Gene expression of calpains and their specific endogenous inhibitor, calpastatin, in skeletal muscle of fed and fasted rabbits

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To investigate the role of calpains in myofibrillar protein degradation in skeletal muscle and the regulation of their activity *in vivo*, we studied the effects of fasting on gene expression of calpains and calpastatin in the skeletal muscle of rabbits. In response to fasting, myofibrillar protein degradation increased 2-fold and mRNA levels of calpain I, calpain II and calpastatin were also increased. However, calpain and calpastatin activities remained unchanged. To investigate this discrepancy, we analysed polysomal calpain mRNA. Results indicated that fasting caused a 2-fold increase in the loading of calpain I and II mRNAs on ribosomes. Thus transcription of genes encoding calpain may be increased during fasting to ensure adequate synthesis of the proteinases needed to mobilize muscle protein reserves. The effect of fasting on calpain and calpastatin mRNA expression is shared by cathepsin D and proteasome C2 but not by β -actin, implying that fasting invokes control of several proteolytic systems in skeletal muscle and underscores the possibility that each proteolytic system plays a role in the adaptation of skeletal muscle to the fasted state.

INTRODUCTION

Calpains (EC 3.4.22.17) participate in numerous cellular functions through limited hydrolysis of various substrates. These observations have been related to the participation of calpains in the rearrangement of submembrane-cytoskeletal connecting protein (Thomas *et al.*, 1983), brain functioning (Zimmerman & Schlaepfer, 1982; Schlaepfer *et al.*, 1985), movement of secretory granules (Perrin *et al.*, 1987), selective limited proteolysis of receptors (Chad & Eckert, 1986) and a number of enzymes such as protein kinase C (Kishimoto *et al.*, 1983), and initiation of myofibrillar protein degradation (Goll *et al.*, 1985, 1989). The present paper focuses on the relationship of the calpain system to muscle protein degradation and on the molecular mechanisms regulating their activities in association with this function.

Evidence supporting a role for calpains in initiating myofibrillar protein degradation is suggestive (Goll *et al.*, 1989). Despite this, no unequivocal data have excluded other proteinases from some role in myofibrillar protein degradation. In fact, growing evidence suggests that, although calpains could initiate the metabolic turnover of myofibrillar proteins, other proteinases may also be involved in this turnover (e.g. Furuno *et al.*, 1990). Clearly, further research is needed to elucidate the role of calpains and other proteinases in myofibrillar protein degradation.

Intracellular proteinases, especially the extralysosomal ones, possess elaborate mechanisms for regulation of their activity. Regulation of enzyme activity may occur at many metabolic stages, including transcriptional, translational and post-translational levels. In the past decade there have been substantial advances in our understanding of the regulatory processes of calpain activity at the post-translational level (Suzuki *et al.*, 1988; Murachi, 1989; Melloni & Pontremoli, 1989; Cong *et al.*, 1989). However, knowledge concerning regulation of calpains at the transcriptional level is limited to the observation by Emori *et al.* (1986) that considerable variability exists in mRNA levels of calpains I and II in various rabbit tissues and to the observations of Ou & Forsberg (1991) that muscle calpain II, but not calpain

I, gene expression is affected by aging. A major obstacle hindering progress in research on transcriptional regulation of calpain activity is the lack of a suitable biological model in which significant changes in calpain activities and mRNA levels occur. For this purpose, we analysed calpain I, calpain II and calpastatin gene expression in the skeletal muscle of rabbits during fasting. Our findings reveal significant changes in the mRNA levels of skeletal muscle calpain I and II and their specific inhibitor, calpastatin, in response to fasting. An implication of this finding is that fasting may provide a suitable experimental model for the elucidation of *trans*-regulatory elements underlying changes in calpain gene expression and changes in muscle protein degradation.

MATERIALS AND METHODS

Animals

Twelve female rabbits (body weight ~ 2 kg) were obtained from the Oregon State University Rabbit Research Center and were individually housed in metabolic cages and assigned to one of two treatments: (1) 8 days of *ad libitum* intake of a commercial rabbit diet consisting of 54 % dehydrated alfalfa, 20 % soybean meal, 21 % wheat mill run, 1.25 % vegetable oil, 3 % molasses, 0.5 % NaCl and 0.25 % dicalcium phosphate, or (2) 8 days of fasting (six animals per treatment). Water was available *ad libitum* and urine was collected daily into 20 ml of 5 M-HCl and frozen. At the end of the study the rabbits were killed by intraperitoneal injection of T-61 Euthanasia solution, after which the biceps femoris was dissected out and frozen in liquid nitrogen. Tissues were stored in plastic bags at -90°C .

