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## Activation of ubiquitin-proteasome pathway is involved in skeletal muscle wasting in a rat model with biliary cirrhosis: potential role of TNF- $\alpha$

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**Lin, Shih-Yi, Wen-Yin Chen, Fa-Yauh Lee, Ching-Jang Huang, and Wayne Huey-Herng Sheu.** Activation of ubiquitin-proteasome pathway is involved in skeletal muscle wasting in a rat model with biliary cirrhosis: potential role of TNF- $\alpha$ . *Am J Physiol Endocrinol Metab* 288: E493–E501, 2005. First published November 2, 2004 doi:10.1152/ajpendo.00186.2004.—Hepatic cirrhosis is associated with negative nitrogen balance and loss of lean body mass. This study aimed to identify the specific proteolytic pathways activated in skeletal muscles of cirrhotic rats. TNF- $\alpha$  can stimulate muscle proteolysis; therefore, a potential relationship between TNF- $\alpha$  and muscle wasting in liver cirrhosis was also evaluated. Cirrhosis was induced by bile duct ligation (BDL) in male adult Sprague-Dawley rats. mRNA and protein levels of various targets were determined by RT-PCR and Western blotting, respectively. The proteolytic rate was measured *ex vivo* using isolated muscles. Compared with sham-operated controls, BDL rats had an increased degradation rate of muscle proteins and enhanced gene expression of ubiquitin, 14-kDa ubiquitin carrier protein E2, and the proteasome subunits C2 and C8 ( $P < 0.01$ ). The muscle protein levels of free ubiquitin and conjugated ubiquitin levels were also elevated ( $P < 0.01$ ). However, there was no difference between the two groups with regard to cathepsin and calpain mRNA levels. Cirrhotic muscle TNF- $\alpha$  levels were increased and correlated positively with free and conjugated ubiquitin ( $P < 0.01$ ). We conclude that the ubiquitin-proteasome system is involved in muscle wasting of rats with BDL-induced cirrhosis. TNF- $\alpha$  might play a role in mediating activation of this proteolytic pathway, probably through a local mechanism.

atrophy; cachexia; catabolism; liver; tumor necrosis factor- $\alpha$

MALNUTRITION OCCURS in 10 to 100% of patients with chronic liver disease and is clinically characterized by anorexia and weight loss (28, 43). Nonalcoholic patients experience depletion of fat and/or skeletal muscle mass at an early cirrhotic stage, which increases as the severity of liver disease progresses (11). Loss of lean body mass may negatively affect the clinical course of acute cirrhotic complications with resultant poor prognosis (28). In experimental and human studies, urinary excretion of 3-methylhistidine (3-MH), a marker of myofibrillar protein degradation, is increased in liver cirrhosis, suggesting that increased protein breakdown may contribute to muscle wasting in the disorder (48, 53, 55). However, the intracellular mechanisms responsible for this increased catabolic response are still not well defined.

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In skeletal muscles, there are at least three pathways of protein degradation: lysosomal, calcium-activated proteases, and ATP-dependent and -independent proteolytic pathways (1, 24, 41). In particular, the ubiquitin-proteasome pathway is vital for breakdown of muscle contractile proteins and may induce muscle loss if inappropriately activated (1, 24, 41). In this pathway, ubiquitin (Ub), containing 76 amino acids, is conjugated to proteins destined for degradation by Ub-activating enzyme (E1), Ub-conjugating enzyme (E2), and Ub-ligase (E3) (26). This process is repeated as multiple Ub molecules are added to form a Ub chain. Ub protein conjugates are recognized by a 26S proteasome complex, composed of two sub-proteasome complexes, a 19S regulatory particle, and a 20S catalytic particle. Ultimately, the ubiquitinated proteins are rapidly degraded by proteasome in an ATP-dependent manner, and the released peptides are further degraded into amino acids in the cytoplasm.

In several catabolic disorders, including sepsis, burns, cancer, renal failure, and uncontrolled diabetes in humans or experimental models, it has been shown that increased levels of mRNAs encoding for Ub, E2-conjugating enzyme, and subunits of proteasome complex are associated with increased protein degradation in skeletal muscle (4, 42, 44, 47, 49, 50). On the basis of these observations, we hypothesized that the ubiquitin-proteasome system may also be activated in skeletal muscle in subjects with liver cirrhosis.

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), a polypeptide secreted from activated macrophages or monocytes, is a pleiotropic cytokine with immunoregulatory and metabolic functions (52). In animal studies, TNF- $\alpha$  has been shown to induce protein degradation in skeletal muscle and participates in protein wasting associated with cachexia (19, 39). Moreover, recent *in vivo* and *in vitro* studies have demonstrated that TNF- $\alpha$  can stimulate proteolysis in skeletal muscle through activation of the ubiquitin-proteasome system (15, 16, 38). In contrast, administration of anti-TNF- $\alpha$  antibodies or inhibitors of TNF- $\alpha$  production to cancerous and septic animals with high blood TNF- $\alpha$  levels suppresses the activity of the ubiquitin-proteasome system and attenuates protein breakdown (8, 10). In human studies, it has been reported that serum TNF- $\alpha$  levels are associated with skeletal muscle loss in patients with cancer and chronic renal failure in association with increased gene expression of critical components of the ubiquitin-proteasome system (4, 44). Increased circulating TNF- $\alpha$  levels are found in

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cirrhotic patients and generally thought to be related to the severity of liver damage (46, 51). We (36) also recently observed that TNF- $\alpha$  levels were associated with malnutrition in cirrhotic patients. The aim of the present study was to determine which proteolytic pathway is involved in alteration of muscle protein turnover in subjects with liver cirrhosis. The relationship between TNF- $\alpha$  and skeletal muscle proteolysis was also evaluated.

