molecular mechanisms responsible for regulation of
calpain expression was conducted by examining effects of a synthetic glucocorticoid ---
dexamethasone on calpains and calpastatin activities and steady-state mRNA
concentrations encoding these proteins.

Female New Zealand White rabbits (1.8-2.1 kg) were treated with 1 mg
dexamethasone/kg BW/day for 0 day, 1 day, 2 days or 4 days by daily subcutaneous
injection. Cranial biceps femoris were taken for analysis of muscle protein concentration,
muscle RNA concentration, ribosomal capacity, N’-methylhistidine (NMH) concentration
and calpains and calpastatin activities. Because glucocorticoids may mediate their actions
indirectly via other hormones, temporal effects of dexamethasone on plasma T₃ and T₄
concentrations were also examined.

Dexamethasone transiently decreased (P < .05) final body weight and total body
weight gain in the 1-day dexamethasone-treated rabbits, but food intake was maintained
in both control and dexamethasone-treated rabbits (P > .05). Muscle protein
concentration was unaffected (P > .05) by dexamethasone, while dexamethasone
decreased (P < .05) muscle RNA concentration in the 4-day dexamethasone-treated
rabbits and tended to decrease ribosomal capacity (P > .05) gradually as duration of
dexamethasone treatment increased.

Although urinary NMH excretion, which serves as an index of myofibrillar protein
degradation, was not affected by dexamethasone (P > .05), the ratio of urinary NMH
excretion to urinary creatinine output was reduced significantly (P < .05) by 4 days of
dexamethasone treatment compared to 1 day of dexamethasone treatment. Also, muscle
NMH concentration was reduced (P < .05) by dexamethasone treatment. These data
indicate that dexamethasone treatment may have reduced muscle protein degradation.
Calpain I, calpain II and calpastatin activities were not affected by dexamethasone (P > .05) although both calpain I and calpain II activities tended to decrease and calpastatin activity had a tendency to increase as duration of dexamethasone treatment increased. Maximum effects of dexamethasone on both calpains, urinary NMH excretion and muscle NMH concentration were detected following 2 days of administration. These results indicated that the temporal decrease in rabbit skeletal muscle protein degradation by dexamethasone was related to calpains and calpastatin. mRNA concentrations encoding calpain I increased (P < .05) in the 1-day dexamethasone-treated rabbits, while mRNA concentrations encoding calpain II decreased (P < .05). These results imply that dexamethasone can affect calpain I and calpain II gene expression in an opposing manner (up-regulation and down-regulation).

Plasma T₃ concentration but not plasma T₄ concentration was significantly reduced (P < .05) by dexamethasone treatment. Because T₃ stimulates myofibrillar protein degradation, its lower concentration in dexamethasone-treated rabbits may account for the apparent reduction in protein degradation caused by dexamethasone.

In this study, the observed effects of dexamethasone on muscle protein degradation may be the combination of direct and indirect responses. Thus, in vitro studies will be needed in order to clarify the direct effects of dexamethasone on muscle protein degradation.
Temporal Effects of Dexamethasone on Skeletal Muscle Protein Metabolism in Rabbits

by

Jan-ying Yeh

A THESIS

Submitted to
Oregon State University

in partial fulfillment of
the requirements for the
degree of
Master of Science

Completed September 4, 1990
Commencement June, 1991
ACKNOWLEDGEMENT

I would like to thank Dr. Neil E. Forsberg for his encouragement, support and assistance. Special thanks are expressed to my committee members, Dr. L. J. Koong, Dr. Frank Moore and Dr. Frank Conklin, for their kindly assistance and support. Also, I would like to thank Dr. Alfred Menino for the generous use of his laboratory equipments.

Many thanks are due to my friends and fellow graduate students in our laboratory, especially Cheng-Chung Liu and Mohamma Alayan. Their friendship and assistance were invaluable. I would also like to thank many other faculty, graduate students and secretaries, especially Helen Chesbrough and Stan Taylor, for their assistance and friendship during this project.

Finally, I would like to thank my family, especially my husband --- Bor-rung Ou who started me toward this degree. Their encouragement and support helped make my degree successful.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>3</td>
</tr>
<tr>
<td>Muscle Protein Turnover</td>
<td>3</td>
</tr>
<tr>
<td>Proteases in Muscle</td>
<td>6</td>
</tr>
<tr>
<td>Lysosomal proteases</td>
<td>6</td>
</tr>
<tr>
<td>ATP-dependent proteases and their endogenous inhibitor</td>
<td>8</td>
</tr>
<tr>
<td>Calcium-dependent proteases and</td>
<td>10</td>
</tr>
<tr>
<td>Glucocorticoids and Muscle Protein Degradation</td>
<td>20</td>
</tr>
<tr>
<td>Effects of glucocorticoids on growth</td>
<td>20</td>
</tr>
<tr>
<td>Effects of glucocorticoids on various tissues and muscle fiber types</td>
<td>21</td>
</tr>
<tr>
<td>Effects of glucocorticoids on muscle protein turnover</td>
<td>22</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>26</td>
</tr>
<tr>
<td>Animal Treatment</td>
<td>26</td>
</tr>
<tr>
<td>Muscle Protein Determination</td>
<td>27</td>
</tr>
<tr>
<td>Muscle RNA Determination</td>
<td>28</td>
</tr>
<tr>
<td>Plasma T₃ and T₄ Concentration</td>
<td>28</td>
</tr>
<tr>
<td>Urinary Creatinine Quantitation</td>
<td>29</td>
</tr>
<tr>
<td>Urinary and Muscle N⁰-methylhistidine (NMH) Determination</td>
<td>30</td>
</tr>
<tr>
<td>Assay of Calpain Activities</td>
<td>31</td>
</tr>
<tr>
<td>Assay of Calpastatin Activities</td>
<td>32</td>
</tr>
<tr>
<td>Preparation of cDNA Probe</td>
<td>33</td>
</tr>
<tr>
<td>Total RNA Extraction and Quantitation</td>
<td>37</td>
</tr>
<tr>
<td>Northern Blotting</td>
<td>38</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>39</td>
</tr>
<tr>
<td>RESULTS</td>
<td>40</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>44</td>
</tr>
<tr>
<td>Effects of Dexamethasone on Animal Growth</td>
<td>44</td>
</tr>
<tr>
<td>Effects of Dexamethasone on Muscle Protein Degradation</td>
<td>44</td>
</tr>
<tr>
<td>Effects of Dexamethasone on Calpains and Calpastatin</td>
<td>46</td>
</tr>
<tr>
<td>Interaction Between Dexamethasone and Thyroid Hormones</td>
<td>48</td>
</tr>
</tbody>
</table>
BIBLIOGRAPHY

APPENDICES

Preparation of Saturated Phenol Solution 84
Preparation of $^{32}$P-labeled cDNA Probe 85
LIST OF FIGURES

Figure 1. Temporal effects of dexamethasone (1 mg/kg BW/d) on rabbit plasma T₃ (triiodothyronine) concentrations. 57

Figure 2. Temporal effects of dexamethasone (1 mg/kg BW/d) on rabbit plasma T₄ (thyroxine) concentrations. 58

Figure 3. Temporal effects of dexamethasone (1 mg/kg BW/d) on muscle N'-methylhistidine (NMH) concentrations of rabbit cranial biceps femoris. 59

Figure 4. Temporal effects of dexamethasone (1 mg/kg BW/d) on steady state mRNA concentrations encoding rabbit muscle calpain I (μ-calpain). 60

Figure 5. Temporal effects of dexamethasone (1 mg/kg BW/d) on steady state mRNA concentrations encoding rabbit muscle calpain II (m-calpain). 61

Figure 6. Temporal effects of dexamethasone (1 mg/kg BW/d) on steady state mRNA concentrations encoding rabbit muscle calpastatin. 62

Figure 7. Scanning densitometry of Northern blots of mRNAs encoding rabbit muscle calpain I (μ-calpain; Fig. 4) and calpain II (m-calpain; Fig. 5). 63

Figure 8. Scanning densitometry of Northern blots of mRNA encoding rabbit muscle calpastatin (Fig. 6). 64

Figure 9. Temporal effects of dexamethasone (1 mg/kg BW/d) on mRNA concentrations encoding rabbit muscle calpain I (μ-calpain; Fig. 4) expressed as scanning densitometry units per gram muscle protein. 65
Figure 10. Temporal effects of dexamethasone (1 mg/kg BW/d) on mRNA concentrations encoding rabbit muscle calpain II (m-calpain; Fig. 5) expressed as scanning densitometry units per gram muscle protein.

Figure 11. Temporal effects of dexamethasone (1 mg/kg BW/d) on mRNA concentrations encoding rabbit muscle calpastatin (Fig. 6) expressed as scanning densitometry units per gram muscle protein.

Figure 12. Plasmid (pLU 1001) cycle map.

Figure 13. Plasmid (pLM 28) cycle map.

Figure 14. Plasmid (pCI 413) cycle map.
LIST OF TABLES

Table 1. Composition of rabbit diet. 50

Table 2. Gradient conditions for urinary and muscle NMH by HPLC. 51

Table 3. Initial body weight of each treatment and effects of dexamethasone (1 mg/kg BW/d) on rabbit skeletal muscle (cranial biceps femoris) weight, total body weight gain and food intake. 52

Table 4. Effects of dexamethasone (1 mg/kg BW/d) protein concentration, RNA concentration and ribosomal capacity of rabbit skeletal muscle (cranial biceps femoris). 53

Table 5. Temporal effects of dexamethasone (1 mg/kg BW/d) on urinary N'-methylhistidine (NMH) excretion and urinary creatinine output. 54

Table 6. Temporal effects of dexamethasone (1 mg/kg BW/d) on activities of calpain I, calpain II and calpastatin in rabbit skeletal muscle (cranial biceps femoris). 55

Table 7. Temporal effects of dexamethasone (1 mg/kg BW/d) on mRNA concentrations encoding calpain I, calpain II and calpastatin in cranial biceps femoris expressed as scanning densitometry units and as scanning densitometry units per gram muscle protein (units/g protein). 56
LIST OF APPENDICES

Preparation of saturated phenol solution. 84

Preparation of $^{32}$P-labeled cDNA probe. 85
INTRODUCTION

Muscle growth is balanced between protein degradation and protein synthesis. It is believed that protein degradation is an important determinant of the rate of muscle growth in domestic animals, but very little is known regarding mechanisms of muscle protein degradation (Allen, 1986).

In general, glucocorticoids are considered to be growth-inhibiting steroids. It has been shown that growth in adrenalectomized rats was not affected by a physiological replacement dose of glucocorticoids (Young, 1980), while large doses of glucocorticoids reduced growth rates and caused muscle atrophy (Morris et al., 1968). In muscle, glucocorticoids exert catabolic effects on muscle growth by altering muscle protein degradation. In studies of glucocorticoid effects on rats, muscle protein degradation was increased during the initial 3-5 days of glucocorticoid treatment, then returned to normal after this period. This implies that glucocorticoids may cause a temporal increase in muscle protein degradation in vivo (Young and Munro, 1978; Tomas et al., 1979; Odedra et al., 1983; Kayali et al., 1987). Several other studies have shown that glucocorticoids may increase, decrease or have no effect on muscle protein degradation (Kayali et al., 1987; McGrath and Goldspink, 1982; Santidrian et al., 1981). Reasons for lack of a consistent effects of glucocorticoids on muscle protein degradation are unknown. These inconsistent results may be due to different methodology and duration of administration (Sharpe et al., 1986). Furthermore, enzymatic and molecular
mechanisms underlying glucocorticoid-dependent up- or down-regulation of myofibrillar protein degradation have not yet been characterized.

Muscle proteins are degraded by a complex mixture of proteinases including extralysosomal mechanisms (ATP-dependent proteinases and calpains) and lysosomal proteinases (cathepsins). It has been proposed that calpains initiate degradation of myofibrillar proteins by dissociation of the Z-disk and that further digestion of myofibrillar proteins is mediated via lysosomal mechanisms. However, recent evidence also indicates that ATP-dependent proteinases may play important roles in degradation of myofibrillar proteins.

To test the hypothesis that calpains are rate-limiting to myofibrillar protein degradation, we chose a model of dexamethasone treatment. Dexamethasone and glucocorticoids, in general, are growth-inhibiting steroids but their actions on muscle protein degradation are equivocal. Although most reports show that glucocorticoids effect a transient increase in muscle protein degradation, other studies have shown that they also do not affect or attenuate this process.