Assessment of 3-methylhistidine (3-MH)

To evaluate the myofibrillar protein degradation rate during fasting, we measured the daily output of 3-MH in the urine of control and fasted rabbits. The method used to determine 3-MH was that of Forsberg & Liu (1989). Results of 3-MH analysis were

Abbreviation used: 3-MH, 3-methylhistidine.

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expressed as $\mu\text{mol/day}$ per 100 g body weight or as the 3-MH/creatinine ratio. Creatinine was determined colorimetrically by the picric acid method (Faulkner & King, 1976).

Preparation of ^{32}P -labelled cDNA probes

A variety of cDNA fragments were provided to us. These included pLU1001, pLM28 and pC121 (Emori *et al.*, 1986, 1987), which encode rabbit muscle calpain I and calpain II (80 kDa subunits) and calpastatin respectively and were provided by Dr. Y. Emori and Dr. K. Suzuki (University of Tokyo); pC2- α , which encodes rat liver proteasome (30.8 kDa C2 subunit; Fujiwara *et al.*, 1989) and was provided by Dr. K. Tanaka (Institute for Enzyme Research, University of Tokushima, Japan); pHcPDEco1.1, which encodes human cathepsin D and was provided by Dr. John Chirgwin (University of Texas Health Science Center, San Antonio, TX, U.S.A.; Faust *et al.*, 1985); and pR β -A1, which encodes rat β -actin and was provided by Dr. Larry Kedes (University of Southern California, Los Angeles, CA, U.S.A.; unpublished work). Plasmids were prepared by transformation of HB101 *Escherichia coli* and amplified and recovered using standard techniques (Maniatis *et al.*, 1982). The identities of plasmids used in this study were checked by establishing their physical maps with restriction enzymes and comparing these maps with the expected ones based on published results. Plasmid DNA was recovered and purified and cDNA inserts were then recovered by restriction enzyme digestion. *RsaI*/*DdeI* and *RsaI* fragments of pLU1001 and pLM28 respectively, which correspond to the 3' non-coding regions of calpain I and II mRNAs, were used to discriminate between these calpain I and II mRNAs. An *EcoRI* fragment of about 1100 bp, corresponding to the coding region of calpastatin, was used to assay for its mRNA levels. A 1000 bp *EcoRI* coding region fragment of pHcPDEco1.1 was utilized to assay cathepsin D mRNA. A 1000 bp *HindIII*/*PvuII* coding region fragment of pC2- α was prepared to assay proteasome C2 mRNA. Finally, a 1057 bp coding region *HindIII*/*PvuII* fragment of pR β -A1 was used to assay β -actin mRNA. cDNA inserts were purified following restriction enzyme digestion via agarose gel electrophoresis, which was followed by electroelution of the cDNA on to a dialysis membrane. The cDNA was recovered from the dialysis bag and then purified by ethanol precipitation and phenol/chloroform (1:1, v/v) extraction. cDNA fragments (25–50 ng) were radiolabelled to high specific radioactivity (1.9×10^9 d.p.m./ μg of cDNA) with [α - ^{32}P]dCTP (6000 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.) using a random primer kit (5-Prime 3-Prime Inc., West Chester, PA, U.S.A.), after which they were purified from unincorporated [α - ^{32}P]dCTP using P60 gel chromatography.

Cellular RNA preparation and Northern analysis

Total RNA was extracted from tissue samples using guanidinium isothiocyanate followed by purification on a caesium chloride gradient as outlined by Maniatis *et al.* (1982). Portions of 50 μg of total RNA were electrophoresed on 2.2 M-formaldehyde-containing agarose gels. The quality of extracted RNA samples was checked by ethidium bromide staining of agarose gels to verify the 28 S and 18 S bands and their intensity. Gels were transferred overnight to a BAS-85 nitrocellulose membrane (Schleicher and Schuell, Keene, NH, U.S.A.). RNA was immobilized by baking the filter for 3–4 h under vacuum at 80 °C, after which the membrane was prehybridized overnight at 42 °C in Stark's buffer (Maniatis *et al.*, 1982) containing 100 μg of sheared salmon sperm DNA/ml (Sigma Chemical Co., St. Louis, MO, U.S.A.). Following prehybridization the immobilized

RNA was hybridized for 48 h at 42 °C to ^{32}P -labelled cDNA probes in Stark's buffer containing dextran sulphate (10 %, w/v) and 5×10^6 c.p.m. of probe/ml of hybridization buffer. Following hybridization, membranes were washed. The washing conditions used for each cDNA are given in the legends of the Figures. Membranes were wrapped in Saran wrap and exposed with two Dupont Cronex Plus intensifying screens for 0.5–4 days to Kodak X-Omat film at -90 °C. Quantification of mRNA was completed via scanning densitometry using a Hoefer GS-350H scanning program.

