A comparative study on gene expression of calpains, calpastatin, cathepsin D, and proteasome in tissues of fed and fasted rabbits

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Molecular mechanisms underlying changes in muscle protein turnover are not fully understood. In this study, effects of fasting on mRNA concentrations encoding several proteinases in skeletal muscle were investigated. Proteinases included calpains I and II, cathepsin D, and proteasome. We also examined effects of fasting on the calpain small subunit, calpastatin, and on β-actin mRNAs for comparative purposes. Fasting increased mRNAs encoding all proteinases, calpain small subunit, and calpastatin in skeletal muscle but reduced β-actin mRNA. This effect was most pronounced for cathepsin D. To determine whether the changes observed in skeletal muscle occurred in other tissues, we examined effects of fasting on proteinase mRNA concentrations in liver, lung, and kidney. Fasting either had no effect or reduced proteinase, calpastatin, and calpain small subunit mRNA concentrations in these tissues. Fasting reduced β-actin mRNA concentration in all tissues. Therefore, proteinase gene expression in skeletal muscle differs from other tissues during fasting. Despite the changes in calpain mRNA concentrations in muscle, changes in calpain activities were not detected. This suggests that either calpain synthesis was concomitantly reduced or calpain turnover was increased during fasting. Differences in calpain mRNA concentrations were detected among tissues and these were related to differences in calpain concentrations and activities among tissues. We conclude that calpain and other proteinase genes are co-regulated in muscle in a manner that differs from other tissues, and that fasting-dependent changes in muscle calpain mRNA serve to maintain calpain concentrations at fixed levels at a time when muscle protein synthesis is reduced or calpain stability is reduced. Finally, we conclude that differences in calpain mRNAs among tissues underlie the differences in tissue calpain concentrations and activities. (J. Nutr. Biochem. 5:529–535, 1994.)

Keywords: calpain; calpastatin; cathepsin D; proteasome; proteinase; muscle; gene expression

Introduction

Several housekeeping proteolytic systems exist in skeletal muscle. These include lysosomal proteinases, the calpains, and the more-recently identified ATP-dependent systems. Functions of the individual proteolytic systems in skeletal muscle are not fully understood. Some investigators[1–3] believe that the ATP-dependent proteolysis plays a key role in mediating skeletal muscle atrophy in diseases or nutritional deficiencies, yet others[4,5] believe that calpains play the more critical role.

Presently, the rate-limiting event underlying myofibrillar protein degradation is unknown. Until the controlling element of this process has been identified, unequivocal identification of the critical proteinase will be difficult. In a previous study,[6] we documented that 8 days of fasting increased myofibrillar protein degradation in rabbits and that this was associated with increased mRNA concentrations encoding calpains, cathepsin D, and a proteasomal subunit. These data suggested that all major proteolytic systems of muscle participate in adaptation to the fasted state, and that each could play some role in myofibrillar protein disassembly and degradation. However, it is difficult to test this because protein degradation in muscle is usually assessed by release of free tyrosine or N-methylhistidine (NMH) from

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muscle. Therefore, inhibition of any protease involved in the process of disassembly and degradation would block the release of free amino acids from the muscle cell.

The goal of this research was to investigate the control of myofibrillar protein degradation in more detail. Specifically, we determined whether fasting brought about coordinated changes in expression of all calpain subunits and determined whether the regulation of proteinase gene expression in muscle was similar to or different from regulation in other tissues (liver, lung, and kidney). Finally, to establish the importance of the regulation of calpain genes in tissues, we examined the effects of fasting on calpain activities and concentrations and examined whether changes in calpain mRNA concentrations brought about corresponding changes in calpain concentrations and activities.

Methods and materials

Animals

Details of animal care and treatment are presented in an earlier report.[6] At the conclusion of the fasting period, rabbits were anesthetized with T61 euthanasia solution and blood was collected in heparinized vacutainer tubes via cardiac puncture. Plasma was separated by centrifugation and was replaced by the same volume of distilled water to lyse blood cells. Samples were frozen at −20° C until analysis. Samples of skeletal muscle (vastus lateralis), liver, lung, and kidney were excised, blotted, and weighed. After their removal, tissues were frozen in liquid nitrogen and stored in a −90° C freezer.