## METHODS AND MATERIALS

**Animal model.** Experimental models of liver cirrhosis in the rat generally are of the toxic type (e.g., carbon tetrachloride induced) or biliary type. To avoid drug-related toxic effects from carbon tetrachloride, this study used a rat model involving bile duct ligation (BDL) (29). This model has been associated with muscle wasting similar to that seen in patients with primary biliary cirrhosis (13, 18, 21), and with an activated TNF- $\alpha$  system (25, 37). Briefly, male Sprague-Dawley rats (purchased from National Science Council Animal Center, Taipei, Taiwan) weighing 250–300 g at the time of surgery were used for this study. Rats were anesthetized with phenobarbital (60 mg/kg body wt im), and the common bile duct was exposed and ligated by two ligatures with 3-0 silk. The first ligature was made below the junction of the hepatic ducts, and the second ligature was made above the entrance of the pancreatic ducts. The common bile duct was then resected between the ligatures. Prophylactic benzathine benzylpenicillin was administered postoperatively (50,000 U im). Vitamin K (8 mg/kg im) was also given after surgery at weekly intervals. During the induction period of liver cirrhosis, the rats were housed in plastic cages at 24°C on a 12:12-h light-dark cycle and allowed to have free access to food and water until the time of experimentation. The mortality rate was ~20%, with the majority of deaths in the first 2 wk. In all experiments, the authors adhered to the *Guiding Principles for the Care and Use of Laboratory Animals* issued by the Taiwan Government.

**Serum and tissue preparations.** At the time of study, cirrhotic rats were anesthetized intraperitoneally with phenobarbital, and intra-arterial catheterization of the left femoral artery was performed for blood sampling. Blood was immediately centrifuged, with serum samples frozen at -70°C until assay. In addition, peripheral muscles, including epitrochlearis, tibialis anterior, soleus, and gastrocnemius muscles, were rapidly dissected and weighed. These tissues were either used immediately to measure proteolytic rate or stored in liquid nitrogen at -70°C until assay. To exclude the confounding effects of infection on muscle proteolysis, blood, bile, and ascites in BDL rats were collected for bacterial culture, and thoracic and abdominal viscera were examined grossly and microscopically by a veterinary pathologist (Y. T. Chiu). Rats with evidence of bacterial infection were excluded from the study.

**Blood substrate assays.** Blood TNF- $\alpha$  levels were determined in duplicate using enzyme-linked immunosorbent assay kits (Biosource, Nivelles, Belgium). Serum levels of alanine aminotransferase, alkaline phosphatase, total bilirubin, albumin, glucose, and creatinine were determined by standard laboratory methods.

**Rate of protein turnover.** To measure protein breakdown rates, freshly dissected epitrochlearis muscle was fixed via tendons to aluminum wire supports at resting length. This mixed-fiber muscle was used because of its large surface area relative to weight, which allows for in vitro incubation with maintained oxygenation and diffusion of substrates into tissue. It also exhibits similar protein turnover to that of bulk muscle in adult rats (6). Epitrochlearis muscle was preincubated for 60 min in oxygenated medium (95% O<sub>2</sub>-5% CO<sub>2</sub>): Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 5 mM glucose, 0.1 U/ml insulin, 0.17 mM leucine, 0.1 mM isoleucine, and 0.20 mM valine. After a 1-h preincubation, muscles were transferred to a fresh medium of identical composition and incubated for a further

2 h with 0.5 mM cycloheximide. The degradation rates of total and myofibrillar proteins were determined by the release of free tyrosine and 3-MH, respectively, in the medium and expressed as nanomoles tyrosine/methylhistidine in medium  $\cdot$  2 h<sup>-1</sup>  $\cdot$  g muscle<sup>-1</sup>. Nonlysosomal and calcium-independent proteolysis was measured in the presence of 10 mM methylamine and 50  $\mu$ M E-64 in a calcium-free medium. The muscles were also homogenized in 0.4 M perchloric acid to determine tissue free tyrosine and 3-MH. In accordance with previous studies, the net production of free tyrosine was calculated as the amount of tyrosine released into the medium plus the increase in tissue free tyrosine determined during incubation, whereas the net production of 3-MH was calculated as the amount of 3-MH in medium minus the decrease in tissue free 3-MH before and after incubation (49). Levels of both tyrosine and 3-MH in medium or tissue samples were determined by high-performance liquid chromatography (HPLC). *o*-Phthaldehyde (OPA) stock solution (0.02 M) was prepared in methanol (10%) containing 0.005%  $\beta$ -mercaptoethanol (vol/vol) and stored at -20°C, if not used immediately. Before measurement, sample medium was first deproteinized by 20% sulfosalicylic acid, and then centrifuged at 13,600 g for 10 min. After neutralization by 1 N sodium bicarbonate, sample medium or standard solution (2:1) was added to a mixture containing the OPA stock solution mixed with 0.25 M sodium borate buffer (1:2) for later derivitization. The solution was then mixed at 4°C for 60 s followed by immediate injection into an HPLC system equipped with a fluorescence detector (excitation wavelength 340 nm, emission wavelength 450 nm). Separation was carried out on an Alltech reversed C-18 column (4.6  $\times$  150 mm), and ternary gradient elution was used. *Mobile phase A* consisted of 93 mM sodium acetate buffer (pH 6.0) in 7% acetonitrile. *Mobile phase B* consisted of 10 mM sodium acetate in 90% acetonitrile. *Mobile phase C* was distilled water, and flow rate was 0.8 ml/min. The elution profile was as follows: 0 min, 93% A, 7% B; 35 min, 80% A, 20% B; 38 min, 80% A, 20% B; 39 min, 10% A, 90% B; 42 min, 10% A, 90% B; 43 min, 93% A, 7% B; 47 min, 93% A, 7% B.