Objectives of this study were to clarify the roles that calpains play in myofibrillar protein degradation using glucocorticoid myopathy as a model. Where regulation of calpain activity was detected, we investigated molecular mechanisms responsible for this regulation by investigating effects of glucocorticoids on steady-state mRNA concentrations encoding these proteins.
LITERATURE REVIEW

Muscle Protein Turnover

Fifty years ago, it was reported that muscle growth and accumulation depended upon both protein degradation and protein synthesis (Schoenheimer and Rittenberg, 1940). The quantitative balance between protein degradation and protein synthesis determines the net rate of accumulation of muscle proteins and net loss of muscle proteins occurs whenever the rate of protein degradation exceeds the rate of protein synthesis.

During normal growth and in many metabolic states the rates of protein degradation and protein synthesis tend to move in tandem (Millward, 1985). Many studies have demonstrated the balance between protein degradation and protein synthesis in animals (Millward et al., 1976; MacDonald and Swick, 1981; McCarthy et al., 1983; Lewis et al., 1984). Rapid growth in young animals is accompanied by increasing both protein degradation and protein synthesis with protein synthesis exceeding protein degradation. When animals reach maturity, both protein degradation and protein synthesis decrease and reach an equivalent rate.

The myofibrillar proteins in skeletal muscle cells consist of two common filaments --- thick filaments in which myosin is the major protein and thin filaments which contain actin (beaded backbone of the thin filament), tropomyosin and troponin (regulatory units of the thin filament). These myofibrils make up the myofibrillar proteins which constitute 50-55% of total muscle proteins and are the major class of muscle proteins (Goll et al., 1976). Other portions of muscle proteins are composed of sarcoplasmic
proteins and stromal proteins (about 35% and 15-20% of total muscle proteins, respectively). Muscle protein is in a constant state of flux. If the rate of muscle protein degradation is 5-8% per day, then animals must synthesize new muscle proteins in the equivalent rate of 5-8% per day in order to maintain muscle mass (Allen, 1986). It had been estimated that about 15-25% of energy ingested by domestic animals is used to synthesize new proteins in order to replace degraded proteins (Young et al., 1975). Consequently, muscle protein turnover is a significant factor in maintenance of animals and the efficiency of converting ingested nutrients to edible muscle should be able to increase by decreasing rate of muscle protein degradation (Goll et al., 1989). Therefore, muscle protein degradation is an attractive target for postnatal growth manipulation research. If muscle protein degradation could be altered in a favorable way, the net accumulation rate of muscle proteins would be increased and less energy would be expended on resynthesizing degraded muscle proteins (Allen, 1986).

Both muscle protein degradation and synthesis are regulated by physiological and nutritional status (Forsberg et al., 1989). Rapid loss of skeletal muscle mass which occurs during various muscle pathologies, such as the muscular dystrophies, is primarily due to an increase in muscle protein degradation and little change in muscle protein synthesis (Li, 1980). Inflammation, burns and fever can also affect muscle protein turnover (Goldberg et al., 1984). Therefore, variations in muscle protein degradation could be a primary determinant of muscle growth in domestic animals (Goll et al., 1989).

It is not clear whether alterations in muscle protein degradation would have any effect on efficiency of muscle growth. During past years, most animal scientists have
focused their research areas on altering muscle growth rate by altering rate of muscle protein synthesis. A great deal is known about the mechanism of muscle protein synthesis and how it is controlled, but little is known about the mechanism of muscle protein degradation. In order to understand the mechanism of muscle protein degradation, it is important to learn what kinds of enzymes are involved in muscle protein degradation and how these enzyme activities are controlled in vivo (Goll et al., 1989).
Proteases in Muscle

Proteolysis is an important component of most muscle diseases and it has generally been assumed that proteolytic enzymes are responsible for protein degradation during metabolic turnover (Goldberg and Dice, 1974; Pontremoli and Melloni, 1986). Therefore, it is important to know which proteolytic enzymes exist inside muscle cells and which are involved in muscle protein degradation (Goll et al., 1989). There are several proteolytic enzymes involved in muscle protein degradation and they can be classified into two groups --- lysosomal proteases and extralysosomal proteases. The extralysosomal proteases include ATP-dependent proteases and calcium-dependent proteases.

Lysosomal proteases

It was traditionally assumed that lysosomal proteases were responsible for intracellular protein degradation. These proteases are located in lysosomes and are active at acidic pH. Also, they are involved in degradation of endocytosed proteins and some endogenous proteins. Several different proteases are grouped into this system and called cathepsins. Not all cathepsins are able to cleave peptide bonds of myofibrillar proteins; only cathepsins B, D, H and L have been found in muscle and have proteolytic activities in skeletal muscle cells (Goll et al., 1983). Cathepsin B is generally distributed in lysosomes of mammalian muscle cells and functions as an endopeptidase. It has a molecular weight of 25 kDa with an -SH active site. It was reported that cathepsin B could degrade myofibrillar proteins --- both actin and myosin (Schwartz and Bird, 1977), but other studies have shown that cathepsin B was unable to degrade myofibrillar proteins.
and suggested that previous results, which showed that cathepsin B could degrade myofibrillar proteins, were due to cathepsin L contamination (Okitani et al., 1980; Matsukura et al., 1981). However, the role of cathepsin B in control of myofibrillar protein degradation remains unclear.

Cathepsin D is widely distributed in lysosomes of all mammalian muscle cells. It is a glycoprotein with approximate 42 kDa molecular weight and has -COOH active site. It functions as an endopeptidase and can digest proteins and some peptides of at least five residues. It was reported that cathepsin D could degrade both actin and myosin (Schwartz and Bird, 1977; Okitani et al., 1981). Cathepsin H is a glycoprotein with approximate 28 kDa molecular weight and has an -SH active site. It is heat-stable and can function as an endopeptidase. Also, it was reported that cathepsin H could degrade myosin (Schwartz and Bird, 1977; Bird and Carter, 1980). Cathepsin L has an approximate 24 kDa molecular weight and is very labile to autolysis. It functions as an endopeptidase and is very active against proteins but less active against peptide substrates. It had been reported that cathepsin L could also degrade myosin heavy chain, actin, α-actinin, troponin T and tropinin I (Matsukura et al., 1981).

It had been reported that skeletal muscle cells contain few lysosomes and, hence, few lysosomal proteases (Goll et al., 1989). Despite this, skeletal muscle cells still have enough cathepsins to degrade all of the muscle proteins within 10 days (Schwartz and Bird, 1977). Although lysosomal proteases can degrade myofibrillar proteins, there is some evidence indicating that myofibrillar protein degradation is not initiated by lysosomal proteases (Lowell et al., 1986): (1) Neither intact myofibrils nor entire thick
and thin filaments have been found inside lysosomes even in muscle cells undergoing dystrophy; however, products of myofibrillar protein degradation have been found in lysosomes (Gerard and Schneider, 1979). (2) Lysosomal cathepsins B, D, H and L can degrade myofibrillar proteins at a pH below 6.0, but they have little or no effect on myofibrillar proteins at the pH of the sarcoplasm (Schwartz and Bird, 1977; Bird et al., 1978; Matsukura et al., 1981; Okitani et al., 1981). (3) Rapid muscle atrophy is not prevented by lysosomal protease inhibitors (Wildenthal et al., 1980) and has been observed in the absence of lysosomes (Tweedle et al., 1974). (4) Treatment of muscle cells with lysosomal enzyme inhibitors failed to inhibit calcium-induced protein degradation (Furuno and Goldberg, 1986). Therefore, it seems that myofibrillar protein degradation is initiated by cytoplasmic proteases and not by lysosomal proteases. Lysosomal proteases may be involved in the secondary digestion of myofibrillar proteins (Goll et al., 1989).

**ATP-dependent proteases**

It has been found that ATP-dependent proteases exist in skeletal muscle, but due to the difficulties associated with their purification, their physiological functions have not been thoroughly examined (Fagan et al., 1987; Kettelhut et al., 1988). This proteolytic system includes an ATP-dependent ubiquitin-dependent proteolytic system and an ATP-dependent ubiquitin-independent proteolytic system.

The ATP-dependent ubiquitin-dependent proteolytic system contains a small, heat-stable protein --- ubiquitin (Ub), which interacts with an activating enzyme in an ATP-dependent process to form a covalent isopeptide bond between the carboxyl group of C-
terminal glycine residue of Ub and an ε-amino group of a lysine residue on target protein. Then, the Ub-protein complex is quickly degraded into peptides and the released Ub can be recycled (Wilkinson et al., 1980; Hershko and Ciechanover, 1982). A neutral protease --- ubiquitin-conjugated degrading enzyme (UCDEN), is involved in this ATP-dependent ubiquitin-dependent proteolytic system. This protease, which has approximate 1500 kDa molecular weight, is inside muscle cells and is composed of several polypeptide subunits. It is heat-labile and its optimal pH is in the range of 7.0-8.5. This protease cleaves only Ub-conjugated proteins and is not inhibited by potent inhibitors of cysteine proteases (Allen, 1986). Although UCDEN can degrade proteins in crude muscle extracts, it is unknown whether it can degrade myofibrillar proteins (Fagan et al., 1987). This ATP-dependent ubiquitin-dependent proteolytic system may be involved in degradation of abnormal and structurally damaged proteins as well as turnover of short-lived proteins in cultured cells (Goldberg and Dice, 1974; Goll et al., 1989). However, the role of this system for intracellular turnover of normal proteins in mammalian cells was unclear.

In addition to ubiquitin-related proteases, muscle also contains an ATP-dependent ubiquitin-independent proteolytic system which is stimulated by the presence of ATP, but does not require ubiquitin (Fagan et al., 1987; Waxman et al., 1987). Two proteases are involved in this proteolytic system. The first protease is multicatalytic protease which is composed of 8 subunits of 19-36 kDa and has an approximate 750 kDa molecular weight. It is located in muscle cells and is optimately activated at pH 8.0-10.0 (Dahlmann et al., 1985; Fagan et al., 1987; Waxman et al., 1987). The multicatalytic protease has very
little proteolytic activity unless stimulated by SDS or lipids, and it can degrade sarcoplasmic protein fractions of rat skeletal muscle (Tanaka et al., 1986). The second protease with approximate 600 kDa molecular weight can degrade both Ub-conjugated and non-Ub-conjugated proteins (Tanaka et al., 1984; Hough et al., 1986; Fagan et al., 1987; Waxman et al., 1987). The ATP-dependent ubiquitin-independent protease seems to be a cysteine protease because high concentrations of leupeptin, a potent cysteine protease inhibitor, are required to inhibit its activity. It is likely that ATP-dependent ubiquitin-independent proteases are involved in muscle protein turnover although the nature of these proteases is still unclear.

**Calcium-dependent proteases and their endogenous inhibitor**

In 1972, Busch et al. discovered that skeletal muscle sarcoplasm contained Ca\(^{++}\)-activated factors which could quickly remove Z-disks from intact myofibrils without causing other large changes in myofibrillar structure. In 1976, these factors, calpains, were purified and described by Dayton et al. Calpains (EC 3.4.22.17), which are also known as calcium-dependent proteases (CDP) and calcium-activated neutral proteases (CANP), are intracellular nonlysosomal cysteine proteases with approximate 110 kDa molecular weight and are distributed widely in the sarcoplasm of skeletal muscle cells as well as many other cell types (Busch et al., 1972; Dayton et al., 1976; Kay, 1984). Calpains, which require Ca\(^{++}\) for activities, are fully active at neutral pH in the presence of reducing agents, such as 2-mercaptoethanol (2-Me), and their activities are inhibited by thiol group-modifying reagents as well as Ca\(^{++}\)-chelating reagents, such as EGTA (Mellgren et al., 1987; Suzuki, 1987; Suzuki et al., 1987). The potent inhibitors of
calpains are leupeptin, antipain and E-64, which is an epoxysuccinyl derivative (Parkes et al., 1985). It has been reported that native calpains may be inactive proenzymes which can be converted to active enzymes by autolysis. The autocatalytic activation of calpains is an intramolecular process which requires Ca\(^{++}\) but is not affected by the presence of substrate or calpain concentrations (Coolican and Hathaway, 1984; Suzuki et al., 1987).

Two forms of calpains, calpain I (\(\mu\)-calpain) and calpain II (m-calpain), are found in mammalian muscle cells with distinct calcium requirements (Dayton et al., 1981; Murachi and Yoshimura, 1985; Yoshimura et al., 1986; Kleese et al., 1987; Sorimachi et al., 1989). It has been reported that calpain I, which has high calcium sensitivity, requires only micromolar Ca\(^{++}\) concentration for activity, while calpain II, which has low calcium sensitivity, requires millimolar Ca\(^{++}\) concentration for activity. Although these two isozymes, which consist of identical small subunits (30 kDa) and different large subunits (80 kDa), are similar in various molecular and enzymatic properties, they are distinctly different proteins which share a high degree of sequence homology (Emori et al., 1986a, b).