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

Preparation of polysomes

The procedure of Buckingham *et al.* (1976) was used to prepare polysomes from muscle. Three muscle samples per treatment were chosen for this analysis. Muscle samples of 1 g (one sample was analysed per animal) were homogenized in ice-cold polysomal buffer (20 mM-Tris/HCl, pH 7.6, 0.25 M-sucrose, 0.25 M-NaCl, 5 mM-magnesium acetate, 1 mg of heparin/ml and 6 mM-2-mercaptoethanol) using a Polytron at half-maximum setting for 3 $\times$ 15 s for 3 times. The homogenate was centrifuged at 10000 g for 10 min to remove debris, plasma membranes, nuclei and mitochondria. Then Triton X-100 was added to the post-mitochondrial supernatant at a 1 % (v/v) final concentration and put on an end-to-end shaker for 10 min while immersed in ice. The mixture was then layered over an equal volume of polysome buffer containing 1.0 M-sucrose in a polyallomer tube (Beckman). The tubes were centrifuged in an ultracentrifuge at 140000 g for 3.7 h at 4 °C using an SW28 rotor (Beckman). After completion of the centrifugal run, the supernatant was aspirated and discarded, and then the tube wall was rinsed with cold polysome buffer lacking sucrose. The polysome pellet was allowed to drain for a few minutes by placing it upside down over tissue paper. Finally, the polysome pellet was suspended in cold sucrose-free polysome buffer for RNA extraction.

Polysomal RNA extraction

The method of Chomczynski & Sacchi (1987) was used to extract RNA from polysomes, with some modification. To the suspension of polysomes (0.5 ml) was added 10 ml of guanidinium thiocyanate (GI) buffer (4 M-GI, 25 mM-sodium citrate, pH 7.0, 0.5 % sarcosyl, 0.1 M-2-mercaptoethanol) and the mixture was homogenized using 15 strokes of a Teflon/glass (Dounce-type) homogenizer. A 0.1 vol. of 2 M-sodium acetate (pH 4) was added, and the RNA was extracted with an equal volume of a 5:1 (v/v) mixture of phenol and chloroform/isoamyl alcohol (49:1, v/v). RNA was collected by two propan-2-ol precipitations. The RNA pellet was given a final wash in 80 % ethanol and was resuspended at 65 °C in 0.5 % SDS by gentle pipetting. The RNA quantity and quality was determined by absorbance analysis at 260/280 nm, and RNA samples were divided into portions at appropriate concentrations and stored at -90 °C until use.

Dot-blot analysis

Polysomal RNA was denatured as described for Northern blots and then diluted with an equal volume of 20 $\times$ SSC (1 $\times$ SSC = 0.15 M-NaCl/0.015 M-sodium citrate). It was then

diluted with $10 \times$ SSC to give serial concentrations of 2.5, 5, 10 and $20 \mu\text{g}$ of polysomal RNA/0.1 ml and applied to a nitrocellulose membrane (BAS-85) in a dot-blot apparatus (Schleicher and Schuell). Each well was washed with 0.1 ml of $10 \times$ SSC. The membrane was allowed to air dry, followed by baking at 80°C for 3 h under vacuum.

Analysis of calpain I and calpain II mRNA in polysomal RNA was performed essentially as described earlier for Northern analysis. Experimental conditions for prehybridization, hybridization, washing and probe preparation to examine the composition of translated calpain I and II mRNAs in muscle tissue were identical to those used for Northern analysis. Finally, dot-blots were scanned by a visible light densitometer (Hoefer TI-350H) and, after establishing that the absorbance and RNA concentration ($2.5\text{--}20 \mu\text{g}$ RNA) were linearly related, the absorbance of dots from fasted groups was expressed as a percentage of the absorbance of dots from the control group. Non-specific hybridization of calpain cDNAs to non-calpain RNA was not detected in earlier Northern analyses.