Calpain and calpastatin assays

Analysis of calpain activity was performed using phenyl-sepharose chromatography as described by Gopalakrishna and Barsky.[7] Details of the analytical procedure are outlined earlier.[6] One unit of calpain activity was defined as the amount of enzyme that produced an increase of one absorbance unit at 280 nm after 30 min incubation at 25° C, corrected by subtracting the activity found in the presence of 1 mM EDTA. Calpastatin activity was assayed as described.[6] One unit of calpastatin inhibited one unit of rabbit muscle calpain II.

Messenger RNA analysis

Determination of steady state mRNA concentrations encoding calpains I and II (large subunits), calpastatin, cathepsin D, proteasome C2, and β-actin were performed by Northern blotting, essentially as outlined previously.[6] RNA extraction via the CsCl method[8] was used to provide an estimate also of tissue RNA content.

For determination of the mRNA concentration encoding the calpain 30 kDa subunit, a 30-base pair oligonucleotide probe was synthesized at the Center for Gene Research of Oregon State University. The probe sequence : 5′ CCG GCG AGG CGG AAG GGG GCG GCT CTG GGG 3′ was complementary to 809 to 838 bp of the 3′ noncoding region of the rabbit calpain small subunit.[9] After synthesis, the oligomer was purified from contaminants using urea denaturing polyacrylamide gel electrophoresis (PAGE) according to Vorndam and Kerschner.[10] The probe was radiolabeled with ^{32}P γ-ATP (6000 Ci/mmol, New England Nuclear, Boston, MA USA) using the 5′-end labeling method,[8] after which it was purified from free γ ^{32}P-ATP using P2 gel chromatography.

Western blotting analysis

Tissues or hemolyzed erythrocytes were homogenized in 5 vol of ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM 2-mercaptoethanol and 150 nM pepstatin A) using a Polytron at half-maximum setting, three times for 15 sec each while keeping the tube immersed in ice. Afterward, samples were centrifuged at 13,000*g* for 30 min and the supernatant was decanted through glass wool to separate lipids. The supernatant was assayed for protein concentration using the Bio-Rad Bradford protein assay solution (Bio-Rad, Richmond, CA, USA) according to the supplier's instructions and a standard curve was constructed using bovine serum albumin fraction IV.

Tissue homogenates (100 μg protein per lane) were processed using sodium dodecyl sulfate-PAGE according to Laemmli[11] in a 7.5% polyacrylamide slab gel. Molecular weight markers were included to determine sizes of immunostained bands. After completion of electrophoresis, the molecular weight marker lane was cut out and visualized with Coomassie brilliant blue P250 stain. Bands were used to construct a standard curve by plotting distance migrated on the gel against log molecular weight of standard proteins. The remaining gel was transferred to a nitrocellulose membrane electrophoretically according to the method of Towbin et al.[12] After transfer, the membrane was blocked with 3% (wt/vol) gelatin in Tris-buffered saline (TBS) for 60 min at room temperature (RT) then washed 2 times for 5 min each in TBS containing 0.05% (vol/vol) Tween-20 (TTBS). Then the membranes were incubated with anti-calpain monoclonal antibodies ($3C_{11}B_{10}$ for calpain I and $1C_5C_8$ for calpain II[13]) diluted with TTBS and 1% gelatin (wt/vol) to 1/1,000. The monoclonal antibodies (mAbs) were donated by Dr. Seiichi Kawashima (Tokyo Metropolitan Institute of Medical Science). The incubation period for primary antibody treatment was 12 hr, followed by two washes with 1 × TTBS for 5 min each at RT. Then the membranes were incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin as the second antibody. The incubation period was 2 hr followed by washing two times with 1 × TTBS for 5 min each at RT. The peroxidase staining was developed using *O*-dianisidine as the substrate.[14]

Partially-purified calpain I and II preparations by phenyl-sepharose chromatography were used to establish the specificity of monoclonal antibodies. We immunoblotted a membrane loaded with 2.5, 5.0, and 7.5 μg protein of partially purified calpain I and calpain II samples with $3C_{11}B_{10}$ to confirm its monospecificity against calpain I. Also, we immunoblotted a membrane loaded with 2.5, 5.0, and 7.5 mg protein of partially purified calpain II, 7.5 μg protein of partially purified calpain I, and 100 and 200 μg protein of hemolyzed erythrocytes, which contain only calpain I, against the calpain II mAb. Determination of calpain concentrations was performed by producing transparencies from membranes and scanning them using a BioRad Model 1650 Scanning Densitometer and a Hoefer-350H scanning densitometric program (Hoefer, San Francisco, CA, USA).