The gene structure of calpain comprises at least 21 exons and is about 10 kbp long. Also, the primary structures of calpains have been determined by cDNA cloning studies and the small subunits and partial structures for large subunits of both rabbit calpains have been elucidated (Emori et al., 1986a, b; Ohno et al., 1986; Imajoh et al., 1988). The small subunits (30 kDa) of calpain I and calpain II originate from the same gene, while the large subunits (80 kDa) of both isozymes originate from separate genes although their amino acid sequences have 52% homology (Emori et al., 1986a).
Therefore, the polypeptides of small subunits for both isozymes are identical and the polypeptides of large subunits for both calpains are homologous but not identical. The small subunits (30 kDa) of calpains are considered to be regulatory subunits, while the large subunits (80 kDa) of calpains are regarded as catalytic subunits (Emori et al., 1986a, b, c; Suzuki, 1987; Sorimachi et al., 1989).

The large subunit is composed of 4 functional domains --- domains I-IV from N-terminus (Ohno et al., 1984; Emori et al., 1986a; Suzuki et al., 1987; Sorimachi et al., 1989). Domain I masks the cysteine residue active-site and is processed during activation of pro-calpains (Suzuki et al., 1987). The large subunit of calpain I is larger than that of calpain II and this may be due to different sizes of domain I. Domain II is a cysteine protease domain which is homologous to thiol proteases, such as papain and cathepsins B, H and L, and is responsible for the calpain activity (Sorimachi et al., 1989). Like other cysteine proteases, domain II should be active without Ca++ when isolated from calpains. Integration of domain II into whole calpain will inhibit or repress the calpain activity through interaction between domain II and other domains, while binding of Ca++ to calcium-binding domains (domain IV and domain IV’) will cause a conformational change and remove the inhibition so that calpain activity can be expressed. Domain IV is a calmodulin-like domain and is homologous to calcium-binding proteins, such as calmodulin. It contains four potential calcium-binding regions which consist of E-F hand structures. Domain IV can bind Ca++ and regulates the activity of domain II. The function of domain III remains unknown.

The small subunit is composed of at least two domains --- domain IV’ and domain
V from N-terminus (Emori et al., 1986b; Suzuki et al., 1987; Sorimachi et al., 1989). Domain IV' is similar to domain IV of large subunit with 50% sequence homology. It is a calmodulin-like domain which can bind Ca\(^{++}\) and regulates domain II activity of the large subunit. Domain V is a glycine-rich hydrophobic domain and is essential for the interaction of the enzymes with phospholipids, liposomes and probably with biological membranes to decrease the Ca\(^{++}\) requirements for calpain autolysis (Suzuki et al., 1988; Sorimachi et al., 1989).

Calpains exist in large amounts in various tissues and cells of vertebrates and may function in various cellular events mediated by Ca\(^{++}\) such as turnover of myofibrillar proteins and regulation of enzyme functions (Suzuki et al., 1987). The typical feature of calpain action is limited proteolysis. Calpains generally cleave only one or a few peptide bonds of a protein in order to hydrolyze protein substrates into large fragments, but not to small peptides or amino acids (Goll et al., 1983; Kay, 1984). Also, calpains have no strict requirement for amino acid sequence in the cleavage sites. Instead, calpains may recognize higher-order structures of proteins (Suzuki et al., 1987). By their limited proteolysis, calpains may regulate functions of several proteins, especially membrane-bound proteins, such as protein kinase-C and phosphorylase. In myofibrils, calpains can affect many regulatory and structural proteins, such as troponin-T, troponin-I, tropomyosin and the Z-disk. But actin and myosin, which are the primary proteins in myofibrils, can not be hydrolyzed by calpains (Goll et al., 1983).

Influx of calcium into cells can increase myofibrillar protein degradation in vitro (Silver and Etlinger, 1985) and lysosomal protease inhibitors can not inhibit this calcium-
induced response. Therefore, the calcium-induced increase in myofibrillar protein
degradation is of nonlysosomal origin (Furuno and Goldberg, 1986). There is much
evidence, which has been summarized by Goll et al. in 1989, indicating that calpains are
involved in the initiation of myofibrillar protein degradation and are responsible for
muscle atrophy in muscular dystrophy (Kay, 1984): (1) The growth and turnover of
myofibrillar protein is achieved by adding and removing filaments from their surface
rather than to their interiors (Morkin, 1970). Myofibrils in rapidly atrophying muscle
typically have diminished diameters rather than being fragmented (Pellegrino and
Franzini, 1963), and it has been found that calpains have specific effects on myofibrils
to cause disassembly of filaments from the myofibril surface (van der Westhuyzen et al.,
1981). (2) It has been found that calpain activity increases during the rapid muscle
atrophy in rabbits and in human (Kar and Pearson, 1976; Dayton et al., 1979). (3) Z-disk
degradation is the most rapid effect of calpains on myofibrils (Dayton et al., 1975, 1976
and 1981), and Z-disk alterations including complete loss of Z-disks are one of the most
consistent features found in rapidly atrophying muscle afflicted with muscle dystrophy
(Cullen and Fulthorpe, 1982). (4) Effects of calpains on proteolytic changes of myofibrils
and the changes occurring in myofibrils during muscle dystrophy are very similar (Sugita
et al., 1980; Obinata et al., 1981).

The mechanism for calpain activation in vivo has been hypothesized by several
investigators. Pro-calpain exists mainly in cytosol. When Ca\textsuperscript{++} concentration increases
to a near micromolar level, the -SH active site of calpain, which is buried inside the
calpain molecule, is exposed to the surface by a conformational change induced by
binding of Ca$$^{++}$$ and then calpain can translocate to the cell membrane. Autolysis of calpain occurs at the cell membrane in the presence of Ca$$^{++}$$ and phospholipid then calpain becomes active as a membrane-bound enzyme (Coolican and Hathaway, 1984). Membrane-bound calpain can be dissociated from the cell membrane by EGTA or by the endogenous calpain inhibitor (calpastatin) which inhibits the binding of calpain and promotes the release of calpain from the cell membrane (Pontremoli et al., 1985; Gopalakrishna and Barsky, 1986). Recently, some studies suggested that proteolysis of calpains is regulated in vivo by associating calpains with a phosphatidylinositol-containing site on the plasma membrane followed by calpain autolysis. This association with phosphatidylinositol decreases the Ca$$^{++}$$ requirement for calpain autolysis and further lowers the Ca$$^{++}$$ requirement for proteolysis. Even so, calpains still can not be proteolytically activated at the Ca$$^{++}$$ concentration which is present inside muscle cell. Therefore, muscle cells must have other mechanisms which differ from membrane association and autolysis in order to activate calpains (Cong et al., 1989).

It has been proposed that calpains initiate myofibrillar protein degradation by releasing thick and thin filaments from the surface of myofibrils (Dayton et al., 1975). Autolyzed calpains degrade the Z-disk by removing C-protein rings from thick filaments and troponin T, troponin I as well as tropomyosin from thin filaments. Degradation of these proteins results in loosening of the myosin molecules from the surface of thick filaments and actin molecules from thin filaments allowing their release into the surrounding sarcoplasm. Because calpains can not degrade myofibrillar proteins to amino acids and have little or no effect on purified actin and myosin, actin and myosin
molecules, which are dissociated into monomers and dimers, must be further degraded to amino acids by other proteases, possibly lysosomal proteases (Dayton et al., 1975).

Two types of mammalian calpains, calpain I (\(\mu\)-calpain) and calpain II (m-calpain), exist in almost all organs but their amounts vary significantly in different tissues. This implies that both calpain isozymes play fundamental roles in different tissues, although the physiological substrates of calpains have not yet been elucidated clearly (Sorimachi et al., 1989). Both calpain I and calpain II are very similar and have similar effects on myofibrillar protein turnover, but it remains unclear whether calpain I, calpain II or both calpain isozymes can degrade myofibrillar proteins in vivo (Dayton et al., 1981; Forsberg et al., 1989). It has been reported that both calpain isozymes have identical reactions with a variety of inhibitors (Szpacenko et al., 1981). Either calpain I or calpain II will rapidly degrade many denatured proteins by hydrolyzing the same peptide bonds (Goll et al., 1989). Different tissues seem to have different relative proportions of calpain I and calpain II. It has been reported that skeletal muscle of most mammalian species contains about 60-80% of total calpain activities as calpain II. However, the physiological significance of these different proportions of both calpain isozymes still remains unknown (Goll et al., 1989).

In general, calpain II degrades proteins only to large fragments rather than to small peptides or free amino acids (Dayton et al., 1975; Goll et al., 1983), and it has a limited and unique specificity. Calpain II can degrade some contractile and cytoskeletal proteins (Dayton et al., 1976; Waxman, 1987), but it does not cause degradation of crude sarcoplasmic proteins from rabbit skeletal muscle. Therefore, it is unlikely that calpain
II is involved in sarcoplasmic protein turnover (Tan et al., 1988). However, in isolated myofibrils, calpain II can release myofilaments from the surface of myofibrils and the release of myofilaments can be stimulated by increasing Ca\(^{++}\) concentration and inhibited by adding leupeptin (van der Westhuyzen et al., 1981). The other isozyme --- calpain I requires lower Ca\(^{++}\) concentrations than that of calpain II from the same species (Goll et al., 1989). It has been reported that calpain I may not be as sensitive to calpastatin as calpain II is. Therefore, myofibrillar protein degradation induced by calpain I may still occur despite the excess of endogenous calpain inhibitor (Kapprell and Goll, 1989). In addition to these two calpain isozymes, a skeletal muscle-specific calpain --- p94 has been found recently. p94 exists only in skeletal muscle, has an approximate 94 kDa molecular weight and it shows sequence homology with the large subunits of both calpain I and calpain II in humans and rats. It contains four different domains which are similar to those of both calpain isozymes. However, the functions and significance of p94 still remain to be identified (Sorimachi et al., 1989).

Calpastatin coexists with calpains in the cytosol of skeletal muscle (Okitani et al., 1976). Calpastatin is a multifunctional inhibitor and plays an important role in regulating calpain activities in vivo. It can inhibit both translocation of calpains to cell membranes and autolysis of calpains. Furthermore, it can remove both active and inactive calpains from cell membranes (Suzuki et al., 1988). It has been reported that calpastatin can only inhibit calpains but not other cysteine proteases and it inhibits both calpain I and calpain II equally by forming a complex in the presence of Ca\(^{++}\) (Suzuki et al., 1987). However, the inhibition of calpains by calpastatin is reversible and both active calpastatin and
Calpains can be reutilized by dissociating the calpain:calpastatin complex (Imajoh and Suzuki, 1985). Calpastatin contains four tandemly-repeated units which may correspond to the number of calpain molecules inhibited by calpastatin, and these repeated units are functional units of inhibition (Imajoh et al., 1987). It has been found that calpastatin has different molecular weights as measured by SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis) and they can be classified into two molecular species --- smaller species and larger species (Cottin et al., 1981; Nakamura et al., 1985). Smaller species with 70 kDa molecular weight exist mainly in erythrocytes, while larger species with 110 kDa molecular weight exist mainly in liver, heart and most other tissues. The properties of both molecular species are similar except for the stoichimetry of inhibition. Therefore, smaller species are presumed to be a derivative of larger species (Takano et al., 1986; Mellgren et al., 1987). In addition to these two molecular species of calpastatin, a molecular species with 34 kDa molecular weight has been found in rabbit skeletal muscle (Takahashi-Nakamura et al., 1981).

It is very difficult to purify calpastatin without using denaturing reagents and heating steps. Although calculation based on Stokes’ radius suggests that the undenatured calpastatin is monomeric, the nature of undenatured calpastatin and its interaction with calpains remain unclear (Otsuka and Goll, 1987). However, calpastatin has no apparent structural homology to other known protease inhibitors, so that the mechanism for inhibition of calpains by calpastatin may be different from those by other protease inhibitors (Emori et al., 1987; Imajoh et al., 1987).

Other soluble proteases may also be important to myofibrillar protein turnover. It has
been reported that several neutral and alkaline proteases can hydrolyze actin or myosin, but most of them are not found in muscle cells (Allen, 1986). Proteases present in muscle cells which are capable of degrading myofibrillar proteins are calpains, cathepsin B and cathepsin D. However, only calpains are active at the pH values which exist inside healthy muscle cells (Bird et al., 1980). Although calpains have been characterized, the nature of calpains and the mechanism by which their activities are regulated are still unclear. It is important to learn more about the cellular location of calpain I, calpain II and calpastatin relative to each other, as well as the regulation of calpains and calpastatin interactions in order to understand how calpain activities are regulated in vivo and how this regulation is related to muscle growth (Goll et al., 1989).
**Glucocorticoids and Muscle Protein Degradation**

A number of metabolic hormones produce either catabolic or anabolic effects on muscle protein turnover (McGrath and Goldspink, 1982; Tomas et al., 1984; Millward, 1985). These effects account for changes in muscle proteolysis associated with a variety of physiological and pathological states (Tischler, 1981).