**RNA preparation and analysis of gene expression of components of ubiquitin-proteasome system.** To obtain sufficient materials for analysis of mRNA expression, and protein levels of the ubiquitin-proteasome pathway, we used gastrocnemius muscle, which, like epitrochlearis, is a mixed-fiber muscle with adequate tissue weight (6). Total mRNA was extracted with TRIzol reagents (Life Technologies), and mRNA concentration was determined by the ultraviolet light absorbency at 260 nm. Measurement of mRNA of various components of the ubiquitin-proteasome system, including polyubiquitin C (UbC), 14-kDa ubiquitin-conjugating enzyme (14-kDa E2), proteasome subunits C2 and C8, and cathepsin B, D, and L, as well as  $\mu$ -calpain, m-calpain, and  $\beta$ -actin, was performed by semiquantitative reverse transcriptase-polymerase chain reaction analyses. Before reverse transcription (RT), 1–5  $\mu$ g of total RNA were reverse transcribed at 42°C for 1 h using standard reagents, which contained 50  $\mu$ l of 1 $\times$  RT buffer, reverse transcriptase (RTase), and poly(dT)<sub>12–18</sub> primer. The RT mixtures were then heated at 100°C for 10 min to inactivate RTase. Each PCR reaction was conducted in a total volume of 50  $\mu$ l of 1 $\times$  PCR buffer containing 5  $\mu$ l of RT template, 200 nM each of sense and antisense primers, 1 unit of *Taq* polymerase, and 200  $\mu$ M each of dNTPs. The solution was covered with 30  $\mu$ l of mineral oil, and PCR was performed in a DNA Thermal Cycler 480 (Perkin-Elmer, Norwalk, CT) with the following profiles. Generally, after initial denaturation at 94°C for 5 min, cycles were performed of denaturation at 94°C for 30 s, annealing at a specific temperature for each product for 1 min, and elongation at 72°C for 1 min. The last cycle was followed by a final extension of 5 min at 72°C. The number of amplification cycles used with each primer pair was optimized to produce a densitometric result that correlated closely with the template. To determine the relative quantities of mRNA encoding UbC, 10  $\mu$ l each of UbC and  $\beta$ -actin PCR products amplified from the same RT template solution were combined and electrophoresed on 2%

agarose gel in Tris-acetate-EDTA buffer for 30 min. After the gel was stained with ethidium bromide for 15 min, the UbC and  $\beta$ -actin bands were measured for densitometry using ImageQuant Analysis software. The relative mRNA levels of UbC in the original RNA extracts from the various skeletal muscle preparation were obtained with UbC expression normalized to  $\beta$ -actin expression. The primer sequences, number of amplification cycle, annealing temperature, and amplification product size for each gene are listed in Table 1.

**Crude, myofibrillar, and sarcoplasmic muscle protein preparation.** For crude protein extracts, gastrocnemius muscles were homogenized in tissue protein extraction reagents (T-PER, Pierce Biotechnology, Rockville, IL) and then centrifuged at 10,000 rpm for 10 min, and supernatants were obtained. To separate myofibrillar and sarcoplasmic muscle proteins, gastrocnemius muscles were homogenized in 4 ml of *buffer A* [10 mM Tris·HCl (pH 7.5)-10 mM EDTA (pH 8.0), 2 mM PMSF, 0.5 mM tosyl-lysylchloromethane, 0.5 mM tosyl-phenylalanylchloromethane, 10 mM *N*-ethylmaleimide, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml pepstatin A, 10  $\mu$ g/ml soybean trypsin inhibitor], and the homogenates were incubated on ice for 10 min. The samples were then centrifuged at 1,500 *g* for 5 min at 4°C to pellet myofibrillar proteins, which were then washed three times in *buffer A* containing 1% Triton X-100. Myofibrillar proteins were then resuspended in 8 M urea and 50 mM Tris·HCl, pH 7.5. The supernatants from the 1,500-*g* spin were recentrifuged for 10 min (10,000 *g*, 4°C), and the resulting supernatants were ultracentrifuged for 3 h (100,000 *g*, 4°C) with the final supernatants containing sarcoplasmic proteins. All of the protein concentrations were determined by Bradford assay (Bio-Rad) before use.

**Western blot analyses.** Variable amounts of myofibrillar, sarcoplasmic, or crude muscle protein were separated on SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. Membranes were then blocked for 1 h in 5% (vol/vol) nonfat dried milk in TTNS [25mM Tris·HCl (pH 7.5), 0.1% (vol/vol) Tween 20, 0.9% (wt/vol) NaCl]. To detect free and conjugated ubiquitin, myofibrillar and sarcoplasmic proteins were incubated for 1 h with a 1:1,000 diluted rabbit polyclonal anti-Ub antibody (DakoCytomation, Glostrup, Denmark). The sarcoplasmic proteins were also incubated with a 1:1,000 diluted anti-14-kDa E2 antibody (kindly donated by Dr. Simon Wing) to quantify this 14-kDa E2 protein. Crude muscle extracts were incubated with a 1:5,000 polyclonal anti-TNF antibodies (PeproTech EC, London, UK) to determine muscle TNF- $\alpha$  levels. After being

washed three times with TTNS, the blots were incubated with secondary horseradish peroxidase-conjugated antibodies (Amersham Life Sciences). The blots were washed four times with TTNS for 20 min, incubated in enhanced chemiluminescence reagent (Amersham Life Sciences), and exposed on radiographic film (Eastman-Kodak, Rochester, NY). Finally, all measured protein levels were quantified by densitometry.