Statistical analysis

Differences ($P < 0.05$) between the two experimental treatments were examined using an unpaired Student's *t* test.[15] Effects of fasting on the changes in mRNA concentrations encoding the calpain subunits were examined by correlation analysis according to the methods of Steele and Torrie.[15]

Results

Fasting for 8 days reduced body weight by 38% ($P < 0.05$) and increased myofibrillar protein degradation (NMH excretion/100 g body weight/d) by twofold.[6] The effects of fasting on organ weights are shown in *Table 1.* Livers of fasted rabbits were 59% smaller ($P < 0.05$) than fed rabbits. Kidneys, vastus lateralis, and lungs were 35, 31, and 30% smaller in fasted rabbits ($P < 0.05$) than in fed rabbits, respectively *(Table 1).*

Table 1 Effect of fasting on organ weights and RNA contents

Parameter (g)	Control	8-day fasted	Change from control (%)
Tissue weights (grams)			
Vastus lateralis	10.0 ± 0.5[a]	6.9 ± 0.2[b]	−31
Liver	99.5 ± 6.3[a]	40.5 ± 1.7[b]	−59
Kidney	18.9 ± 1.0[a]	12.3 ± 0.5[b]	−35
Lung	10.4 ± 0.5[a]	7.3 ± 0.5[b]	−30
RNA contents (μg RNA/g wet weight)			
Vastus lateralis	933 ± 42[a]	600 ± 36[b]	−35.7
Liver	4861 ± 164[a]	4497 ± 290[a]	−8.5
Kidney	1856 ± 112[a]	1395 ± 98[b]	−24.8
Lung	1604 ± 182[a]	1090 ± 156[b]	−32.0

Initial body weights were 2003 ± 72 and 2008 ± 45 for control and fasted groups, respectively. Each value is an average ± SEM of six observations. Total RNA in tissues was determined as the yield of RNA calculated from UV absorption.
Values in the same row with different superscripts differ significantly ($P < 0.05$).

The effects of fasting on skeletal muscle, liver, kidney, and lung RNA concentrations are also shown in *Table 1*. After 8 days of fasting, RNA concentrations in skeletal muscle, lung, and kidney were 35.7, 32.0, and 24.8% lower than respective RNA concentrations in control animals ($P < 0.05$). However, the liver response was less profound. Liver RNA concentration was reduced by only 8.5% ($P > 0.05$) by fasting. Among tissues, liver had the highest concentration of RNA, followed by lung and kidney, then skeletal muscle.

Effects of fasting on protease mRNA concentrations in various tissues

The utility of using cDNA probes for calpain I, calpain II, calpastatin, cathepsin D, and β-actin for specificity and quantitative Northern analysis was established earlier.[6] Validation studies for the calpain small subunit oligonucleotide probe were performed in this study (data not shown). Specifically, a dose-response curve and cross-species validation analysis using 12.5, 25, and 50 μg of rabbit and rat muscle RNA were performed. Results of the dose-response analysis showed a linear response of image density to gradations in RNA concentrations for the rabbit. Cross-species validation analysis indicated that the 3′ noncoding region selected for the oligomer hybridized only calpain small subunit mRNA in rabbits. A single hybridization band of about 1.5 kbp was detected. This result closely matched the predicted size for calpain small unit mRNA reported by Emori et al.[9]

Effects of fasting on calpain subunit mRNA concentrations in various tissues are shown in *Figure 1*. Because blots were processed simultaneously, for each proteinase mRNA the relative concentrations of mRNAs among the four tissues may be compared. Among tissues of control animals, the highest calpain I mRNA concentration was detected in lung, followed by kidney and muscle, then liver. Effects of fasting on calpain I mRNA concentration differed among tissues. Whereas fasting increased calpain I mRNA concentration twofold ($P < 0.05$) in skeletal muscle, it reduced the concentration of calpain I mRNA significantly ($P < 0.05$) in liver and lung but had no effect ($P > 0.05$) in kidney.