**Effects of glucocorticoids on growth**

Glucocorticoids are generally regarded as growth-inhibiting hormones, and can maintain normal blood pressure and cardiac output in animals (Sharpe et al., 1986). Several studies have shown different relationships of plasma glucocorticoid levels with animal growth. One study has shown that plasma glucocorticoid levels were negatively related to growth rate in sheep (Purchas, 1973). On the other hand, some studies have shown that plasma glucocorticoid levels were positively related to growth rate in cattle (Trenkle and Topel, 1978; Purchas et al., 1980). One study has shown that plasma glucocorticoid levels were unrelated to growth rate in sheep (Purchas et al., 1980). It is worth mentioning that these studies relied upon total plasma glucocorticoids as an index of glucocorticoid levels, while the total concentration of plasma glucocorticoids might not reflect the concentration of the physiologically-active hormone (Sharpe et al., 1986). In addition to growth rate, high doses of glucocorticoids generally produce weight loss in animals. It has been reported that endogenous glucocorticoids exert different effects on animal body weight, either maintaining body weight (Tomas et al., 1979) or losing body weight (Kelly and Goldspink, 1982; Clark et al., 1986; Woodward and Emery, 1989). Also, food consumption in glucocorticoid-treated animals does not change (Bellamy,
1964; Odedra et al., 1983; Kayali et al., 1987). Although these results are not wholly in agreement, the overall effect of high doses of glucocorticoids on animal growth is usually catabolic (Long et al., 1940; Loeb, 1976; Tomas et al., 1979).

**Effects of glucocorticoids on various tissues and muscle fiber types**

Various body tissues respond quite differently to glucocorticoids. Glucocorticoids can cause the sparing of glucose and the tendency toward hyperglycaemia. This increases protein catabolism in muscle, adipose, skin and lymphoid tissues, and further results in release of amino acids from these tissues and a decrease of glucose uptake in these tissues (Baxter and Forsham, 1972). Glucocorticoids generally have anabolic effects in liver by increasing both glucose production and protein synthesis (Sharpe et al., 1986). In contrast to their anabolic effects on liver, glucocorticoids generally have catabolic effects on muscle.

Skeletal muscle and smooth muscle are more susceptible to catabolic actions of glucocorticoids by increasing protein degradation and decreasing protein synthesis (Kelly and Goldspink, 1982). But studies of glucocorticoid effects on cardiac muscle are contradictory. There are studies which have shown that glucocorticoids have little or no effect on cardiac muscle (Bullock et al., 1972; Rannels et al, 1978), or cardiac muscle shows little anabolic response to glucocorticoids by decreasing protein degradation and slightly increasing or having no effect on protein synthesis (Griffin and Wildenthal, 1978; Kelly and Goldspink, 1982; Nichols et al., 1984; Clark et al., 1986).

Muscle fibers can be grouped into three general categories based on their contraction speed and metabolic pathways used to provide energy for contraction (Peter et al., 1972).
The first fiber type is a white fiber (fast-twitch, glycolytic fiber) which has fast contraction speed and depends on anaerobic or glycolytic energy metabolic pathway. The second fiber type is a red fiber (slow-twitch, oxidative fiber) which has slow contraction speed and depends on oxidative metabolic pathway. The third fiber type is intermediate between white fiber and red fiber (fast-twitch, oxidative-glycolytic fiber) which has glycolytic metabolic capacity and a significant capacity for oxidative metabolism.

Individual muscles vary in fiber type composition and different muscle fiber types show different responses to glucocorticoids (Goldberg, 1969; Shoji and Pennington, 1977; Kelly and Goldspink, 1982). The white muscles, such as tibialis anterior, plantaris and extensor digitorum longus, are more susceptible to glucocorticoids and protein synthesis in white muscle fiber is inhibited by glucocorticoids (Rannels and Jefferson, 1980; Kayali et al., 1987). The red muscles, such as soleus in which protein degradation was directly inhibited by various glucocorticoids in vitro for more than 4 hr (McGrath and Goldspink, 1982), show little response to glucocorticoids and seem to be protected from the catabolic action of glucocorticoids (Kelly and Goldspink, 1982). These different responses have not been clearly defined in terms of the steroid’s actions in vivo on both protein degradation and protein synthesis. However, the different susceptibilities of different muscle fiber types may be an adaptation which protects the most physiologically active muscle against glucocorticoids (Sharpe et al., 1986).

Effects of glucocorticoids on muscle protein turnover

Myofibrillar proteins, which constitute 50-55% of total muscle proteins, are the major muscle cell proteins and are highly conserved across species. Therefore,
myofibrillar proteins play an important role in muscle protein metabolism and many developmental studies of muscle have focused on myofibrillar proteins. Effects of glucocorticoids on muscle are generally catabolic and glucocorticoid-induced muscle atrophy is caused by altering both protein synthesis and protein degradation with a larger effect on protein synthesis (McGrath and Goldspink, 1982). Several studies have shown that catabolic doses of corticosterone decrease muscle protein synthesis and transiently increase myofibrillar protein degradation in rats (Odedra et al., 1983; Kayali et al., 1987). Although these results indicate that glucocorticoids exert their effects on both muscle protein synthesis and myofibrillar protein degradation, some studies have shown that glucocorticoids only affect either muscle protein synthesis or myofibrillar protein degradation and the effects of glucocorticoids on muscle protein synthesis are more consistent than those on myofibrillar protein degradation (Rannels and Jefferson, 1980; Kelly and Goldspink, 1982; Santidrian et al., 1981; Ballard and Francis, 1983).

The suppressive effect of glucocorticoids on muscle protein synthesis was previously found both in vivo (Young, 1970; Rannels and Jefferson, 1980; Kelly and Goldspink, 1982; Odedra et al., 1983) and in vitro (McGrath and Goldspink, 1982). It was reported that these reductions in muscle protein synthesis are due to decreased DNA synthesis (Baxter and Forsham, 1972), decreased RNA content (Rannels and Jefferson, 1980), decreased efficiency of protein synthesis (Kelly and Goldspink, 1982) or interference with the initiation step in protein synthesis (Rannels and Jefferson, 1980). However, the mechanism of inhibition by glucocorticoids on muscle protein synthesis is initially at the translational level with a subsequent alteration in transcription (Bullock et al., 1972;
Rannels and Jefferson, 1980).

Although many studies have examined effects of glucocorticoids on myofibrillar protein degradation, these results are not as definitive as the effects of glucocorticoids on muscle protein synthesis. Several studies show that high doses of glucocorticoids can temporally increase myofibrillar protein degradation both in vivo (Goldberg, 1969; Tomas et al., 1979; Santidrian et al., 1981; Kayali et al., 1987) and in vitro (Ballard and Francis, 1983). Other studies showed that glucocorticoids either decreased myofibrillar protein degradation in vitro (McGrath and Goldspink, 1982) or had no effect on myofibrillar protein degradation in vivo (Rannels and Jefferson, 1980; Santidrian et al., 1981).

Four possible reasons which could account for these different observations: (1) Different methods of assessing myofibrillar protein degradation were used. These included indirectly estimating myofibrillar protein degradation by the difference between rates of muscle protein accretion versus muscle protein synthesis, by measuring urinary N\(^7\)-methylhistidine excretion and by detecting protease activities. (2) Different routes of administration of glucocorticoids (i.e. subcutaneous injection versus intraperitoneal injection) can result in different effects of glucocorticoids on myofibrillar protein degradation (Santidrian et al., 1981). (3) Different duration of glucocorticoids administration may cause different observations. The increase of myofibrillar protein degradation by glucocorticoids is only temporal. If the initial increase in myofibrillar protein degradation is missed, then the effects of glucocorticoids on myofibrillar protein degradation will not be observed (Odedra et al., 1983). (4) Different types of muscle
fibers were examined. Different muscle fibers respond differently to glucocorticoids.

Different plasma concentrations of glucocorticoids have different effects on myofibrillar protein degradation. It has been reported that plasma glucocorticoid concentration within the normal range may not regulate myofibrillar protein degradation in rats, but excessive plasma glucocorticoid concentration will increase myofibrillar protein degradation (Tomas et al., 1979; Young, 1980). Therefore, glucocorticoids have no effects on myofibrillar protein degradation unless plasma glucocorticoid concentration increases to the values observed in severe stress. Also, it has been shown that glucocorticoids can not activate muscle lysosomal hydrolases (Buchanan and Schwartz, 1967), but can increase activity of a myofibrillar protease --- calpains (Mayer et al., 1976; Kayali et al., 1985) and several nonlysosomal proteases (Mayer and Rosen, 1977). However, no direct evidence indicates that treatment of glucocorticoids actually affect myofibrillar protein degradation in vivo and the mechanism of glucocorticoids on myofibrillar protein degradation remains unknown (Kayali et al., 1985).

The primary objectives of our research were first to develop a model in which muscle protein degradation was changed and then to investigate the molecular and enzymatic basis for the regulation associated with this. Because glucocorticoids have been reported to usually affect muscle protein degradation, we chose this as a model. Conduct of this study, therefore, provides insight into the enzymology and molecular biology underlying muscle atrophy in general but of mechanisms by which glucocorticoids regulate muscle protein homostasis specifically.
MATERIALS AND METHODS

Animal Treatment

Forty-five female New Zealand White rabbits, initially weighing 1.8-2.1 kg, were obtained from the Rabbit Research Center of Oregon State University and were randomized to three blocks with fifteen rabbits each. Rabbits were caged in individual metabolic cages and kept in a temperature-controlled room with a 12-hr light-dark cycle upon arrival, and fed daily with a pelleted diet (Table 1). Feed and water were provided ad libitum.

After 3 days of adaptation, each block was divided into five groups of three and each group was randomly assigned to one of the five treatments:

(A) Initial control
(B) Vehicle injection control
(C) 1-Day dexamethasone injection
(D) 2-Day dexamethasone injection
(E) 4-Day dexamethasone injection

The initial control group was injected with T-61 euthanasia solution (1 ml/10 lbs BW) and killed on the first day of experiment. The vehicle control group received only subcutaneous injection of vehicle (corn oil : ethanol = 5 : 1) for 4 days. The other three groups were daily-injected subcutaneously for 1 day, 2 days and 4 days respectively with dexamethasone (1 mg/kg BW) which was dissolved in the vehicle solution. The 1-day dexamethasone-treated group received 3 days of vehicle injection following by 1 day of dexamethasone injection. The 2-day dexamethasone-treated group received 2 days of
vehicle injection following by 2 days of dexamethasone injection. The 4-day
dexamethasone-treated group received only 4 days of dexamethasone injection. Body
weights and food intakes were measured daily.

Urine of control and dexamethasone-injected groups was collected in bottles
containing 2 ml of 6 N HCl for 24 hr starting after the last injection. Total urine volume
was determined, and an aliquot was kept in -20°C for urinary N'-methylhistidine (NMH)
determination and urinary creatinine assays. At the end of experiments, rabbits were
injected with T-61 euthanasia solution and blood samples were taken. Cranial biceps
femoris was quickly removed and weighed, then stored frozen in -80°C until analysis.

**Muscle Protein Determination**

Muscle protein concentration was measured by the dye-binding method of Bradford
(1976). Muscle tissue was homogenized in distilled deionized water, then an equal
volume of .1 N NaOH was added to the tissue homogenate and this mixture was
incubated at 37°C for 30 min to dissolve insoluble proteins. The mixture was neutralized
with .1 N HCl and then Bio-Rad dye reagent (Bio-Rad) was added. After incubating at
room temperature for 10 min, the absorbance of each muscle sample versus blank was
measured by spectrophotometer (Shimadzu UV-160) at 595 nm based on the observation
that the maximum absorbance for Bio-Rad Dye Reagent which is an acidic solution of
Coomassie Brilliant Blue G-250 shifted from 465 nm to 595 nm wavelength when
binding to protein occurs. By using bovine serum albumin (BSA) as a standard, the
absorbance value of muscle samples was converted to protein concentrations according
to the standard curve.
Muscle RNA Determination

Determination of muscle RNA concentration was according to the method of Munro and Fleck (1969). Muscle tissue (0.5 g) was homogenized in 6 volumes (3 ml) of distilled deionized water, then 0.5 volume (1.5 ml) of tissue homogenate was taken and added to an equal volume (1.5 ml) of ice-cold 20% (w/v) trichloroacetic acid (TCA) and mixed immediately. The mixture was placed on ice for 1 hr and was then centrifuged at 5000 x g for 15 min at 4°C. The supernatant was discarded and the pellet was washed twice in 2 ml of ice-cold 10% (w/v) TCA. After discarding the supernatant, the pellet was resuspended in 2 ml of 5% (w/v) TCA and this suspension was heated at 90°C for 15 min. After centrifugation at 2000 x g for 5 min at 25°C, 0.5 ml of the final supernatant was mixed with 6 volumes (3 ml) of Orcinol Reagent (Sigma; O-1875), then the mixture was heated at 95°C for 30 min and cooled down to room temperature (RT). The absorbance of the mixture versus blank was measured by using a spectrophotometer at 695 nm wavelength. Standard curves of RNA were developed using Escherichia Coli (E. Coli) RNA (Sigma; R-1753).