**Nuclear extraction and electrophoretic mobility shift assay.** Gastrocnemius muscle nuclei were prepared according to Kumar and Boriek (30), with some modifications. In brief, the muscle tissues (~300–500 mg) were washed in ice-cold PBS and then homogenized in ice-cold hypotonic buffer [10 mM HEPES (pH 7.9), 10 mM KCl, MgCl<sub>2</sub> 1.5 mM, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonylfluoride (PMSF), 2.0  $\mu$ g/ml leupeptin, 2.0  $\mu$ g/ml aprotinin, 0.5 mg/ml benzamide] and incubated on ice for 15 min. The sample tissues were then lysed by the addition of 0.5% Nonidet P-40 with vigorous vortexing for 30 s. The nuclei were pelleted by centrifugation at 16,000 *g* for 1 min at 4°C and resuspended in ice-cold extraction buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 25% glycerol, 0.5 mM PMSF, 2.0  $\mu$ g/ml leupeptin, 2.0  $\mu$ g/ml aprotinin, 0.5 mg/ml benzamide). After 15 min on ice, lysates were centrifuged at 12,000 *g* for 10 min at 4°C. Supernatants (nuclear extracts) were obtained and stored at -70°C until analysis. For electrophoretic mobility shift assay (EMSA), double-stranded oligonucleotides encoding binding sites for NF- $\kappa$ B [5'-AGTTGAGGGGACTTTC-CCAGGC-3' (sense), and 5'-GCCTGGGAAAGTCCCCTCAACT-3' (antisense)] were end labeled with biotin. Binding reaction was performed for 20 min at 30°C with 20  $\mu$ l of buffer [10 mM (pH 7.6) HEPES, 50 mM NaCl, 0.5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 1 mM DTT, 5% glycerol], 100 pmol of biotin-labeled DNA, 1  $\mu$ g nonspecific DNA competitor (dI-dC), and 10  $\mu$ g of nuclear protein extracts. Finally, the DNA-protein complex was subjected to electrophoretic separation on a nondenaturing 6% acrylamide gel at 80 V using TBE buffer and quantified by densitometry. To ensure the specificity of the gel shifts, an excess of unlabeled NF- $\kappa$ B consensus oligonucleotide or unlabeled mutant NF- $\kappa$ B oligonucleotide [5'-AGTTGAGGCGACTT-TCCCAGGC-3' (sense) and 5'-GCCTGGGAAAGTCCGCTCAACT-3' (antisense); 1 bp substitution underlined] was also added for competition reactions.

Table 1. Ubiquitin C, 14-kDa E2, proteasome subunits C2 and C8, cathepsin B, D, and L, and  $\mu$ -calpain, m-calpain, and  $\beta$ -actin primer sequences

Name	Primer Sequences	PCR Cycles	Annealing Temperature, °C	Amplification Length, bp
Ubiquitin C	5'-TCTTCGTGAAGACCCTGACC-3' 5'-CAGGTGCAGGGTTGACTCTT-3'	35	54	200
14-kDa E2	5'-GTGCACCATCTGAAAACAA-3' 5'-ATCGGTTCTGCAGGATGTCT-3'	30	50	221
C2	5'-GGCTGCTCATTGCTGTTAG-3' 5'-CCAACAATCCCAATGGAAC-3'	30	52	256
C8	5'-AGGTTTGTGGCAGATGCTC-3' 5'-GGCAGGTCATTTCCCTTCATC-3'	30	52	326
Cathepsin B	5'-GAAGCCGGTGATGTGATGGGAGGC-3' 5'-CAAACCTTGCTGAAAGCTTCATGC-3'	30	58	347
Cathepsin D	5'-GAGTAAGGAACTACAGAAGG-3' 5'-AGTTAGAGTGTGGCAGCCTTGG-3'	30	55	295
Cathepsin L	5'-CCCCAAGACTGTGGACTGGAGAGA-3' 5'-TTTACAAGATCCATCCTTTGCTTC-3'	30	58	322
$\mu$ -Calpain	5'-CCGTACACTTGAAGCGTGACTT-3' 5'-CGCATCTCGTAGGCACTCA-3'	40	58	553
m-Calpain	5'-CCCTCCCAACCTGTTCAAG-3' 5'-GCCTCCAGTTCATCCCA-3'	40	55	440
$\beta$ -Actin	5'-TCCTGTGGCATCCAGGAACT-3' 5'-GGAGCAATGATCTTGATCTTC-3'	28	58	180

E2, ubiquitin-conjugating enzyme.

**Statistical analyses.** Data are expressed as means  $\pm$  SE, and statistical analysis was performed using the unpaired Student's *t*-test or ANOVA, whichever was appropriate. The difference was determined by the Mann-Whitney nonparametric *U*-test (comparisons between 2 groups) or Kruskal-Wallis one-way analysis (comparison among  $>2$  groups). The correlation between two variables was analyzed by Spearman's rank order correlation. All data were analyzed by SPSS software (Statistical Package for the Social Sciences, version 6.0, for Windows, SPSS, Chicago, IL). *P* values  $< 0.05$  were considered significant.

## RESULTS

**Effects on body and tissue weights.** In this study, the rats typically developed liver cirrhosis 5 wk after ligation, which was confirmed biochemically and histologically (Table 2 and Fig. 1). A comparison of food intake between BDL rats and sham-operated controls showed no significant difference throughout the induction period except during the 1st wk. Body weight and fat mass in BDL rats were significantly lower than those of controls (Table 2), whereas liver weight was higher ( $32.9 \pm 1.6$  vs.  $18.1 \pm 0.9$  g,  $P < 0.01$ ). In addition, the mass of all muscles studied was significantly lower in cirrhotic rats compared with controls (Table 2).