Effects of fasting on calpain II mRNA concentrations and on the small (30 kDa) calpain subunit in various tissues are also shown in *Figure 1*. Essentially, a similar distribution pattern among tissues of control animals and a similar response to fasting among tissues to that of calpain I mRNA was observed.

Effects of fasting on calpastatin mRNA concentrations in skeletal muscle, liver, kidney, and lung are shown in *Figure 1*. Three calpastatin mRNA bands of 3.8, 3.0, and 2.5 kbp were detected (bands I, II, and III, respectively), as has been previously reported.[6,16,17] These bands arise from the same gene and differ only in processing of their 3′ noncoding regions.[8] Calpastatin mRNA was expressed at high levels in kidney and lung and at lower levels in muscle and liver. Calpastatin mRNA band patterns, which were constitutively expressed, and effects of fasting on calpastatin mRNA concentrations and band patterns differed among tissues. Skeletal muscle expressed bands I and II constitutively in control and fasted rabbits, but with slightly higher levels ($P < 0.05$) for the fasted animals. Fasting, however, caused expression of band III in skeletal muscle. Thus, muscle total calpastatin mRNA (summation of bands I–III) increased ($P < 0.05$) in response to fasting. Liver expressed the three forms of calpastatin mRNA constitutively in control

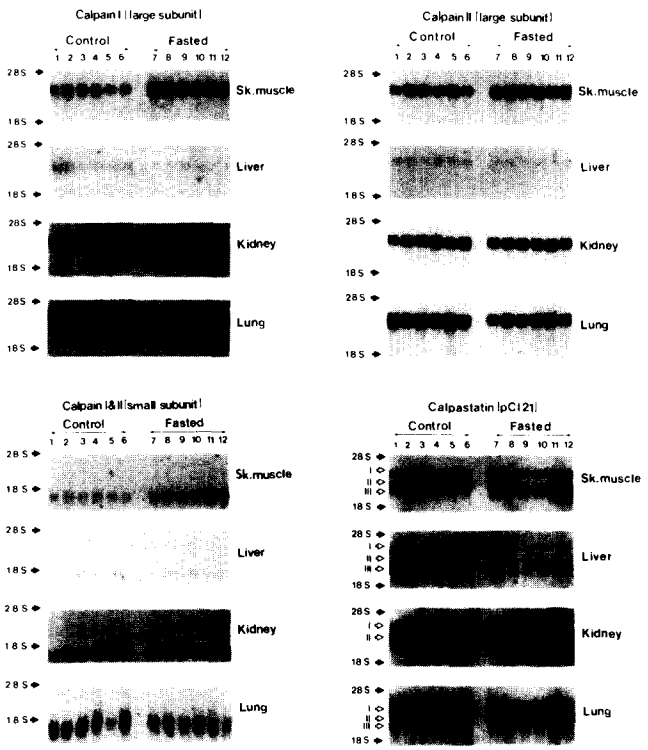

Figure 1 Concentrations of calpain I (upper left panel), calpain II (upper right panel), calpain 30 kDa subunit (lower left panel), and calpastatin (lower right panel) mRNAs in skeletal muscle (vastus lateralis), liver, kidney, and lung of control and fasted rabbits. Washing conditions for calpain I and II and the 30 kDa subunit were four times with 0.1 × SSC/0.1% SDS at 50°C. Washing conditions for calpastatin membranes were 1 × SSC/0.1% SDS four times at 25°C followed by 0.1 × SSC/0.1% SDS two times at 50°C. In all blots, each lane represents one animal. pCI21 is the plasmid name used to derive calpastatin cDNA.[16] Positions of ribosomal RNAs (28S and 18S) are indicated by solid arrows.

and fasted animals. Fasting dramatically reduced total calpastatin mRNA concentration in liver ($P < 0.05$) by reducing the concentrations of each of the three bands. Fasting did not affect ($P > 0.05$) calpastatin mRNA concentrations or band distribution in kidney. In lung, fasting caused expression of calpastatin band III mRNA in some of the experimental animals (*Figure 1*, lanes 8 and 10 of lower left panel) in addition to the constitutively expressed bands I and II. However, fasting reduced ($P < 0.05$) total calpastatin mRNA concentrations in lung.