Isolation and quantification of calpains and calpastatin

Phenyl-Sepharose chromatography was utilized to separate calpains and calpastatin according to the procedure of Gopalakrishna & Barsky (1985). Skeletal muscle samples (5 g) were homogenized in 5 vol. of ice-cold 50 mM-Tris/HCl, pH 7.5, containing 1 mM-EDTA, 10 mM-2-mercaptoethanol and 150 mM-pepstatin A, in a Polytron at half-maximum setting for 3×15 s while immersing the tube in ice. All separation and chromatography steps were performed at 4°C . The homogenate was centrifuged at $13000 g$ for 30 min and the supernatant was decanted through glass wool to separate lipids. The supernatant was divided into three portions: 1 ml for protein analysis, 1 ml for calpastatin determination and 20 ml for calpain isolation. To the supernatant (20 ml) was added $40 \mu\text{l}$ of 1 mM-leupeptin, 4 ml of phenyl-Sepharose beads [previously washed with buffer A (20 mM-Tris/HCl, pH 7.5, 0.1 mM- CaCl_2 , 10 mM-2-mercaptoethanol, $20 \mu\text{M}$ -leupeptin) containing added 0.25 M-NaCl], and 1.2 ml of 5 M-NaCl. After shaking the mixture for 5 min while immersed in ice, 0.8 ml of 0.1 M- CaCl_2 was added and the shaking was continued for another 10 min. Then the gel suspension was packed into a 10 ml plastic column (Bio-Rad Laboratories, Richmond, CA, U.S.A.). The packed column was then washed successively with 8 ml of buffer A with added 0.2 M-NaCl, buffer A without NaCl, and buffer A without leupeptin. Calpain II and calpain I were eluted successively with 16 ml of buffer B (20 mM-Tris/HCl, pH 7.5, 1 mM-EGTA, 10 mM-2-mercaptoethanol) with added 0.1 M-NaCl, and 8 ml of buffer B alone.

Calpastatin was partially purified by boiling the supernatant (1 ml) for 5 min, followed by centrifugation in a microcentrifuge to precipitate heat-denatured proteins. The calpastatin-containing supernatant was collected using a Pasteur pipette and stored at -20°C until activity analysis.

Calpain and calpastatin assays

Ca^{2+} -dependent proteolytic activity was determined by measuring the release from Hammersten casein of trichloroacetic acid-soluble peptides, absorbing at 280 nm (Yoshimura *et al.*, 1984). Proteolytic activity was measured in triplicate. Samples of eluants (3 ml) were combined with 1.5 ml of Ca^{2+} assay medium [100 mM-Tris, pH 7.5, 2 mM-2-mercaptoethanol, 1 mM- NaN_3 , 0.05 mM- CaCl_2 (for calpain I) or 1 mM- CaCl_2 (for calpain II), and 5 mg of casein/ml] and incubated for 30 min at 25°C . The reaction was stopped by adding 0.25 ml of ice-cold 50% trichloroacetic acid. The soluble digestion products were measured spectrophotometrically at 280 nm. One unit of calpain activity

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

Values are means $\pm$ S.E.M. of six observations, except for polysomal RNA analysis, with only three observations. * Significantly different from controls ($P < 0.05$). RNA concentrations were determined spectrophotometrically following purification as described in the Materials and methods section (Maniatis *et al.* 1982).

Parameter	Control	Fasted	Change (%)
Body wt. (g)			
Initial	2003 ± 72	2008 ± 45	—
Final	2284 ± 66	$1420 \pm 20^*$	—38
Cranial biceps femoris			
Weight at end of study (g)	15.4 ± 0.8	$10.0 \pm 0.4^*$	—35
Total cellular RNA ($\mu\text{g/g}$ of tissue)	1377 ± 94	$972 \pm 57^*$	—29
Polysomal RNA ($\mu\text{g/g}$ of tissue)	550 ± 59	$311 \pm 79^*$	—43
Polysomal RNA/total RNA	0.40 ± 0.08	$0.32 \pm 0.09^*$	—20

Fig. 1. Effects of fasting on daily urinary excretion of 3-MH and creatinine

Results are means $\pm$ S.E.M. ($n = 6$). Values for 3-MH (a) and creatinine (b) secretion are expressed as $\mu\text{mol/day}$ per 100 g body wt.

was defined as the amount of enzyme which produced an increase of 1 absorbance unit at 280 after 30 min of incubation at 25°C , corrected by subtracting the activity found in the presence of 1 mM-EDTA.

Calpastatin activity was assayed on the basis of its inhibition

Fig. 2. Dose-response curve and validation results for cDNA probes of calpain I and calpain II large subunits and calpastatin against RNA samples of rabbit and rat skeletal muscle

Various amounts of rabbit or rat skeletal muscle RNA were electrophoresed, transferred to nitrocellulose and then probed using cDNA probes from the 3' untranslated ends of the rabbit calpain I and II cDNAs and from a rabbit coding-region calpastatin probe. Details of the preparation of these cDNAs and their labelling are included in the Materials and methods section. The dose response of image density versus RNA concentration is shown for each probe. Northern analyses are shown in insets in which positions of 28 S and 18 S RNAs are indicated. Although Northern blots have been trimmed, non-specific binding of the cDNA probes elsewhere in the lanes was not detected. For calpastatin mRNA, multiple mRNAs were detected and identified as bands I, II and III. For both calpains and calpastatin, rabbit cDNAs did not hybridize to rat RNA.