**Change in protein degradation rate.** As described previously in other cachectic models, enhanced proteolysis of total and myofibrillar proteins was observed in the culture medium of isolated epitrochlearis muscle of cirrhotic rats with and without addition of inhibitors of lysosomal function and calcium-dependent proteinases (Table 3). After correction for the change of tissue free tyrosine and 3-MH levels, net free tyrosine and 3-MH production in the cirrhotic group was still greater than that in the sham animals (Table 3)

**Expression of ubiquitin-proteasome system.** Because previous studies suggest that the ubiquitin-proteasome system is stimulated in the muscles of cachectic experimental animal models, we measured the gene and protein expression of various components of this proteolytic pathway in BDL rats. The mRNA levels for ubiquitin, 14-kDa E2, and C2 and C8 proteasome subunits were increased by 217, 166, 164, and 150%, respectively, in gastrocnemius muscle of cirrhotic rats compared with sham controls (Fig. 2). However, cathepsin B,

Table 2. Weights of body and various muscles and liver biochemical function in sham-operated and bile-duct-ligated rats

	Sham Operated (n = 12)	Bile Duct Ligated (n = 12)
Body weight, g		
Initial	282.2 $\pm$ 7.9	275.8 $\pm$ 6.2
Final	486.1 $\pm$ 9.7	438.1 $\pm$ 15.0*
Epitrochlearis, mg	96 $\pm$ 8	65 $\pm$ 6*
Gastrocnemius, mg	3167 $\pm$ 198	2230 $\pm$ 160*
Soleus, mg	859 $\pm$ 54	678 $\pm$ 46*
Extensor digitorum longus, mg	475 $\pm$ 24	383 $\pm$ 18*
Creatinine, mg/dl	0.61 $\pm$ 0.03	0.43 $\pm$ 0.04*
AST, U/l	99 $\pm$ 9	801 $\pm$ 110*
ALT, U/l	44 $\pm$ 5	189 $\pm$ 20*
Alk-P, U/l	276 $\pm$ 14	575 $\pm$ 34*
Albumin, g/dl	3.53 $\pm$ 0.07	2.73 $\pm$ 0.05*
Total bilirubin, mg/dl	0.03 $\pm$ 0.01	7.69 $\pm$ 0.37*

Data are expressed as means  $\pm$  SE. AST, aspartate aminotransferase; ALT, alanine aminotransferase; Alk-P, alkaline phosphatase \**P*  $< 0.01$  vs. control.

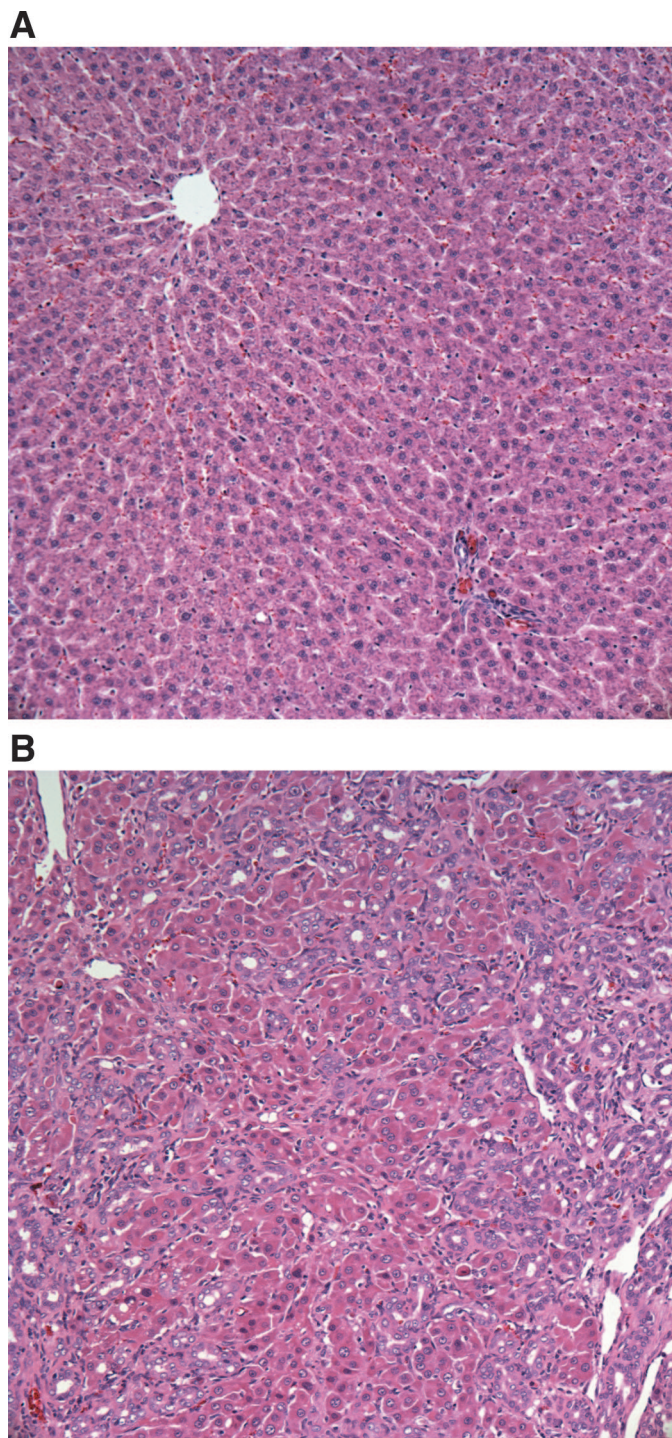


Fig. 1. Hematoxylin and eosin preparations of representative liver samples of control (A) and cirrhotic rats (B). Cirrhotic liver shows ductular proliferation and expansion of portal tracts with portoportal and centroportal linkage leading to separation of small nodules of parenchyma.