To determine whether the fasting-dependent increase in proteinase subunit mRNAs in muscle was unique, we examined effects of fasting on expression of mRNAs encoding proteinases in liver, kidney, and lung. Effects of fasting on cathepsin D mRNA levels in various tissues of control and fasted rabbits are shown in *Figure 2*. The tissue containing the highest endogenous concentration of cathepsin D mRNA was kidney, followed by lung, liver, and skeletal muscle, respectively. Despite the low levels of cathepsin D mRNA expressed in fed animals, fasting markedly increased skeletal muscle cathepsin D mRNA several fold and had no effect ($P > 0.05$) in liver, kidney, and lung.

Effects of fasting on proteasome C2 mRNA concentrations in various tissues are also shown in *Figure 2* (right panel). Proteasome C2 mRNA was expressed at its highest level in kidney, followed by lung, liver, then skeletal muscle. Fasting increased ($P < 0.05$) proteasome C2 subunit mRNA concentration twofold in skeletal muscle; however, proteasome mRNA was either unaffected ($P > 0.05$) in liver and kidney or reduced ($P < 0.05$) in lung. β-actin mRNA concentration was reduced by approximately 30% ($P < 0.05$) by fasting (*Figure 3*) in all tissues. Scanning densitometry, typical of the analyses used for other mRNA species, is shown in this figure.

Effects of fasting on calpain subunit concentrations

We assessed calpain I and II large subunit concentrations using Western blot analysis and also examined effects of fasting on muscle calpain I and II activities. Specificities of

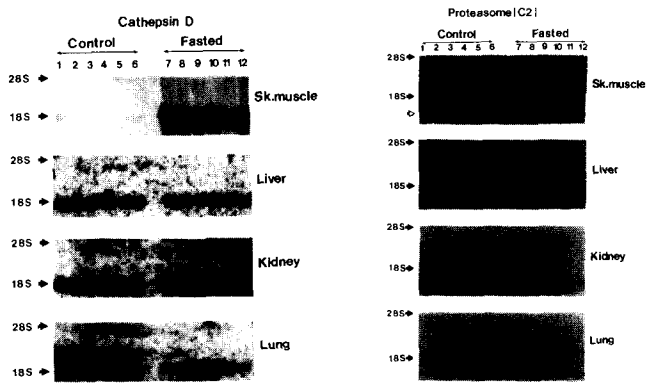

Figure 2 Concentration of cathepsin D and proteasome C2 mRNAs in skeletal muscle (vastus lateralis), liver, kidney, and lung of control and fasted rabbits. Washing conditions for all blots were four times with 1 × SSC/0.1% SDS at RT. Positions of ribosomal RNAs (28S and 18S) are indicated by solid arrows. The position of proteasome C2 mRNA is indicated in the upper right panel with a hollow arrow. Backgrounds in liver, kidney, and lung on the cathepsin D blots are higher due to re-probing of these membranes.

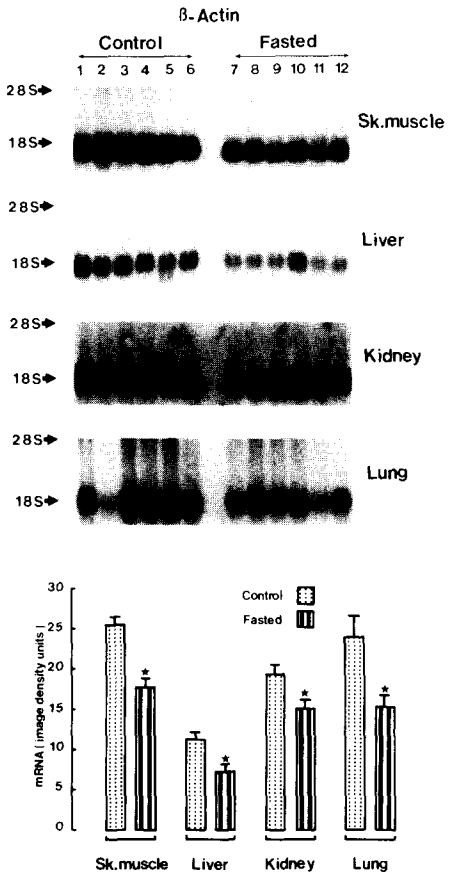

Figure 3 Concentration of β-actin mRNA in skeletal muscle (vastus lateralis), liver, kidney, and lung of control and fasted rabbits. Washing conditions were 0.5 × SSC/0.1% SDS at 60° C four times. Position of ribosomal RNAs (28 S and 18 S) are indicated by solid arrows. The lower panel represents scanning densitometry (mean ± SEM, n = 6). Differences ($P < 0.05$) between control versus fasted animals are indicated by a "star" above scan bars. Lane 2 in lung was excluded from analysis because RNA leaked from this lane during loading.

the mAbs for the calpains were examined using Western blot analysis. Both were isozyme-specific. Scanning densitometry indicated that staining intensity increased linearly with gradations in partially purified antigens (2.5 to 7.5 μg protein; data not shown).