of calpain II activity (Ou & Forsberg, 1991). Inhibitor activity was measured by introducing portions (150 μl) of boiled fractions to a calpain II preparation from rabbit tissues (0.2 enzyme unit) and preincubated for 10 min at 25 °C. After preincubation the remaining (uninhibited) proteinase activity was determined by the proteinase assay described above. In this assay system, 1 unit of calpastatin activity completely inhibited 1 unit of calpain II. Inhibitory units were calculated only from the dilution which showed 40–50% inhibition.

Western blot analysis

Tissues were homogenized in 50 mM-Tris/HCl (pH 7.5) containing 1 mM-EDTA, 10 mM-β-mercaptoethanol and 150 mM-pepstatin. Samples of protein (150 μg/lane) were electrophoresed on a 12% polyacrylamide gel, transferred to nitrocellulose, blocked with 3% gelatin, incubated in calpain isoenzyme-specific antibody (1C₅C₈ for m-calpain and 3C₁₁B₁₀ for μ-calpain; Kasai *et al.*, 1986) washed twice with TTBS buffer [20 mM-Tris (pH 7.5)/500 mM-NaCl/0.05% Tween-20] then incubated with secondary antibody conjugated to horseradish peroxidase. Substrate solution (4-chloro-1-naphthol) was then added to visualize the complex.

Statistical analysis

Differences ($P < 0.05$) between the two experimental treat-

ments were examined using an unpaired Student's *t* test (Steel & Torrie, 1980). For all comparisons, a 5% level of significance was adopted.

RESULTS

Characterization of fasted rabbits

Effects of fasting on body weight and the weight of the cranial biceps femoris and its RNA content are shown in Table 1. Rabbits allowed access to food continued to grow during the 8-day experimental period. Fasted rabbits were 38% smaller than their fed counterparts at the conclusion of the experiment. Biceps femoris weight was decreased ($P < 0.05$) by fasting to a similar degree as was body weight. Fasting reduced total and polysomal RNA concentrations in biceps femoris by 29% and 43% respectively. Polysomal RNA expressed as a proportion of total RNA was also decreased ($P < 0.05$) by fasting.

Urinary 3-MH excretion increased gradually as fasting progressed and rose to about 200% of the control level on day 8 (Fig. 1). Creatinine excretion was slightly higher, but not significantly different ($P > 0.05$), in fasted rabbits. The 3-MH/creatinine ratio for fasted rabbits compared with controls during the 8-day experimental period changed in a similar way to absolute rates of 3-MH excretion.

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

Validation studies were carried out as outlined in the legend for Fig. 2, except that rat liver RNA was used for the validation of the β -actin probe. Details of the preparation of the cDNA probes and hybridization conditions are given in the Materials and methods section. Dose-response curves of image density for rabbits (■) and rats (▼) are indicated. Insets are Northern blots with positions of 28 S and 18 S RNA indicated. Each of the cDNA probes hybridized with both rabbit and rat mRNA.

Effect of fasting on proteinase mRNA levels

To investigate the characteristics of the transcriptional regulation of calpain and calpastatin expression under normal conditions, and under conditions of accelerated myofibrillar protein degradation, we determined steady-state concentrations of mRNA encoding calpain I, calpain II and calpastatin in control and fasted rabbits. Furthermore, to gain insight into the nature of the responses (i.e. specific versus general) of mRNAs of the calpain system to fasting, we examined the effects of fasting on levels of mRNAs encoding cathepsin D, proteasome C2 subunit and β -actin in skeletal muscle.

Dose-response curves for calpain I, calpain II, calpastatin, cathepsin D, proteasome and β -actin, using 12.5, 25 and 50 μ g of total RNA, showed linear responses of image density to gradations in RNA concentrations (Figs. 2 and 3). Consequently, 50 μ g of total RNA was routinely processed for Northern blot analysis to enhance detection sensitivity. The cross-species validation study indicated that the 3' non-coding region cDNA probes prepared from pLU1001 and pLM28 were species specific, as they hybridized to RNA isolated from rabbit muscle but not from rat muscle (Fig. 2). Coding region cDNA probes for human cathepsin D, rat proteasome C2 and rat β -actin hybridized to both rat and rabbit mRNAs (Fig. 3). These corresponded to bands of molecular sizes of 2.2, 1.35 and 2.1 kb respectively. These results closely match the predicted sizes of mRNAs encoding these proteins in human liver (Faust *et al.*, 1985), rat liver (Fujiwara *et al.*, 1989) and rat kidney respectively. Dose-response curves to gradations of RNA concentration indicated that image density increased linearly up to 50 μ g total RNA.