Plasma T3 and T4 Concentration

Total circulating T3 (triiodothyronine) and T4 (thyroxine) concentrations in serum were measured quantitatively by using solid-phase 125I-radioimmunoassay kits (DPC; Diagnostic Products Corporation). In the presence of blocking agents which serve to liberate bound T3 and T4 from carrier proteins (hormone-binding proteins), 125I-labeled T3 and T4 will compete with T3 and T4 in serum samples for antibody binding sites during a fixed time (Walfish, 1981; Wenzel, 1981).
After adding 100 μl or 25 μl of serum into each T₃ or T₄ antibody-coated tubes respectively, 1 ml of ^125I-T₃ or ^125I-T₄ was added to each tube, and vortexed briefly and gently. These tubes were incubated in a 37°C waterbath for 2 hr (for T₃ assay) or 1 hr (for T₄ assay). After incubating, the tubes were decanted thoroughly and counted for 1 min in a gamma counter. Then the concentrations of T₃ and T₄ could be read from a standard curve.

**Urinary Creatinine Quantitation**

Creatinine concentration in urine was quantitated according to the method of Sigma Diagnostic Creatinine Kits (Sigma; 555-A). Urine sample was diluted 10-15 fold. Then 3 ml of alkaline picrate solution, which was prepared by mixing 5 volumes of creatinine color reagent (Sigma; 555-1) and 1 volume of sodium hydroxide solution (Sigma; 930-65), was added to .3 ml of urine dilution, mixed and incubated at 25°C for 8-12 min. The absorbance of mixture was measured by spectrophotometer at 500 nm wavelength. Then, to each mixture was added exactly .1 ml of acid reagent (Sigma; 555-2). The sample was mixed immediately and thoroughly and sat at 25°C for 5 min. The absorbance of mixture after adding acid was measured at the same wavelength (500 nm).

Yellow or orange color forms when creatinine was mixed with alkaline picrate, but a number of substances including proteins could interfere. Under acidic conditions, the creatinine-picrate color faded faster than other interfering chromogens. Therefore, the difference in color intensity measured at 500 nm wavelength before and after acidification was proportional to creatinine concentration (Heinegard and Tiderstorm, 1973). By using creatinine standards (Sigma; 925-3 and 925-15) and calibration curve, the absorbance
value of each sample could be converted to creatinine concentration.

**Urinary and Muscle N\(^\prime\)-Methylhistidine (NMH) Determination**

NMH is the methylated form of histidine residues associated with myofibrillar proteins --- actin and myosin. After protein degradation, it can neither be reutilized for protein synthesis, nor undergo further metabolism, but is quantitatively excreted to urine in some animals such as rabbits (Harris et al., 1977), rats (Young and Munro, 1978) and cattle (Harris and Milne, 1981). Therefore, NMH can be used as an index of muscle protein degradation at least in these animals. \(N\(^\prime\)-methylhistidine\) was measured by the method of Forsberg and Liu (1989) and Forsberg et al. (1989). Two major compounds which are \(N\)-acetyl-3-methylhistidine and unchanged 3-methylhistidine account for the total urinary \(N\(^\prime\)-methylhistidine\) (NMH; 3-methylhistidine; 3-MeHis), so that a hydrolysis step prior to determination of urinary NMH is needed in some animals (Young et al., 1972).

After centrifugation, 2 ml of each urine sample was mixed with 3 ml of 10 N HCl and heated at 100°C for 2 hr to deacetylate NMH. Then .5 ml of the mixture was lyophilized in a Virtus Uni-Top Model 600 SL freeze dryer (Virtus) until dry, then resuspended in .5 ml of freshly-prepared derivatization solution which was a 7:1:1:1 mixture of ethanol : triethylamine (Aldrich; 239623) : distilled deionized water : phenylisothiocyanate (Pierce; 26922) respectively, and incubated at 25°C for 20 min to produce phenylthiocarbamyl (PTC) amino acids. This mixture was lyophilized again and resuspended in .5 ml of sample diluent which was composed of 5 mM phosphate buffer (pH 7.4) with 5% (v/v) acetonitrile (J. T. Baker; 9011-03). A 2.5 mM NMH standard
(Sigma; M-3879) was prepared identically as urine samples. Muscle samples (.5 g) were homogenized in 6 volumes (3 ml) of distilled deionized water and centrifuged at 5000 x g for 30 min at 4°C. Then half the volume (1.5 ml) of the homogenate was taken, mixed with 3 ml of 10 N HCl and processed as urine samples.

After adding sample diluent, the derivatized sample was put in a limited volume insert (Waters; 72030) for injecting 50 µl into a C-18 Pico-Tag 30-cm HPLC (high performance liquid chromatography) column (Waters) by using a WISP 710B sample anto-injector (Waters), and column temperature was maintained at 44°C. A Model 680 automated gradient controller (Waters) was used, and eluent 1 (Waters; 10960) and eluent 2 (Waters; 10985) were delivered by two Model 510 solvent delivery pumps (Waters). Derivatized NMH was detected at 254 nm wavelength with a Model 441 fixed UV-detector (Waters). Solvent delivery conditions and gradient program used for separating NMH from other urinary PTC-reactive components were shown in Table 2.

Assay of Calpain Activities

Activities of calpains were measured by the method of Gopalakrishna and Barsky (1985). Muscle tissue (3 g) was homogenized in 5 volumes (15 ml) of homogenization buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM 2-mercaptoethanol and 150 nM pepstatin A (Sigma; P-4265). Tissue homogenate was centrifuged at 10000 x g at 4°C for 30 min and the supernatant (15 ml) was mixed with 30 µl of 1 mM leupeptin (Boehringer Mannheim Biochemicals; BMB; 1017128), .9 ml of 5 M NaCl and 1 ml of phenyl sepharose CL-4B (Sigma; P-7982) which was previously equilibrated with Buffer A (20 mM Tris-HCl [pH 7.5], .1 mM CaCl₂, 10 mM 2-mercaptoethanol and 20
μM leupeptin) containing .25 M NaCl. After shaking for 5 min, the gel suspension was added to .6 ml of .1 M CaCl₂ and agitated for another 10 min. Then the gel suspension was poured into a .8 X 4 cm column (Bio-Rad; 731-1550) and the packed column was washed first with 2 ml of Buffer A containing .25 M NaCl, then subsequently washed with Buffer A and Buffer A without leupeptin. By washing with 4 ml of Buffer B (20 mM Tris-HCl [pH 7.5], 2 mM EGTA and 10 mM 2-mercaptoethanol) containing .1 M NaCl, calpain II was eluted and collected. Then the column was washed with 2 ml of Buffer B with NaCl and calpain I was eluted by 4 ml of Buffer B. All the procedures were conducted at 4°C.

Calpain activities were measured by using Hammarstem casein (EM Science; 2242) as a substrate. The eluted calpain I and calpain II were individually combined with 2 mM CaCl₂ and 1 ml casein solution (8 mg/ml casein in the solution of 20 mM Tris-HCl [pH 7.5] and 10 mM 2-mercaptoethanol), and incubated at 25°C for 30 min. Then 1 ml of 36% (w/v) ice-cold TCA was added to stop the reaction and the mixture was centrifuged at 3500 x g for 15 min at 4°C. TCA-soluble digested products were measured by the Bio-Rad protein assay (Bradford, 1976). One unit of calpain activity was defined as the amount of calpain which caused an increase of .1 absorbance unit at 595 nm wavelength after incubation at 25°C for 30 min.

Assay of Calpastatin Activity

Calpastatin activity was determined by modifying the method of Nakamura et al. (Nakamura et al., 1988). Muscle tissues were homogenized in homogenization buffer composed of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM 2-mercaptoethanol and
150 nM pepstatin A (Sigma; P-4265). Tissue homogenate was centrifuged at 10000 x g for 20 min at 4°C. The supernatant was heated at 100°C for 10 min in order to inactivate calpains and other proteases, then centrifuged at 10000 x g for 10 min at 4°C. To the supernatant was added a fixed amount of calpain II, which was partially purified from rabbit muscle, and 2 mM CaCl₂, and incubated at 25°C for 5 min to allow calpastatin react with calpain II. Then to this mixture was added the casein solution (8 mg/ml casein in the solution of 20 mM Tris-HCl [pH 7.5] and 10 mM 2-mercaptoethanol) and incubated at 25°C for 30 min. After adding 36% (w/v) TCA to stop the reaction, the mixture was centrifuged at 10000 x g for 15 min at 4°C and the TCA-soluble digested products were measured by Bio-Rad protein assay (Bradford, 1976). The remaining calpain II activity was determined as previously described. One unit of calpastatin was defined as the amount of enzyme which inhibited one unit of rabbit muscle calpain II.

**Preparation of cDNA Probe**

The method used for preparing rabbit muscle calpains and calpastatin cDNA probes followed the methods of Maniatis et al. (1982) and Davis et al. (1986). Plasmids containing calpain I (pLU 1001), calpain II (pLM 28) and calpastatin (pCI 413) cDNA fragments (Figures 12, 13 and 14, respectively) were kindly provided by Dr. Y. Emori from Japan. The cDNA fragments encoding portions of calpain I (pLU 1001) and calpain II (pLM 28) from rabbit muscle were prepared from the 3’-noncoding regions (Emori et al., 1986a), while the cDNA fragment of calpastatin (pCI 413) from rabbit muscle was prepared from the coding region (Emori et al., 1987).

After transforming bacteria (HB101-E. Coli) with plasmids, a single bacterial colony
was incubated at 37°C in 10 ml of LB (Luria-Bertani) medium (1% [w/v] Bacto-tryptone, .5% [w/v] Bacto-yeast extract and .5% [w/v] NaCl; pH 7.5) with appropriate antibiotic (tetracycline hydrochloride and ampicillin for calpains and calpastatin respectively) and shaken vigorously overnight. On the following day, .1 ml of the overnight culture was added to 25 ml of LB medium containing appropriate antibiotic, and incubated at 37°C with vigorous shaking until the culture reached late log phase (OD$_{600}$ ≈ .6). Then the late log culture (25 ml) was added to 500 ml of LB medium prewarmed to 37°C with the appropriate antibiotic and incubated at 37°C for exactly 2.5 hr with vigorous shaking (OD$_{600}$ ≈ .4). After that, to this culture was added chloramphenicol (170 µg/ml) and further incubated at 37°C for 12-16 hr with vigorous shaking to amplify the plasmids.

The bacterial cells were harvested by centrifuging at 4000 x g for 10 min at 4°C. The supernatant was discarded and the bacterial pellet was washed with 100 ml of ice-cold STE solution which was composed of .1 M NaCl, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. After centrifugation at 4000 x g for another 10 min at 4°C, the bacterial pellet was resuspended in 10 ml of Solution A (50 mM glucose, 25 mM Tris-Cl [pH 8.0] and 10 mM EDTA) containing ice-cold lysozyme (5 mg/ml; Sigma; L-6876), then transferred to a Beckman SW 27 polyallomer tube and allowed to incubate at 25°C for 5 min. To the mixture was added 20 ml of freshly-prepared Solution B which was composed of .2 N NaOH and 1% (w/v) SDS. This was mixed gently and sat on ice for 10 min. Then 15 ml of an ice-cold potassium acetate solution (60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of glass-distilled water; pH 4.8) was added and the contents were mixed sharply and allowed to sit on ice for another 10 min. After
centrifugation at 20000 rpm for 20 min at 4°C, the cell DNA and bacterial debris formed a tight pellet on the bottom of tube. The supernatant was transferred into a Corex tube and was mixed with .6 volumes of isopropanol and allowed to incubate at 25°C for 15 min. Plasmid DNA was recovered by centrifugation at 12000 x g for 30 min at 25°C. The supernatant was discarded and the pellet was washed with 70% ethanol at 25°C. After discarding ethanol, the pellet was dried in a vacuum desicator.

The dried pellet was dissolved in small volume of TE buffer (10 mM Tris-Cl [pH 8.0] and 1 mM EDTA [pH 8.0]). RNase (0.1 mg/ml; BMB; 1119-915) was added and the mixture was incubated at 37°C for 30 min to digest RNA. After this mixture was extracted once with phenol (Appendix 1) and twice with chloroform, .1 volume of 3 M sodium acetate (pH 5.2) and 2.2 volume of ethanol were added to the aqueous phase and this mixture was placed in the freezer (-20°C or -80°C) for several hr to precipitate plasmid DNA. After centrifugation for 10 min at 4°C, the supernatant was discarded and the plasmid pellet was dried again in a vacuum desicator.