D, and L and  $\mu$ - and m-calpain gene expression did not significantly differ between the two groups of animals (Fig. 2). In addition, a parallel change of protein expression of various components of the ubiquitin-proteasome pathway in muscles from cirrhotic rats was observed. Free ubiquitin and 14-kDa E2 protein levels in the sarcoplasmic fraction of cirrhotic muscles were increased by 148 and 570%, respectively (Fig. 3). More-

Table 3. Effects of biliary cirrhosis on total protein (tyrosine release) and myofibrillar protein (3-MH) breakdown in muscles of sham-operated and cirrhotic rats

	Controls (n = 6)				Cirrhotics (n = 6)			
	Tissue levels		Medium	Net release	Tissue level		Medium	Net release
	0 h	2 h			0 h	2 h		
Tyrosine, nmol·g <sup>-1</sup> ·2 h <sup>-1</sup>								
(-)	55.8±14.5	63.1±14.1	210.0±12.9	218.2±13.1	86.2±14.5	114.9±15.5	320.0±15.8*	348.3±15.1*
(+)	40.6±15.2	51.0±13.6	153.7±16.3	164.1±15.0	73.2±11.7	93.5±13.2	273.1±11.6*	293.3±12.2*
3-MH, nmol·g <sup>-1</sup> ·2 h <sup>-1</sup>								
(-)	4.5±0.4	3.5±0.5	2.9±0.3	1.9±0.4	7.5±0.5	5.6±0.4	6.3±0.6*	4.4±0.5*
(+)	4.3±0.5	3.0±0.3	2.6±0.2	1.3±0.4	6.9±0.5	5.1±0.4	5.3±0.5*	3.5±0.5*

Data are means  $\pm$  SE. 3-MH, 3-methylhistidine. Proteolysis was measured in epitrochlearis muscles from control and cirrhotic rats, which were incubated in the presence or absence of 10 mM methylamine and 50  $\mu$ M E-64 in Ca<sup>2+</sup>-free medium. \* $P$  < 0.05 vs. control.

over, the ubiquitination of high-molecular-weight proteins in myofibrillar fraction was also enhanced (Fig. 3).

**TNF- $\alpha$  signaling pathway in skeletal muscle.** In BDL-induced cirrhotic rats, the serum TNF- $\alpha$  concentration was 11.1  $\pm$  4.1 pg/ml but was undetectable in all sham controls. Moreover, TNF- $\alpha$  levels in whole muscle homogenates were significantly elevated in experimental rats compared with those in controls (500%; Fig. 4). To eliminate blood contamination of skeletal muscles, we performed intra-arterial saline perfu-

sion in cirrhotic rats. The increased skeletal muscle TNF- $\alpha$  level remained the same (data not shown). Interestingly, a significant positive correlation between muscle TNF- $\alpha$  and free ubiquitin and myofibrillar conjugated ubiquitin levels ( $r = 0.86$ ,  $r = 0.87$ , respectively,  $P < 0.01$ ; Fig. 5) was observed. To examine whether this locally increased TNF- $\alpha$  exerted any possible effects on muscle wasting, a downstream transcription factor involved in mediating catabolic responses of TNF- $\alpha$ , NF- $\kappa$ B, was analyzed by EMSA. Nuclear extracts of whole muscle lysates from cirrhotic rats showed significantly increased NF- $\kappa$ B DNA binding activity not present in sham controls (Fig. 4). The NF- $\kappa$ B band disappeared with the addition of excess unlabeled cold competitor to binding reactions, but not with mutant competitor, confirming its specificity.

## DISCUSSION

Several mechanisms, such as protein calorie deprivation and alteration of protein metabolism in skeletal muscle, have been proposed to explain muscle wasting in chronic liver disease (28, 43, 48). In this study, BDL rats, despite food intake comparable to that of a control group, had, on average, a

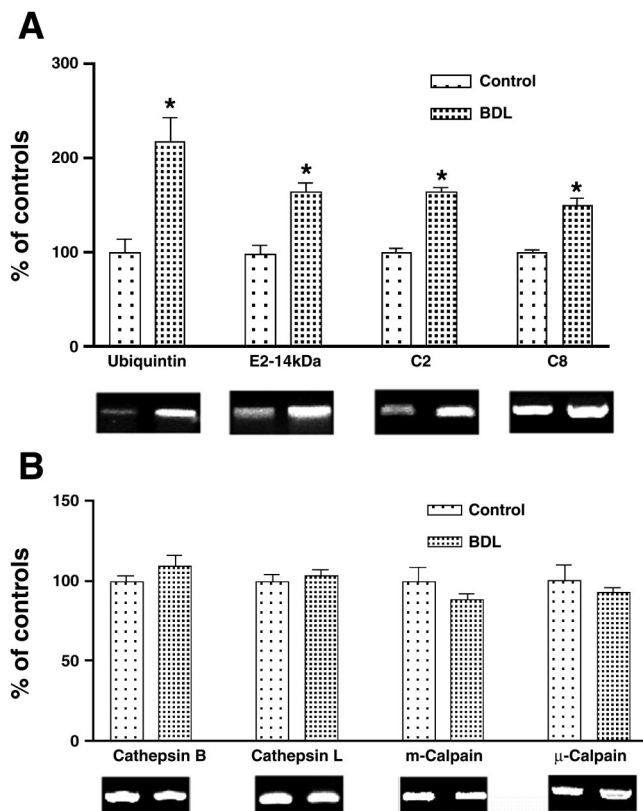


Fig. 2. Marked upregulation of transcripts for components of the ubiquitin-proteasome proteolytic pathway in gastrocnemius muscles was noted in bile duct-ligated (BDL) rats ( $n = 12$ ; A), but cathepsin B, cathepsin L, m-calpain, and  $\mu$ -calpain mRNA in gastrocnemius muscle showed no difference from sham-operated rats ( $n = 12$ ; B). E2 14-kDa, 14-kDa ubiquitin-conjugating enzyme. mRNA levels were measured by semiquantitative RT-PCR with normalization of  $\beta$ -actin. Data are means  $\pm$  SE and expressed as percentage of control values. \* $P$  < 0.01 vs. sham-operated controls.