The effects of fasting on the expression of mRNAs encoding calpain I, calpain II and calpastatin are shown in Fig. 4. Fasting increased the concentrations of mRNAs encoding calpain I, calpain II and calpastatin by 4.2-, 4.5- and 3.2-fold respectively. The effects of fasting on the expression of calpains I and II were very similar. Muscle samples containing low levels of calpain I mRNA also contained low levels of calpain II mRNA.

Analysis of the total RNA for calpastatin mRNA revealed three bands, which we identified as bands I, II and III (Fig. 4), of 3.8, 3.0 and 2.5 kb respectively, as has been previously reported (Emori *et al.*, 1987). These arise from the same gene (Emori *et al.*,

1987). Concentrations of both bands I and II increased during fasting. The most intriguing observation, however, was the fasting-dependent appearance of band III (short species) which contributed to the fasting-dependent increase in total calpastatin mRNA.

To determine whether the effects of fasting on calpain mRNA levels were unique or whether fasting effected changes in expression of other proteinase genes, the effects of fasting on cathepsin D and proteasome C2 subunit mRNA concentrations were examined (Fig. 5). Cathepsin D and proteasome were selected for this comparison because they represent lysosomal and ATP-dependent proteolytic systems respectively (Faust *et al.*, 1985; Fujiwara *et al.*, 1989) and because both have been implicated in myofibrillar protein degradation (Gerard *et al.*, 1988; Kettelhut *et al.*, 1988). The proteasome C2 cDNA, in addition to binding to proteasome C2 mRNA, also hybridized non-specifically to the 28 S ribosome RNA and to a 3.5 kb mRNA. Fasting caused 6- and 2-fold increases in mRNAs encoding cathepsin D and proteasome C2 respectively. Thus fasting increases gene expression of multiple proteolytic systems in skeletal muscle. This effect is not a response of typical house-keeping genes because fasting decreased the concentration of β -actin mRNA by about 30% ($P < 0.05$), as shown in Fig. 5.

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

Calpain activities, whether expressed on per g of tissue or per g of protein, were unaffected ($P > 0.05$) by fasting. Calpain I activities (units/g of protein) in control and fasted rabbits were 21.2 ± 2.2 and 25.1 ± 3.4 respectively. Calpain II activities in control and fasted rabbits were 36.5 ± 2.7 and 40.1 ± 3.1 units/g of protein respectively, and calpastatin activities were 111.3 ± 11.5 and 149.9 ± 31.5 units/g of protein respectively. The calpastatin/calpain I + calpain II ratios were essentially similar in the tissues of control and fasted rabbits.

Polysomal mRNA analysis

To determine whether the lack of change in calpain activity was due to lack of loading of polysomes with calpain mRNAs in the proportions in which they existed in the unbound mRNA pool, we examined calpain I and II mRNA concentrations

Fig. 4. Effects of fasting on steady-state concentration of mRNAs encoding calpain I and calpain II large subunits and calpastatin

The panels present Northern blots. Washing conditions for all probes were $1 \times \text{SSC}/0.1\% \text{ SDS}$ (four times at room temperature), followed by $0.1 \times \text{SSC}/0.1\% \text{ SDS}$ (twice at 50°C). The positions of ribosomal RNAs (28 S and 18 S) are indicated by solid arrows. Positions of calpastatin mRNA multiple bands are indicated by open arrows. The lower panel represents results of quantification of mRNAs by scanning densitometry, expressed as percentages of controls. Each bar is the mean $\pm$ S.E.M. ($n = 6$), and differences ($P < 0.05$) between control and fasted animals are indicated by *.

associated with polysomes using dot-blot analysis (Fig. 6). Fasting caused a 2-fold increase ($P < 0.05$) in calpain I and II mRNA concentrations associated with polysomes.