The dried pellet was resuspended in small volume of 4B buffer (50 mM Tris-Cl [pH 7.5], 10 mM EDTA and .5 M NaCl) and was purified through a Sepharose-4B (Pharmacia; 17-0120-01) column which was pre-equilibrated with 4B buffer. Ten or twelve 1-ml fractions were collected, then 10 µl of each fraction were mixed with bromophenol blue-sucrose mixture and applied to a 1% agarose gel which was composed of 1% (w/v) agarose in TAE (Tris-acetate) buffer (40 mM Tris-acetate and 2 mM EDTA) with ethidium bromide (.5 µg/ml). Electrophoresis allowed identification of fractions containing plasmid DNA. All plasmid-containing fractions were pooled and
extracted once with phenol:chloroform (1:1). Then 2 volumes of ethanol were added to
the aqueous phase and the mixture was placed in the freezer (-20°C or -80°C) for at least
10 min to precipitate plasmid DNA. After centrifugation, the supernatant was discarded
and the pellet was resuspended in .4 ml of .3 M sodium acetate (pH 7.0) and .8 ml of
ethanol then again placed in the freezer for at least 10 min to precipitate the plasmid
DNA. This mixture was centrifuged again to discard the supernatant and the pellet was
rinsed with .5 ml of 80% ethanol. Then the ethanol was discarded by centrifugation and
the pellet was dried again in a vacuum desiccat.

The dried pellet was resuspended in TE buffer. The approximate plasmid DNA
concentration was determined by spectrophotometry (OD_{260}). To recover the cDNA from
plasmid, an appropriate amount of restriction enzyme (Rsa I and Dde I for pLU 1001,
Rsa I for pLM 28 and EcoR I for pCI 413) was added and this mixture was incubated
at 37°C for 1-1.5 hr to digest plasmid DNA. Generally, one unit of restriction enzyme
can digest 1 µg of DNA at 37°C for 1 hr and about 2-3 fold as many units of restriction
enzyme as needed were used to ensure complete digestion. After restriction enzyme
digestion, cleaved plasmid DNA and DNA standards were electrophoresed on a 2%
agarose gel to separate digested DNA fragments. After electrophoresis, the desired
cDNA fragments (494 bp for calpain I, 639 bp for calpain II and 1300 bp for calpastatin)
were cut out from the agarose gel, put into individual dialysis bags and electroeluted with
.2 X TAE buffer in a horizontal agarose gel apparatus at 300 V for 1-3 hr at 4°C to
recover cDNA fragments. After electroelution, the solution was carefully taken out of
dialysis bags, purified by phenol:chloroform (1:1) extraction and precipitated with
ethanol as previously described. The pellet was dried again in a vacuum desiccat or and the dried cDNA fragments were quantitated as previously described by spectrophotometry (OD\textsuperscript{260}). These cDNA fragments can be stored at -80°C until use.

cDNA probes for Northern blotting should be labelled freshly prior to use. The cDNA fragments were labelled with \textsuperscript{32}P-dCTP (New England Nuclear; NEG-013H) to high specific activity by using a random primer DNA labeling kit (United States Biochemical; USB; 70020) and further purified through Bio-gel P-60 (Bio-Rad; 150-1640) chromatography (Appendix 2).

**Total RNA Extraction and Quantitation**

Extraction of total RNA was followed the method of Chomczynski and Sacchi (1987). Muscle tissue (0.5 g) was homogenized at 25°C in 10 volumes (5 ml) of Buffer A (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% [w/v] of sarcosyl and 10 mM 2-mercaptoethanol). This tissue homogenate was mixed with 0.5 ml of 2 M sodium acetate (pH 4.0), 5 ml of phenol (Appendix 1) and 1 ml of chloroform:isoamyl alcohol (49:1) mixture, and mixed well after each addition. Then this mixture was centrifuged at 10000 x g for 20 min at 4°C. The aqueous phase was transferred to a new tube with 5 ml of isopropanol and incubated at -20°C for 1 hr. After that, this mixture was centrifuged at 10000 x g for 20 min at 4°C. The supernatant was discarded and the RNA pellet was resuspended in 0.5 ml of Buffer A. After adding 1 volume (0.5 ml) of isopropanol, this mixture was incubated for another 1 hr at -20°C. The mixture was then centrifuged at 12000 x g for 10 min at 4°C and the supernatant was discarded. The RNA pellet was washed with 75% ethanol and dried in a vacuum desiccator. The dried RNA
pellet was dissolved in diethyl-pyrocarbonate (DEPC) treated-water and quantitated by spectrophotometer at 260 nm wavelength.

Northern Blotting

The procedures used for Northern blotting mainly followed methods of Davis et al. (1986) and Ausubel et al. (1989). Total RNA was extracted from rabbit muscle as described above. Each RNA sample (40 μg of total RNA) was denatured at 55°C for 15 min in 3.4 fold of 50 X master mix (MM) solution, which was composed of 22.6 % (v/v) of deionized formaldehyde, 64.5 % (v/v) of deionized formamide and 12.9 % of 10 X MOPS (3-[N-morpholino] propane-sulfonic acid) solution (.4 M MOPS [Sigma; M-9381], 100 mM sodium acetate and 10 mM EDTA; pH 7.0), and electrophoresed on a formaldehyde-containing agarose gel which was composed of 1.1 % (w/v) of agarose, 10.2 % of 10 X MOPS solution and 18.2 % of deionized formaldehyde. After electrophoresis, the RNA was transferred to a .45 μm nitrocellulose membrane (Schleicher & Schuell; 21640) for 24 hr at 25°C with 20 X SSC (3 M NaCl and .3 M sodium citrate; pH 7.0). After completing transfer, the nitrocellulose membrane was baked in a vacuum oven at 80°C for 2 hr to immobilize RNA. Then the baked membrane was prehybridized at 42°C overnight with Stark’s solution which was composed of 5 X SSC (.75 M NaCl and 75 mM sodium citrate; pH 7.0), 25 mM KPO₄ (pH 7.0), 5 X Denhardt’s solution, 50% (v/v) of deionized formamide and .02% (w/v) of salmon sperm DNA. On the next day, the membrane was hybridized at 42°C for 48 hr with ³²P-labelled cDNA probe in a solution containing 80% of Stark’s solution and 20% (w/v) of dextran sulfate (Sigma; D-6001). After completing hybridization, the membrane was washed with
buffer composed of .1 X SSC and .1% (w/v) SDS at 25°C for three times of 15 min each and then washed with the same buffer at 50°C for three times of 15 min each. Then the membrane was autoradiographed to a Kodak X-film with intensifying screens at -80°C for 1-2 days. The hybridized bands on X-film were quantitated by scanning densitometry (Bio-Rad) with Hoefer program (Hoefer; GS350H).

**Statistical Analysis**

All of the data in this experiment were analyzed as randomized block design by using analysis of variance (ANOVA; Steel and Torrie, 1980). Normal distribution and equal variance were checked as basic assumptions prior to conduct of statistical analysis. Mean differences between each treatments were compared with LSD (least significant difference) multiple range test and a significance level of 5% was used. More than one autoradiographic exposure was made for each Northern blot conducted. This allowed selection of exposures between blocks with similar back ground and similar intensity of exposure. Differences in exposure between blocks were examined as a block effect in analysis of variance.
RESULTS

Initial body weight, final body weight, total body weight gain, food intake and proportion of cranial biceps femoris of each treatments are shown in Table 3. Rabbit initial body weights of each treatments did not differ significantly (P > .05). Dexamethasone treatment did not significantly affect (P > .05) food intake, muscle weight and proportion of cranial biceps femoris in treated groups, but caused temporal reductions (P < .05) on final body weight and total body weight gain in the 1-day dexamethasone-treated rabbits. However, there was a tendency for the 4-day dexamethasone-treated rabbits to have smaller muscle proportion.

Plasma T₃ (triiodothyronine) concentrations (Fig. 1) were reduced significantly by dexamethasone (P < .05) and reached a minimum in the 2-day treated rabbits. Dexamethasone had no effect (P > .05) on plasma T₄ (thyroxine) concentrations (Fig. 2), although plasma T₄ concentrations tended to decrease to a minimum in the 2-day dexamethasone-treated rabbits and recovered in the 4-day dexamethasone-treated rabbits.

Effects of treatments on muscle RNA concentration, muscle protein concentration and ribosomal capacity (mg RNA/g protein) are shown in Table 4. Muscle protein concentration was not significantly affected by dexamethasone (P > .05). Ribosomal capacity was unaffected by treatments (P > .05), but tended to decrease gradually as the duration of dexamethasone treatment increased. Muscle RNA concentration did not differ (P > .05) among control, 1-day dexamethasone-treated and 2-day dexamethasone-treated rabbits. However, muscle RNA concentration of the 4-day dexamethasone-treated rabbits was significantly lower than the control rabbits which received no dexamethasone (P <
Effects of treatments on urinary N\textsuperscript{\textprime}-methylhistidine (NMH) excretion and urinary creatinine output are shown in Table 5. Urinary NMH excretion expressed on a body weight basis was unaffected by dexamethasone treatment (P > .05). Dexamethasone tended to increase urinary creatinine output as duration of dexamethasone treatment increased, these differences were not significant (P > .05). The ratio of urinary NMH excretion to urinary creatinine output decreased significantly (P < .05) as duration of dexamethasone treatment increased. Effects of dexamethasone on muscle NMH concentration are shown in Fig. 3. Muscle NMH concentrations of 1-day and 2-day dexamethasone-treated rabbits were significantly lower than the control rabbits (P < .05) and then recovered in the 4-day dexamethasone-treated rabbits. No significant differences (P > .05) were found among the dexamethasone-treated rabbits.

Effects of dexamethasone on activities of calpains and calpastatin in cranial biceps femoris were shown in Table 6. Activities of calpain I and calpain II did not differ significantly among treatments (P > .05). Calpastatin activities tended to increase as duration of dexamethasone treatment increased, but no significant differences (P > .05) were found among control and dexamethasone-treated rabbits.

Due to mRNA degradation, only two blocks of rabbit muscle Northern blots were shown. Northern blots for rabbit muscle calpain I (\(\mu\)-calpain), calpain II (m-calpain) and calpastatin are shown in Figures 4, 5 and 6, respectively. Scanning densitometry of Northern blots for rabbit muscle calpain I (Fig. 7), calpain II (Fig. 7) and calpastatin (Fig. 8) are shown in Table 7. Calpain I mRNA (Fig. 7; Table 7) tended to increase in
the 1-day dexamethasone-treated rabbits then decreased to normal gradually. However, no significant differences were found among control and dexamethasone-treated rabbits (P > .05). Calpain II mRNA (Fig. 7; Table 7) decreased significantly to a minimum in the 1-day dexamethasone-treated rabbits (P < .05) then tended to recover, however, no significant differences were found between 2-day and 4-day dexamethasone-treated rabbits (P > .05). Calpastatin band I mRNA (Fig. 8; Table 7) was unaffected by treatments (P > .05). However, calpastatin band II mRNA (Fig. 8; Table 7) increased significantly (P < .05) in the 2-day dexamethasone-treated and the 4-day dexamethasone-treated rabbits.

The mRNA concentrations of calpain I, calpain II and calpastatin were also expressed as arbitrary densitometry units per gram muscle protein (Units/g muscle protein). This provides an estimate of mRNA per unit of muscle protein. In the face of changing total RNA concentration and stable protein concentration, this value provides a more accurate estimate of mRNA concentration whereas mRNA expressed on an RNA basis provides insight into specific regulation of mRNA expression. The mRNA concentrations encoding calpain I, when expressed on a protein basis (Fig. 9; Table 7), increased significantly and reached a maximum in the 1-day dexamethasone-treated rabbits (P < .05), then decreased significantly from 2-day dexamethasone-treated rabbits to 4-day dexamethasone-treated rabbits (P < .05). While mRNA concentrations encoding calpain II (Fig. 10; Table 7) decreased significantly and reached a minimum in the 1-day dexamethasone-treated rabbits (P < .05). However, no significant differences were found among dexamethasone-treated rabbits (P > .05). Although mRNA concentrations
encoding calpastatin band I, when expressed on a protein basis (Fig. 11; Table 7), did not differ significantly (P > .05) among treatments (Fig. 11; Table 7). mRNA concentrations encoding calpastatin band II increased significantly (P < .05) in the 2-day dexamethasone-treated and the 4-day dexamethasone-treated rabbits.
DISCUSSION

Effects of Dexamethasone on Animal Growth

Dexamethasone is a synthetic glucocorticoid with a relatively long half-life and is not rapidly metabolized in vivo. As an anti-inflammatory agent, dexamethasone is approximate 25-40 times more potent than natural glucocorticoids, such as corticosterone and cortisone (Kelly and Goldspink, 1982). Thus, the dexamethasone-treatment in our study is roughly equivalent in potency to a catabolic dose of glucocorticoids.