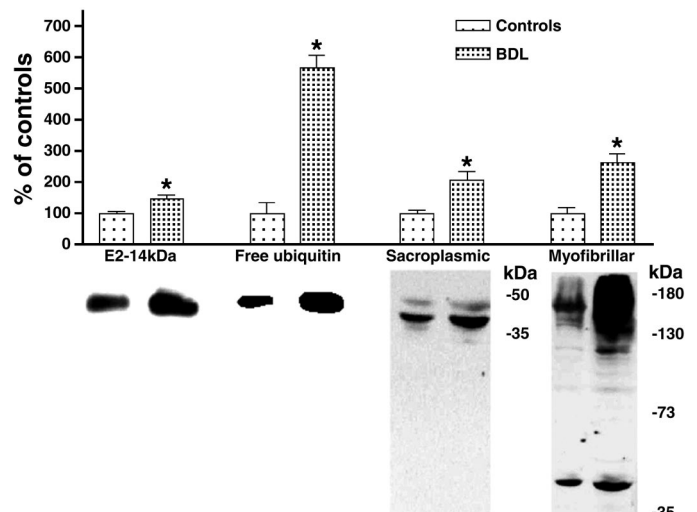


Fig. 3. Protein levels of 14-kDa E2, free ubiquitin, and conjugated ubiquitin in sarcoplasmic and myofibrillar protein pools of muscles were increased in BDL rats ( $n = 12$ ) compared with sham-operated rats ( $n = 12$ ). Data are means  $\pm$  SE and expressed as percentage of control values. \* $P$  < 0.01 vs. sham-operated controls.

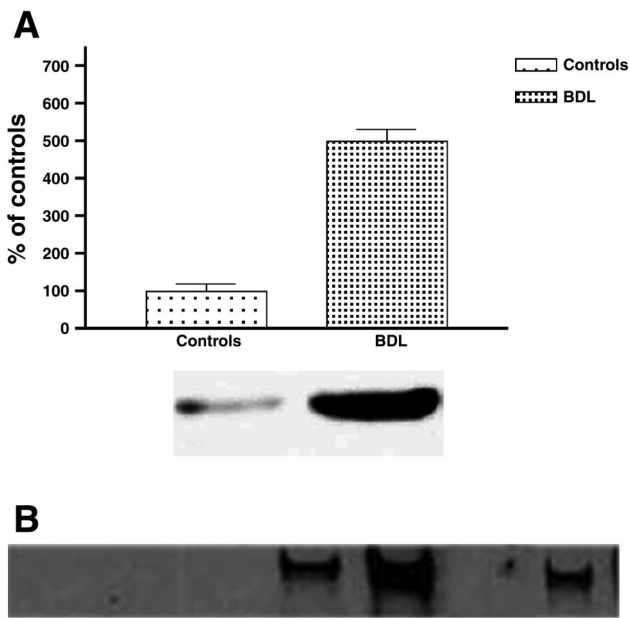


Fig. 4. *A*: Western analysis showed increased expression of TNF- $\alpha$  in muscle of BDL rats. Data are means  $\pm$  SE and expressed as percentage of control values.  $*P < 0.01$  vs. sham-operated controls. *B*: NF- $\kappa$ B binding activity in nuclear proteins of gastrocnemius muscle determined by electrophoretic mobility shift assay showed marked increase in BDL rats ( $n = 8$ ) compared with sham-operated rats ( $n = 8$ ). Lane 1, free probe only; lanes 2 and 3, sham-operated controls; lanes 4 and 5, BDL rats; lane 6 unlabeled competitor oligonucleotide; lane 7, mutant competitor.

10–15% reduction in weight associated with peripheral muscle wasting. Moreover, it was shown that total and myofibrillar protein degradation, as indicated, respectively, by tyrosine and 3-MH release in isolated muscles, were enhanced. This result was compatible with the previous studies using a carbon tetrachloride-induced model (3, 53) and indicated that altered protein turnover (e.g., increased degradation) might play a role in muscle wasting in both of these cirrhotic animal models. To further elucidate which proteolytic system is directly responsible for this increased proteolytic process, we studied the three major proteolytic pathways: lysosomal-, calcium-, and ubiquitin-dependent systems. It was shown that excessive proteolysis was not significantly inhibited in BDL rats after addition of inhibitors of lysosomal proteases and calpains in cultured muscle tissues. Furthermore, mRNA expression of multiple components of the ubiquitin-proteasome pathway, including ubiquitin, 14-kDa E2, and proteasome subunits C2 and C8, are all coordinately increased in cirrhotic rat muscles. The protein levels of 14-kDa E2, free ubiquitin, and ubiquitin-protein conjugates were also elevated. Conversely, the gene expressions of cathepsin and calpain were similar to those in sham-operated controls. These data, together with observed increased muscle proteolysis, indicate that the ubiquitin-proteasome system might, at least to some extent, play a major role in the development of muscle wasting in BDL-induced cirrhotic rats. The other two proteolytic pathways, lysosomal- and calcium-dependent systems, seem less likely to be involved (9).