DISCUSSION

Characteristics of fasted rabbits

Successful metabolic adaptation to fasting depends on the ability of animals to mobilize body energy reserves to support their energetic requirements and to maintain the required levels of blood glucose for proper functioning of brain and erythrocytes (Owen *et al.*, 1967). Many studies have examined the effects of fasting on muscle protein metabolism in the rat (Long *et al.*, 1988; Emery *et al.*, 1986). The results of these studies and others (Li & Wassner, 1984; Lowell *et al.*, 1986a,b) revealed that fasting causes a preferential acceleration in the degradation of myofibrillar proteins over sarcoplasmic proteins. Results in the present study on the effects of fasting on urinary 3-MH meta-

Fig. 5. Effect of fasting on steady-state mRNA concentrations encoding cathepsin D, proteasome C2 and β -actin

Filters hybridized to cathepsin D and proteasome C2 cDNAs were washed four times with $1 \times \text{SSC}/0.1\% \text{ SDS}$ at room temperature. Washing conditions for the β -actin membrane were $0.5 \times \text{SSC}/0.1\% \text{ SDS}$ at 60°C (four times). The positions of ribosomal RNAs (28 S and 18 S) are indicated by solid arrows; positions of putative mRNAs of cathepsin D, proteasome C2 and β -actin are shown by open arrows. The lower panel represents results of quantification of mRNAs by scanning densitometry as percentages of control. Each bar is the mean $\pm$ S.E.M. ($n = 6$), and differences ($P < 0.05$) between control and fasted animals are indicated by *.

bolism and RNA content in skeletal muscle in rabbits are essentially similar to results observed in the rat (Lowell *et al.*, 1986b; Emery *et al.*, 1986). The present results indicate that fasting in young rabbits causes marked changes in protein metabolism in skeletal muscle. In this tissue, a fall in muscle protein synthetic capacity (RNA content) and a rise in myofibrillar protein degradation were observed in rabbits fasted for 8 days.

Gene expression of the calpain system during fasting

Information on the regulation of expression of calpain I,

Fig. 6. Dot-blot hybridization analysis of calpain I and calpain II mRNAs charged to ribosomes in skeletal muscle of control and fasted rabbits

Serial dilutions of polysomal RNA (2.5–20 µg) were spotted on to nitrocellulose filters. The lower panel represents results of quantification of mRNAs by scanning densitometry, which are expressed as percentages of controls. Each bar is a mean $\pm$ S.E.M. ($n = 3$), and differences ($P < 0.05$) between control and fasted animals are indicated by *.

calpain II and calpastatin is limited. Only two studies have examined gene expression of the calpain system *in vivo* (Samis *et al.*, 1991; Ou & Forsberg, 1991). Based on the work of Samis *et al.* (1991) it was concluded that calpain II mRNA is constitutively expressed in the rat uterus regardless of its physiological condition (i.e. non-pregnant, pregnant or post-partum). Ou & Forsberg (1991) reported age-related changes in muscle calpain II and calpastatin mRNA expression, but not in calpain I mRNA. These changes, however, followed changes in 'house-keeping' gene expression.

Regulation of enzymes at the transcriptional level is usually employed only for enzymes that are needed in special circumstances or at a particular stage of development. Enzymes that perform housekeeping functions are generally not regulated in this manner (Mathews & van Holde, 1990). The ubiquitous distribution of the calpain-calpastatin system in animal cells lends support to the notion that calpains are housekeeping enzymes (Miyake *et al.*, 1986; Suzuki *et al.*, 1987). On the other hand, a distinct unevenness of the absolute and relative amounts of calpain I and II and calpastatin in different cells (Murachi *et al.*, 1989) and large variations in levels of calpain I and II mRNAs among various tissues (Emori *et al.*, 1986) could be interpreted as an indication of the relative importance of the calpain system in different cells. Processing of protein kinase C to

modified PKC (PKM) by calpain (Kishimoto *et al.*, 1983) may represent a housekeeping function of calpains, whereas the calpain-catalysed initiation of myofibrillar protein degradation in muscle may belong to a category of tissue-specific functions. With this in mind, we examined the effects of fasting on muscle calpain gene expression, as we believed that this may provide a model in which the tissue-specific control of calpain gene expression may be elucidated. Furthermore, the model could provide us with an insight into the molecular events underlying muscle protein degradation.

Expression of the genes encoding calpains was increased by fasting. An implication of this observation is that fasting may be used as a model to characterize the regulatory mechanisms controlling expression of the calpain system. Another interesting result pertains to the specific induction of calpastatin mRNA (band III) expression. The structural elements responsible for the appearance of three forms of mRNA for calpastatin have become clear after the cloning and sequencing of calpastatin in rabbits (Emori *et al.*, 1987). That study revealed that the calpastatin gene contains three 3' processing sites (AAATTA) which account for the expression of three mRNA species of varying size but which are identical in the coding region. The physiological significance of the expression of three calpastatin mRNA isoforms is unknown, although it has been suggested that the size of the 3' non-coding region of mRNA species influences their stability (Sheiness & Darrell, 1973; Brawerman, 1976) or transport from nucleus to cytoplasm (Brawerman & Diez, 1975). Physiological significance is not apparent in the present study, as fasting did not affect calpastatin activity.