Results of Western analysis of calpain I in skeletal muscle, erythrocytes, kidney, and lung are shown in *Figure 4*. Fasting did not affect ($P > 0.05$) calpain I concentrations in kidney, muscle, or erythrocytes; however, lung calpain I concentration was lower ($P < 0.05$) in fasted animals compared with controls. Among the tissues of control rabbits, lung possessed the highest calpain I concentration. Erythrocytes also contained high calpain I concentrations. Skeletal muscle and kidney contained similar quantities of calpain I. Calpain I was not detectable in liver, and this tissue also contained the least calpain I activity and least calpain I mRNA.

Western analysis of calpain II in various tissues is also presented in *Figure 4* (lower panel). Calpain II was not detectable in erythrocytes. Fasting did not affect ($P > 0.05$) calpain II concentrations in any of the tissues. When comparing concentrations of calpain II among tissues, lung was found to have the highest concentration, followed by kidney, skeletal

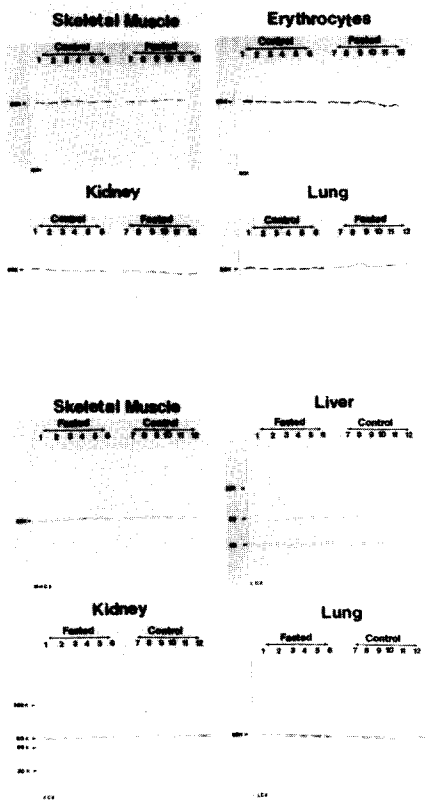

Figure 4 Western blot analysis of calpain I (upper panel) and calpain II (lower panel) in tissues of control and fasted rabbits. Homogenates (100 μg protein/well) were subjected to SDS-PAGE and then transferred to a nitrocellulose filter that was stained with anti-rabbit calpain I or calpain II monoclonal antibodies. Molecular weight standards are indicated by solid arrows (K = KDa). Liver calpain I and erythrocyte calpain II were not detectable.

muscle, and liver. Although the calpain II mAb was monospecific in muscle and lung, it reacted with several proteins in liver and kidney. In liver these were identified as 252, 80, and 30 kDa, and in kidney as 252, 80, 56, and 30 kDa. Identities of the non-80 kDa proteins that reacted with the antibodies are unknown. Calpains I and II were detected only as 80 kDa isoforms. Autolyzed isoforms were not evident.

Effects of fasting on calpain activities

Fasting did not effect changes ($P > 0.05$) in calpain or calpastatin activities in vastus lateralis or in other tissues (*Table 2*). The highest calpain activities were detected in the lung and kidney, while other tissues (skeletal muscle, blood cells and liver) had lower activities of the enzymes. The levels of calpastatin in different tissues followed that of calpains closely. Activity of calpastatin was highest in lung, followed by liver, kidney, erythrocytes, and muscle.