Generally, glucocorticoids have catabolic effects on animal growth. In our study, we found that dexamethasone temporally decreased total body weight gain in the 1-day dexamethasone-treated rabbits. Although growth was suppressed transiently, food intake was maintained in both control and dexamethasone-treated rabbits (Table 3). However, muscle proportion as cranial biceps femoris was unaffected by dexamethasone (Table 3). These results are consistent with other studies done with glucocorticoid-treated rats (Tomas et al., 1979; Odedra et al., 1983; Kayali et al., 1987).

Dexamethasone-induced changes in the synthesis of new proteins could arise from alteration in either muscle RNA content or from ribosomal capacity. We found that both muscle RNA content and ribosomal capacity (Table 4) tended to decrease gradually as duration of dexamethasone treatment increased. This is consistent with the study done with corticosterone-treated rats (Odedra et al., 1983). These results imply that dexamethasone may inhibit transcription and further decrease translational capacity.

Effects of Dexamethasone on Muscle Protein Degradation

In vivo studies of muscle protein degradation are made difficult by amino acid
reutilization. Various isotopic methods have been proposed to minimize effects of recycling of labeled amino acids, but no one has provided practical solutions to the quantitative examination of muscle protein degradation in vivo. Measurement of urinary NMH excretion was suggested to be an index of myofibrillar protein degradation in vivo (Young and Munro, 1978). Urinary creatinine output has been used as an index of muscle mass. Therefore, some investigators suggested that the ratio of urinary NMH excretion to urinary creatinine output should provide an index of muscle protein degradation per unit muscle mass (Young and Munro, 1978; Tomas et al., 1979).

In our study, urinary NMH excretion (Table 5) tended to decrease to a minimum in the 2-day dexamethasone-treated rabbits and then recovered in the 4-day dexamethasone-treated rabbits. Also, the ratio of urinary NMH excretion to urinary creatinine output was reduced by dexamethasone as duration of dexamethasone treatment increased. These results suggest that muscle protein degradation was decreased by dexamethasone although the decreases in urinary NMH excretion alone and the ratio of urinary NMH excretion to urinary creatinine output were not significant.

Due to possible contributions of skin and intestine to urinary NMH output, urinary NMH concentration may mask the real effect of dexamethasone on muscle protein degradation. Therefore, we also measured muscle NMH concentration of cranial biceps femoris. Dexamethasone transiently decreased muscle NMH concentration which reached a minimum in the 2-day dexamethasone-treated rabbits, then recovered in the 4-day dexamethasone-treated rabbits (Fig. 3). This transient decrease in muscle NMH concentration indicated that dexamethasone may cause a transient decrease in muscle
protein degradation.

Abundant evidence suggests that calpains and calpastatin are involved in myofibrillar protein degradation (Goll et al., 1989). It has been reported that glucocorticoid-induced myofibrillar protein degradation was related to calpains --- nonlysosomal proteases, but not cathepsin D --- a lysosomal protease (Kayali and Young, 1985). From the activities of calpains and calpastatin (Table 6), we found that both calpain I and calpain II activities were decreased to a minimum in the 2-day dexamethasone-treated rabbits, then recovered in the 4-day dexamethasone-treated rabbits. Additionally calpastatin activity tended to increase to a maximum in the 2-day dexamethasone-treated rabbits and this increase of calpastatin activity may have inhibited both calpain I and calpain II activities. Thus, it is possible that the temporal decrease of muscle protein degradation by dexamethasone was mediated by calpains and calpastatin.

Effects of Dexamethasone on Calpains and Calpastatin

mRNA concentration (Unit/g muscle protein) encoding calpain I in the 1-day dexamethasone-treated rabbits was increased significantly compared to control, then recovered in the 4-day dexamethasone-treated rabbits. This result suggests that dexamethasone may transiently enhance the transcription of calpain I mRNA.

The activity of calpain I did not differ significantly, but tended to decrease to a minimum in the 2-day dexamethasone-treated rabbits. The reduction of calpain I activity in the 2-day dexamethasone-treated rabbits may be due to inhibition of calpain I translation and to post-translational processes directly by dexamethasone or indirectly by other hormones, such as thyroid hormones (Rupprecht et al., 1989), insulin (Tomas et
al., 1984) and growth hormone (Rousseau et al., 1987), which are affected by dexamethasone in vivo.

mRNA concentration (Unit/g muscle protein) encoding calpain II in the 1-day dexamethasone-treated rabbits was decreased significantly compared to control; however, no differences among dexamethasone-treated rabbits were found. It has been reported that differential expression of calpain II in tissues may be attributed to tissue-specific expression of an uncharacterized trans-element which negatively regulates transcription of the calpain II gene by interaction with the negative cis-element (Hata et al., 1989). The reduction of calpain II mRNA concentration implies that dexamethasone can induce the trans-element which, in turn, inhibits mRNA transcription of calpain II. Because the effects of dexamethasone on mRNA transcriptions of calpain I and calpain II are opposite, it suggests that dexamethasone regulate individual gene expression in an opposing manner. The upstream region of calpain I has not been cloned and sequenced. The opposing regulation of calpain I versus calpain II implies that calpain I promotor differs from the calpain II promotor region.

The activity of calpain II did not change significantly, but tended to decrease gradually as duration of dexamethasone treatment increased. This observation of a decrease in mRNA concentration with no change in enzyme activity may be due to the stability (half-life) of calpain II or to a large calpain II pool in vivo. However, other hormones which were affected by dexamethasone may also regulate calpain II in vivo.

Although mRNA concentrations encoding calpastatin band II in the 2-day dexamethasone-treated and the 4-day dexamethasone-treated rabbits increased
significantly compared to control, no differences were found in calpastatin activities among treatments. These results imply that transcription rate of calpastatin mRNA was regulated by dexamethasone. It is possible that the actions of dexamethasone may have been masked or offset by dexamethasone-dependent changes in other hormones which regulate translational processes of protein synthesis, therefore, no changes in calpastatin activities were observed.

**Interaction Between Dexamethasone and Thyroid Hormones**

Thyroid hormone is believed to elevate intracellular free Ca$^{++}$ concentration through stimulation of Ca$^{++}$ release from mitochondria. Thus, the action of thyroid hormones is partly related to Ca$^{++}$ metabolism (Shears and Bronk, 1981). It was reported that thyroid hormones could affect calpastatin (Miyamoto et al., 1988). Therefore, thyroid hormones may play an important role in regulating calpains and calpastatin.

Glucocorticoids may have a pathophysiological role in modulating the peripheral metabolism of thyroid hormones in stress (Burr et al., 1976). It was reported that dexamethasone could suppress endogenous thyroid-releasing hormone (TRH) when given for a short period of time, and could cause an acute reduction in plasma T$_3$ concentration of human subjects (Duick et al., 1974; Chopra et al., 1975; Williams et al., 1975; Burr et al., 1976; Gómez-Balaguer et al., 1986; Rupprecht et al., 1989). In our study, we found that plasma T$_3$ concentrations were significantly decreased in the 1-day dexamethasone-treated rabbits and stayed low in the 2-day dexamethasone-treated rabbits as well as the 4-day dexamethasone-treated rabbits, while dexamethasone did not significantly affect plasma T$_4$ concentration. These results were consistent with those
findings of Burr et al. (1976) and Gómez-Balaguer et al. (1986). In the studies of Burr et al. (1976) and Chopra et al. (1975), the fall in plasma T₃ concentration was synchronous with an increase in plasma rT₃ (reverse T₃) concentration after dexamethasone administration. These results imply that dexamethasone may not alter overall T₄ catabolism, but instead may lead to decreased formation of metabolically active T₃ (3,5,3'-Triiodothyronine) and increased formation of metabolically inactive rT₃ (3,3',5'-Triiodothyronine). Thus, high levels of glucocorticoids in stress may play a role in decreasing the concentration of the active thyroid hormone --- T₃, which is available for tissue uptake and metabolic activity, and a rapid reduction of thyroid hormones may limit or alter the catabolic effect produced by glucocorticoids (Burr et al., 1976). Because T₃ stimulates muscle protein degradation, its associated loss in glucocorticoid-treated animals may account for the apparent reduction in myofibrillar protein degradation detected in our study.

In our study, the overall effects of dexamethasone on muscle protein degradation may be due to the interaction of dexamethasone and thyroid hormone --- T₃ or other hormones in vivo and be the combination of both direct and indirect responses. However, in vitro studies will be needed for future research to clarify the direct effects of dexamethasone on muscle protein degradation.
Table 1. Composition of rabbit diet.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>% Dry Matter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa meal</td>
<td>38.00</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>10.00</td>
</tr>
<tr>
<td>Wheat mill run</td>
<td>19.00</td>
</tr>
<tr>
<td>Ground corn</td>
<td>25.00</td>
</tr>
<tr>
<td>Vegetable oil</td>
<td>2.13</td>
</tr>
<tr>
<td>Ground corn cobs</td>
<td>2.00</td>
</tr>
<tr>
<td>Molasses</td>
<td>2.75</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>.25</td>
</tr>
<tr>
<td>Limestone</td>
<td>.12</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>.50</td>
</tr>
<tr>
<td>Vitamin-mineral premix*</td>
<td>.25</td>
</tr>
</tbody>
</table>

Calculated composition:

- Crude protein (%) 16.00
- Digestible energy (Mcal/kg) 2850.00

* Vitamin and mineral premix at .25 % of dietary dry matter resulted in the following concentrations of vitamins and minerals: 2 ppm of carotene, 15 IU/g of vitamin A, 4.41 IU/g of vitamin D, 35 ppm of α-tocopherol and 14 IU/g of thiamine.
Table 2. Gradient conditions for urinary and muscle NMH by HPLC.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (ml/min)</th>
<th>% Eluent 1</th>
<th>% Eluent 2</th>
<th>Gradient Curve*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>1.0</td>
<td>100</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>13.5</td>
<td>1.0</td>
<td>97</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>24.0</td>
<td>1.0</td>
<td>94</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>30.0</td>
<td>1.0</td>
<td>91</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>50.0</td>
<td>1.0</td>
<td>66</td>
<td>34</td>
<td>6</td>
</tr>
<tr>
<td>62.5</td>
<td>1.0</td>
<td>0</td>
<td>100</td>
<td>6</td>
</tr>
<tr>
<td>67.0</td>
<td>1.0</td>
<td>100</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

* Gradient curves refer to gradient condition available on Waters Model 680 gradient controller.
Table 3. Initial body weight of each treatment and effects of dexamethasone (1 mg/kg BW/d) on rabbit skeletal muscle (cranial biceps femoris) weight, total body weight gain and food intake.

<table>
<thead>
<tr>
<th></th>
<th>initial control</th>
<th>vehicle control</th>
<th>1-day dexamethasone</th>
<th>2-day dexamethasone</th>
<th>4-day dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Initial BW (kg)</td>
<td>1.93 ± .08</td>
<td>1.96 ± .05</td>
<td>1.91 ± .05</td>
<td>1.93 ± .06</td>
<td>1.97 ± .04</td>
</tr>
<tr>
<td>Final BW (kg)</td>
<td>-</td>
<td>2.08 ± .05(^a)</td>
<td>1.94 ± .06(^b)</td>
<td>2.06 ± .06(^a)</td>
<td>2.06 ± .06(^a)</td>
</tr>
<tr>
<td>Muscle weight (g)</td>
<td>12.5 ± .5</td>
<td>13.2 ± .7</td>
<td>12.2 ± .8</td>
<td>13.0 ± .7</td>
<td>12.0 ± .7</td>
</tr>
<tr>
<td>Muscle proportion (% body weight)</td>
<td>.65 ± .01</td>
<td>.63 ± .03</td>
<td>.62 ± .02</td>
<td>.62 ± .02</td>
<td>.58 ± .03</td>
</tr>
<tr>
<td>Body weight gain (g/4 days)</td>
<td>-</td>
<td>123 ± 17(^a)</td>
<td>35.6 ± 17.7(^b)</td>
<td>130 ± 17(^a)</td>
<td>90.9 ± 18.6(^b)</td>
</tr>
<tr>
<td>Food intake (g/d)</td>
<td>-</td>
<td>119 ± .6</td>
<td>119 ± .4</td>
<td>119 ± .5</td>
<td>120 ± .2</td>
</tr>
</tbody>
</table>

Values are means of each treatments ± SE.

Values in the same row which do not share a common superscript differ significantly (P < .05).
Table 4. Effects of dexamethasone (1 mg/kg BW/d) on protein concentration, RNA concentration and ribosomal capacity of rabbit skeletal muscle (cranial biceps femoris).