Several studies indicated that muscle atrophy involves coordinated changes in several proteolytic systems, each of which may function in the breakdown of different cell components (Furuno *et al.*, 1990). To determine whether the effects of fasting on mRNAs of the calpain system were unique to calpain I, calpain II and calpastatin, or whether fasting effected changes in the concentrations of mRNAs encoding other proteinases, we determined the concentrations of mRNAs encoding cathepsin D and proteasome C2. mRNAs encoding both proteasome and cathepsin D were also increased by fasting. Of interest was that fasting effected the largest increase in cathepsin D mRNA concentration. These effects are atypical of housekeeping gene expression, as fasting reduced β -actin gene expression. Observations that fasting increased the concentrations of mRNAs encoding several proteolytic systems indicate that each proteolytic system plays a role in the adaptation of muscle to the fasted state. An important adaptation to fasting in animals is the mobilization of body protein reserves, mainly myofibrillar proteins in muscle, to support the organism's energy requirements. Myofibrillar proteins must be degraded completely to amino acids to provide energy and to support gluconeogenesis during fasting, but the complete hydrolysis of myofibrillar proteins to amino acids cannot be performed by the calpain system, as calpains are endopeptidases. Thus it is possible that co-regulation of the proteolytic systems reflects a requirement for the presence of a cascade of proteolytic enzymes needed for complete hydrolysis of muscle proteins to free amino acids.

Hata *et al.* (1989) have determined that the human calpain II gene is under the control of four negative enhancer-like *cis* elements and have proposed that the relative tissue concentrations of calpains are determined, in part, by concentrations of an uncharacterized negative *trans*-acting element. If this is true, the concentrations of this element would be lower in the skeletal muscle of fasted animals compared with controls. For example, fasting may reduce the concentration of this *trans*-acting factor in skeletal muscle and result in the release of the negative control on gene expression. The sequence of the 5'-flanking region of the

calpain I gene has not been published; however, based on the similarity of the gene expression response to fasting of the calpain I and calpain II mRNAs, it is likely that the calpain I gene possesses features similar to those of the calpain II 5' flanking region.

Proteolytic activity and polysomal mRNAs of the calpain system during fasting

We were surprised to find that the large increases in calpain mRNA were not associated with increases in calpain I and II activities. Because calpain activities were determined under conditions in which they would be maximally activated, these observations indicate that calpain concentrations were unaffected by fasting. A limitation of our assays is that other metallo-proteinases would be inhibited by inclusion of EDTA in control assay tubes; however, we are confident that no changes in calpain activities were caused by fasting, as Western blotting analysis using monoclonal antibodies supplied by Dr. S. Kawashima (Tokyo Metropolitan Institute of Medical Science) showed that fasting did not affect calpain concentrations. Our approach would not allow us to detect changes in calpain activities which may arise from their post-translational modification. Hence we cannot exclude calpains from a role in the turnover of muscle protein during fasting. We believe, however, that the large increase in proteinase mRNA is necessary to circumvent the decrease in protein synthesis which is typically associated with muscle during fasting. Specifically, the increased proteinase mRNA would ensure the continued synthesis of proteinases and maintenance of proteinase concentrations, which would allow continued mobilization of muscle protein reserves. This hypothesis is supported, in part, by the observation that fasting increased the concentrations of calpain I and II mRNAs associated with the polysomes. The specific control of the activities of the individual proteinases during fasting could be effected post-translationally, as has been suggested by others (Kapprell & Goll, 1989). Alternatively, proteolysis may not be increased in rabbit skeletal muscle and the changes in 3-MH excretion may have arisen from changes in the turnover of non-muscle 3-MH pools. For example, gut and skin contain a 3-MH pool (Li & Wassner, 1984); however, we believe that this possibility is unlikely, as 3-MH excretion has been validated for this purpose in rabbits (Harris *et al.*, 1977) and changes in protein degradation are a well-characterized response of muscle to fasting in other species. A second reason why calpain activity may have remained unchanged despite large changes in calpain mRNA is that the fasting-dependent changes in proteinase mRNA had recently occurred and sufficient time for changes in calpain concentrations and activities had not been permitted by sampling muscle on day 8. Temporal analysis of the effects of fasting would be needed to assess this possibility. Alternatively, the lack of an effect of fasting on calpain activities could also arise from an altered stability of calpains and other proteins. It would not be surprising if calpains, like other proteins, were degraded at a greater rate in fasted animals. This would also counter the consequences of the fasting-dependent increase in calpain mRNA concentrations.

Note added in proof

It has recently been recommended that calpain I be designated as u-calpain and calpain II as m-calpain (Suzuki, 1991).

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