Discussion

Fasting increases myofibrillar protein degradation and reduces protein synthesis.[18–21] In an earlier study, we documented that an 8-day fast increased urinary NMH excretion, an index of myofibrillar protein degradation, in rabbits. Although effects of fasting on muscle and liver have been extensively studied, effects of fasting on protein homeostasis in other tissues are not as well known. The reductions in weights of kidney and lung in addition to their losses in RNA concentrations suggest that they may resemble skeletal muscle in their biochemical responses to fasting. The reductions in RNA content in muscle, kidney, and lung indicate a reduction in capacity for protein synthesis. Ability of liver to maintain its RNA concentration indicates that the fasting-dependent reduction in its mass occurred either via reduced efficiency of protein synthesis (i.e., reduced protein synthesis per ribosomal unit) or via enhanced proteolysis or both.

In a previous study,[6] we investigated effects of fasting on calpain large subunit mRNA concentrations in biceps femoris. Because fasting had similar effects on muscle calpain I and II mRNAs, we proposed that other members of the calpain family, specifically the 30 kDa small subunit, may be similarly regulated. Using a different muscle, vastus lateralis, we examined effects of fasting on steady-state mRNA concentrations encoding each of the calpain subunits.

Fasting increased mRNA concentrations encoding each of the calpain subunits (calpain I, calpain II, 30 kDa subunit, and calpastatin) in skeletal muscle, indicating that the entire calpain system is coordinately regulated at the gene level during fasting. In this study, fasting caused a twofold increase in muscle calpain mRNA concentrations, whereas in biceps femoris[6] the effect was much larger. This difference between individual muscles may in part be due to differences in fiber type properties of the two muscles. Specifically, we previously determined that rabbit biceps femoris contained 10% Type I fibers, whereas vastus lateralis contained only 1% Type I fibers. Other factors may also influence the response of the calpain system to fasting. Intensity of use of individual muscles could alter the pattern of protease gene expression during fasting. Alternatively, protease gene expression during fasting could differ temporally among individual muscles.

Individual calpastatin mRNA bands responded differently to fasting. We reported that newborn rabbits expressed only an intermediate-sized calpastatin mRNA (band II), that this declined in concentration during aging, and that calpastatin mRNA band I in muscle was detectable only in adults.[17] Fasting caused expression of the third calpastatin mRNA isoform (band III) in adults. The variable expression of calpastatin mRNA bands arises from existence of three 3′ processing sites in the calpastatin gene.[16] It is not unusual for multiple mRNAs to arise from the same gene product. Although these results are interesting, their physiological meaning is unknown.

We examined whether the fasting-dependent changes in calpain subunit mRNA concentrations were unique to the calpain system or were shared by other proteolytic systems. For this comparison we selected cathepsin D and the proteasomal C2 subunit. Cathepsin D activity has been used in previous studies as an index of muscle proteolysis.[22] The proteasome was chosen because there is evidence that this complex plays an important role in degradation of myofibrillar proteins,[1–3] although this is equivocal.[4,5]

In both this study and in our previous study,[6] fasting increased cathepsin D mRNA concentration to a much greater extent than its effects on mRNAs encoding other proteinases. Proteasome C2 mRNA concentration was increased by fast-

Table 2 Calpain I, calpain II, and calpastatin activities in various tissues of control and fasted rabbits

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

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

ing but the effect, compared with to cathepsin D, was small. A portion of this effect was due to a concentration effect because fasting also reduced muscle total RNA content *(Table 1)* and equivalent amounts of total RNA were loaded for Northern blotting. We believe that the fasting-dependent changes in concentrations of mRNAs encoding several proteolytic system in vastus lateralis indicates that each proteinase plays a role in coordination of muscle protein homeostasis during fasting. Because fasting increased all proteinase mRNAs, we cannot ascribe particular significance to a specific proteinase, but it is intriguing that the response of cathepsin D, a proteinase that is now regarded as having only marginal importance to myofibrillar protein degradation, was the largest among the proteinase mRNAs investigated. Because the magnitude of this effect was large, it would be of interest to determine whether the mRNAs encoding other lysosomal enzymes (e.g., cathepsins B, H, and L) change as markedly during fasting and to identify the molecular mechanisms (i.e., signal transduction system and *cis*-elements) accounting for such a large change in cathepsin D gene expression. Furthermore, it would be of interest to determine whether this change in cathepsin D gene expression increases muscle cathepsin D activity and to identify the role that cathepsin D performs in skeletal muscle during fasting.