<table>
<thead>
<tr>
<th></th>
<th>initial control</th>
<th>vehicle control</th>
<th>1-day dexamethasone</th>
<th>2-day dexamethasone</th>
<th>4-day dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Muscle [RNA] (mg/g tissue)</td>
<td>2.38 ± .16ᵃ</td>
<td>2.38 ± .13ᵃ</td>
<td>2.17 ± .11ᵇ</td>
<td>2.07 ± .13ᵇ</td>
<td>1.89 ± .14ᵇ</td>
</tr>
<tr>
<td>Muscle [protein] (g/g tissue)</td>
<td>.27 ± .01</td>
<td>.26 ± .01</td>
<td>.26 ± .01</td>
<td>.26 ± .01</td>
<td>.25 ± .01</td>
</tr>
<tr>
<td>Ribosomal capacity (mg RNA/g protein)</td>
<td>8.66 ± .50</td>
<td>9.44 ± .74</td>
<td>8.36 ± .43</td>
<td>7.96 ± .51</td>
<td>7.80 ± .69</td>
</tr>
</tbody>
</table>

Values are means of each treatments ± SE.

Values in the same row which do not share a common superscript differ significantly (P < .05).
Table 5. Temporal effects of dexamethasone (1 mg/kg BW/d) on urinary N\textsuperscript{-}methylhistidine (NMH) excretion and urinary creatinine output.

<table>
<thead>
<tr>
<th></th>
<th>vehicle control</th>
<th>1-day dexamethasone</th>
<th>2-day dexamethasone</th>
<th>4-day dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Urinary N\textsuperscript{-}methylhistidine (µmole/100 g BW/d)</td>
<td>2.52 ± .40</td>
<td>2.58 ± .34</td>
<td>1.93 ± .41</td>
<td>2.15 ± .58</td>
</tr>
<tr>
<td>Urinary creatinine (µmole/100 g BW/d)</td>
<td>4.02 ± .58</td>
<td>3.40 ± .34</td>
<td>4.06 ± .99</td>
<td>5.92 ± 1.17</td>
</tr>
<tr>
<td>Urinary NMH / creatinine (ratio; µmole/µmole)</td>
<td>.52 ± .09\textsuperscript{ab}</td>
<td>.75 ± .08\textsuperscript{b}</td>
<td>.62 ± .08\textsuperscript{ab}</td>
<td>.39 ± .08\textsuperscript{a}</td>
</tr>
</tbody>
</table>

Values are means of each treatments ± SE.

Values in the same row which do not share a common superscript differ significantly (P < .05).
Table 6. Temporal effects of dexamethasone (1 mg/kg BW/d) on activities of calpain I, calpain II and calpastatin in rabbit skeletal muscle (cranial biceps femoris).

<table>
<thead>
<tr>
<th></th>
<th>initial control</th>
<th>vehicle control</th>
<th>1-day dexamethasone</th>
<th>2-day dexamethasone</th>
<th>4-day dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Calpain I activity (unit/g protein)</td>
<td>6.92 ± .32</td>
<td>6.99 ± .37</td>
<td>6.44 ± .20</td>
<td>5.93 ± .44</td>
<td>7.07 ± .53</td>
</tr>
<tr>
<td>Calpain II activity (unit/g protein)</td>
<td>11.3 ± .2</td>
<td>12.3 ± .5</td>
<td>11.5 ± .5</td>
<td>11.2 ± .4</td>
<td>11.4 ± .8</td>
</tr>
<tr>
<td>Calpastatin activity (unit/g protein)</td>
<td>240 ± 33</td>
<td>273 ± 30</td>
<td>292 ± 31</td>
<td>302 ± 18</td>
<td>295 ± 20</td>
</tr>
</tbody>
</table>

Values are means of each treatments ± SE.
Table 7. Temporal effects of dexamethasone (1 mg/kg BW/d) on mRNA concentrations encoding calpain I, calpain II and calpastatin in cranial biceps femoris expressed as scanning densitometry units and as scanning densitometry units per gram muscle protein (units/g protein).

<table>
<thead>
<tr>
<th></th>
<th>initial control</th>
<th>vehicle control</th>
<th>1-day dexamethasone</th>
<th>2-day dexamethasone</th>
<th>4-day dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Calpain I (X 10^3 units)</td>
<td>4.62 ± .82</td>
<td>4.14 ± .65</td>
<td>8.36 ± 1.76</td>
<td>7.61 ± 2.01</td>
<td>4.51 ± 1.22</td>
</tr>
<tr>
<td>(X 10^6 units/g protein)</td>
<td>10.3 ± 1.9</td>
<td>8.80 ± 1.23</td>
<td>17.1 ± 3.6</td>
<td>13.6 ± 3.1</td>
<td>7.19 ± 1.02</td>
</tr>
<tr>
<td>Calpain II (X 10^4 units)</td>
<td>2.37 ± .31</td>
<td>2.17 ± .34</td>
<td>1.04 ± .22</td>
<td>1.35 ± .20</td>
<td>1.28 ± .27</td>
</tr>
<tr>
<td>(X 10^6 units/g protein)</td>
<td>4.25 ± .80</td>
<td>3.78 ± .68</td>
<td>2.20 ± .52</td>
<td>2.56 ± .41</td>
<td>2.23 ± .35</td>
</tr>
<tr>
<td>Calpastatin:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Band I (X 10^4 units)</td>
<td>1.18 ± .18</td>
<td>1.30 ± .22</td>
<td>1.38 ± .26</td>
<td>1.53 ± .41</td>
<td>1.94 ± .47</td>
</tr>
<tr>
<td>(X 10^6 units/g protein)</td>
<td>2.51 ± .29</td>
<td>2.84 ± .57</td>
<td>2.92 ± .62</td>
<td>3.06 ± .93</td>
<td>3.79 ± 1.23</td>
</tr>
<tr>
<td>Band II (X 10^4 units)</td>
<td>3.26 ± .58</td>
<td>3.10 ± .52</td>
<td>2.87 ± .31</td>
<td>5.02 ± 1.01</td>
<td>5.91 ± .29</td>
</tr>
<tr>
<td>(X 10^6 units/g protein)</td>
<td>5.96 ± 1.11</td>
<td>7.00 ± 1.62</td>
<td>6.02 ± .86</td>
<td>11.8 ± 2.1</td>
<td>11.2 ± 1.9</td>
</tr>
</tbody>
</table>

Values are means of each treatments ± SE.

Values in the same row which do not share a common superscript differ significantly (P < .05).
Fig. 1. Temporal effects of dexamethasone (1 mg/kg BW/d) on rabbit plasma $T_3$ (triiodothyronine) concentrations. Five treatments include initial control which is indicated as an arrow (●), vehicle control, 1-day dexamethasone injection, 2-day dexamethasone injection and 4-day dexamethasone injection. Values shown represent means of each treatments ± SE.
Fig. 2. Temporal effects of dexamethasone (1 mg/kg BW/d) on rabbit plasma T₄ (thyroxine) concentrations. Five treatments include initial control which is indicated as an arrow (●), vehicle control, 1-day dexamethasone injection, 2-day dexamethasone injection and 4-day dexamethasone injection. Values shown represent means of each treatments ± SE.
Fig. 3. Temporal effects of dexamethasone (1 mg/kg BW/d) on muscle N\textsuperscript{\textprime}-methylhistidine (NMH) concentrations of rabbit cranial biceps femoris. Four treatments include vehicle control, 1-day dexamethasone injection, 2-day dexamethasone injection and 4-day dexamethasone injection. Values shown represent means of each treatments ± SE.
Fig. 4. Temporal effects of dexamethasone (1 mg/kg BW/d) on steady state mRNA concentrations encoding rabbit muscle calpain I (μ-calpain). Five treatments include initial control (A; Lanes 1-3), vehicle control (B; Lanes 4-6), 1-day dexamethasone injection (C; Lanes 7-9), 2-day dexamethasone injection (D; Lanes 10-12) and 4-day dexamethasone injection (E; Lanes 12-15).
Fig. 5. Temporal effects of dexamethasone (1 mg/kg BW/d) on steady state mRNA concentrations encoding rabbit muscle calpain II (m-calpain). Five treatments include initial control (A; Lanes 1-3), vehicle control (B; Lanes 4-6), 1-day dexamethasone injection (C; Lanes 7-9), 2-day dexamethasone injection (D; Lanes 10-12) and 4-day dexamethasone injection (E; Lanes 12-15).
Fig. 6. Temporal effects of dexamethasone (1 mg/kg BW/d) on steady state mRNA concentrations encoding rabbit muscle calpastatin. Five treatments include initial control (A; Lanes 1-3), vehicle control (B; Lanes 4-6), 1-day dexamethasone injection (C; Lanes 7-9), 2-day dexamethasone injection (D; Lanes 10-12) and 4-day dexamethasone injection (E; Lanes 12-15).
Fig. 7. Scanning densitometry of Northern blots of mRNAs encoding rabbit muscle calpain I (μ-calpain; Fig. 4) and calpain II (m-calpain; Fig. 5). Five treatments include initial control which is indicated as an arrow (for calpain I and for calpain II respectively), vehicle control, 1-day dexamethasone injection, 2-day dexamethasone injection and 4-day dexamethasone injection. Values shown represent means of each treatments ± SE. △-△: Calpain I. ○-○: Calpain II.
Fig. 8. Scanning densitometry of Northern blots of mRNA encoding rabbit muscle calpastatin (Fig. 6). Five treatments include initial control which is indicated as an arrow ( קורה for band I and orderBy for band II respectively), vehicle control, 1-day dexamethasone injection, 2-day dexamethasone injection and 4-day dexamethasone injection. Values shown represent means of each treatments ± SE. △-△: Band I. ○-○: Band II.
Fig. 9. Temporal effects of dexamethasone (1 mg/kg BW/d) on mRNA concentrations encoding rabbit muscle calpain I (μ-calpain; Fig. 4) expressed as scanning densitometry units per gram muscle protein. Five treatments include initial control which is indicated as an arrow (↑), vehicle control, 1-day dexamethasone injection, 2-day dexamethasone injection and 4-day dexamethasone injection. Values shown represent means of each treatments ± SE.
Fig. 10. Temporal effects of dexamethasone (1 mg/kg BW/d) on mRNA concentrations encoding rabbit muscle calpain II (m-calpain; Fig. 5) expressed as scanning densitometry units per gram muscle protein. Five treatments include initial control which is indicated as an arrow (△), vehicle control, 1-day dexamethasone injection, 2-day dexamethasone injection and 4-day dexamethasone injection. Values shown represent means of each treatments ± SE.
Fig. 11. Temporal effects of dexamethasone (1 mg/kg BW/d) on mRNA concentrations encoding rabbit muscle calpastatin (Fig. 6) expressed as scanning densitometry units per gram muscle protein. Five treatments include initial control which is indicated as an arrow (< for Band I and < for Band II respectively), vehicle control, 1-day dexamethasone injection, 2-day dexamethasone injection and 4-day dexamethasone injection. Values shown represent means of each treatments ± SE. Δ-Δ: Band I. ○-○: Band II.
Fig. 12. Plasmid (pLU 1001) cycle map. pLU 1001 is constructed in the Pst I site of pBR 322.
Fig. 13. Plasmid (pLM 28) cycle map. pLM 28 is constructed in the Pst I site of pBR 322.
Fig. 14. Plasmid (pCI 413) cycle map. pCI 413 is constructed in the EcoRI site of pUC 18.
BIBLIOGRAPHY


Preparation of Saturated Phenol Solution

1. Add .5 g of 8-hydroxyquinoline (Sigma; H-6878) into a 2 L glass beaker.

2. Melt solid phenol in 65°C water bath.

3. Pour 500 ml of liquified phenol into beaker.

4. Add 500 ml of 50 mM Trizma base solution and stir slowly at room temperature for 10 min.

5. Stop stirring and let phases separate at room temperature.

6. Gently discard the upper phase into a suitable waste container.

7. Substitute 50 mM Trizma base solution with 50 mM Tris-Cl solution (pH 8.0) and repeat procedure 4-6 twice.

8. Add 250 ml of 50 mM Tris-Cl solution (pH 8.0) and store this saturated phenol solution in dark at 4°C.
Preparation of $^{32}$P-labeled cDNA Probe

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

2. Add 4.0 µl of Klenow enzyme, 50 µCi of $^{32}$P-dCTP, and 7.5 µl of dATP, dGTP and dTTP (1:1:1) mixture into tube B.

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

4. Add 5 µl of .2 M EDTA solution to stop reaction and make volume to 100 µl by deionized distill water.

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

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

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

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

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

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

11. Collect 12 fractions of 300 µl each.

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

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

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

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