To identify the mechanism underlying the changes in proteinase gene expression, we have completed several studies with cultured L8 myotubes in which we tested effects of endocrine factors on protease (calpain and cathepsin D) gene expression. To date, we have evaluated effects of IGF-1,[23] dexamethasone, and phorbol ester in vitro on protease gene expression.[24] We have also investigated effects of exogenous porcine somatotropin on cathepsin D gene expression on porcine muscle in vivo.[25] To date, none of these well-studied endocrine factors have demonstrated ability to regulate protease gene expression to the extent demonstrated in this and in our previous[6] study. Other factors are under investigation. It remains possible that intensity of muscle use (i.e., fasted rabbits were very inactive) or neuronal myotrophic factors could account for these observations.

Further objectives of this study were to determine whether the fasting-dependent changes in muscle proteinase gene expression were a general response, which could be detected in all tissues, or were unique to muscle. Proteinase subunit mRNAs, which were consistently elevated in skeletal muscle, were either unaffected or reduced by fasting in liver, lung, and kidney. This difference was particularly large for cathepsin D where, in muscle, fasting caused a very large increase in its expression but had no effect in other tissues. We conclude that effects of fasting on proteinase gene expression in the other tissues may differ temporally from skeletal muscle, or that there may exist in muscle a distinct signal transduction mechanism that coordinates proteinase gene expression during fasting. Perhaps these changes underlie the ability of skeletal muscle to mobilize its protein reserves during fasting.

We examined effects of fasting on steady-state concentrations of calpain subunit mRNAs, calpain subunit concentrations, and calpain activities in muscle, liver, kidney, and lung as a means of assessing the importance of transcriptional regulation to expression of calpain activities. Although fasting increased calpain mRNAs in muscle, it did not alter calpain concentrations or activities. These results are similar to those in which we determined that large changes in muscle calpain mRNA concentrations also did not bring about changes in calpain activities.[6] However, we did observe that tissues that contain high concentrations of the calpain subunit mRNAs (e.g., lung) also possessed high calpain activities. Tissues with low calpain subunit mRNAs (e.g., liver) contained low or negligible calpain activities. Hence, we believe that the variable activity of calpains among tissues is determined by the relative expression of calpain subunit mRNAs, and that additional regulation of transcription of calpain subunit genes, as is seen in muscle during fasting, is intended to ensure that calpain subunit concentrations are maintained at levels which are appropriate to the needs of that tissue rather than to change calpain concentrations. We propose that the purpose of fasting-dependent transcriptional regulation of proteinases is to counter the concomitant fasting-dependent reduction in protein synthesis such that proteinase concentrations may be maintained at constant levels. Although we did not assess protein synthesis in this study, it is well known that fasting reduces muscle protein synthesis.[18]

An alternative explanation for the lack of change in calpain activities in muscle during fasting is that muscle calpains may turn over more rapidly during a fast. Specifically, calpains may be activated to degrade muscle proteins during fasting and, if the model for calpain activation/inactivation is correct,[26] this would be followed by their auto-degradation. A similar interpretation has been offered for why proteasome levels remain unchanged in tumors despite a large increase in proteasome gene expression.[27]

In the previous analysis, one discrepancy was noted. Lung calpain I activity was reduced, by fasting, but lung calpain I concentration, as assessed by Western analysis, was unaffected. The basis for this discrepancy is uncertain.

In conclusion, fasting increases proteinase gene expression in skeletal muscle and has little effect or the opposite effect on proteinase gene expression in other tissues. The divergent regulation of proteinase genes in muscle relative to beta-actin and relative to total RNA indicates that non-housekeeping elements are responsible. These observations underscore the importance of skeletal muscle to whole-body protein and energy homeostasis during fasting and demonstrate that the control of proteolysis in muscle differs from other tissues. Perhaps muscle possesses a unique signal transduction pathway that causes muscle proteinase gene expression to differ from that in other tissues during fasting.

In addition, this study has shown that differences in magnitudes of calpain subunit gene expression among tissues account for the different calpain concentrations that we detected among tissues. Fasting-dependent changes in calpain gene expression serve to maintain constant levels of calpains in muscle at a time when protein synthesis and calpain stability may be reduced. The similar responses of several proteolytic systems to fasting implies that a common mechanism influences transcription of several proteinases in muscle. Based on our other studies,[23,24] we believe that IGF-1, glucocorticoids, and hormones, that rely on protein kinase C for signal transduction, do not account for these changes.

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