Among the multiple activated components of the ubiquitin-proteasome system, 14-kDa E2, a member of the large E2 enzyme family, has been shown to be stimulated in several

experimental cachectic models, including starvation, cancer, and sepsis as well as liver cirrhosis in this study (42, 47, 49). It can catalyze the E3-dependent ubiquitin conjugate formation (26) and is thought to play an important regulatory role in the initial steps of the ubiquitin proteolytic pathway in skeletal muscle (27). In BDL-induced cirrhotic rats, most of the ubiquitinated proteins in the myofibrillar fraction were of high molecular mass. Similar findings have also been reported in other catabolic models, such as starvation, denervation, sepsis, or cancer (8, 49, 54). These high-molecular-mass conjugates in skeletal muscle might represent the ubiquitinated proteins destined for degradation, as in vitro studies have found that the 26S proteasome complex preferentially degrades large conjugates (54). After ubiquitin conjugation, the muscle proteins were then rapidly recognized by a 26S proteasome complex and degraded. Like the process for ubiquitin conjugation of various proteins by 14-kDa E2, the degradation of ubiquitinated proteins by proteasome could be another rate-limiting step, as the levels of ubiquitin conjugates were shown to be accumulated.

In this study, however, we did not provide exact quantitative information for each component of the ubiquitin-proteasome pathway contributing to muscle atrophy. We used only two mixed-fiber muscles, epitrochlearis and gastrocnemius, for analysis. It is not known whether similar proteolytic mecha-

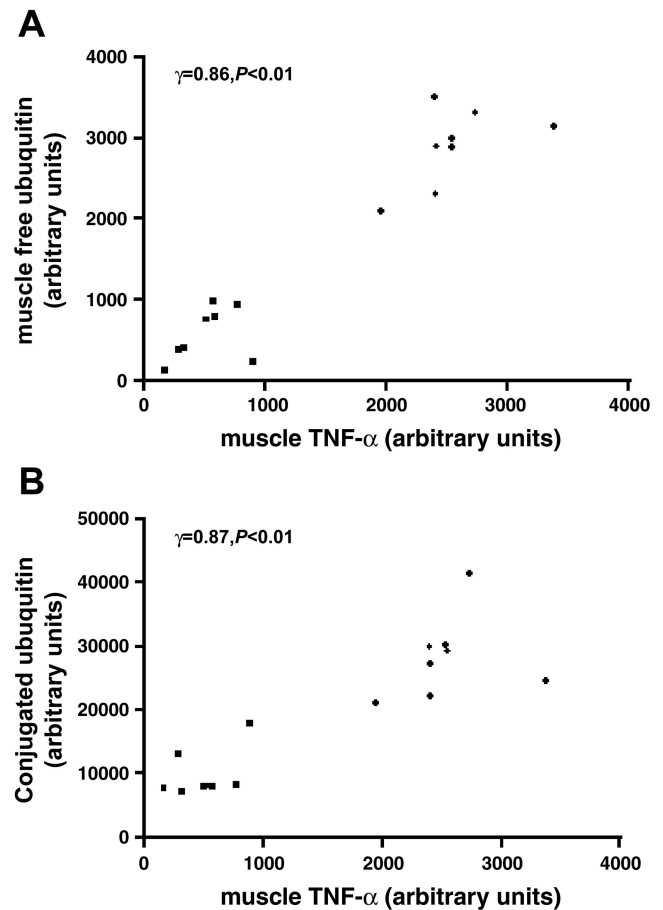


Fig. 5. Relation of muscle TNF- $\alpha$  to free ubiquitin (*A*) and conjugated ubiquitin (*B*) levels in sham-operated ( $\blacksquare$ ;  $n = 8$ ) and BDL ( $\bullet$ ;  $n = 8$ ) rats.

nisms were activated in other fiber types of skeletal muscle, although a general decrease of weight was observed in all types of muscles [e.g., soleus (red fiber) and extensor digitorum longus (white fiber)]. Further study is necessary to address these questions.

This study also demonstrated that muscle TNF- $\alpha$  levels are increased in liver cirrhosis with or without saline perfusion of muscle samples. This result suggests enhanced production of this cytokine locally. It has been reported that the source of TNF- $\alpha$  in liver cirrhosis is either from macrophage stimulation by cirrhotic endotoxemia or from decreased hepatic TNF- $\alpha$  clearance (12, 36). Because macrophages can circulate to several organs, they may contribute to increased muscle TNF- $\alpha$  levels in BDL-induced cirrhotic rats. In addition, it has recently been found that TNF- $\alpha$  is expressed in skeletal muscle cells and can be upregulated by exogenous TNF- $\alpha$  or endotoxin (14). Because increased circulating endotoxin and TNF- $\alpha$  are commonly seen in liver cirrhosis, myocytes might be another possible source of elevated TNF- $\alpha$  levels in skeletal muscle of BDL rats. Although it was not known whether the upregulation of TNF- $\alpha$  in skeletal muscle of BDL rats derived from infiltrating macrophages or resident cells, or even from blood contamination, this increased cytokine level would be expected to alter muscle metabolism within the local environment regardless of its exact sources.

In this study, skeletal muscle TNF- $\alpha$  correlated with free and conjugated ubiquitin levels, and DNA binding activity of NF- $\kappa$ B, a transcription factor signaled by TNF- $\alpha$ , was also increased. These data suggest that the signal transduction pathway of TNF- $\alpha$  was activated in cirrhotic rat muscle and might play a role in regulating the skeletal muscle ubiquitin-proteasome system, probably through a local mechanism. It has been shown that TNF- $\alpha$  can stimulate a wide variety of postreceptor signaling events that evoke pleiotropic and cell-specific responses (52). Among these multiple transduction pathways, TNF- $\alpha$  activates NF- $\kappa$ B, causing it to translocate to the nucleus and bind to promoters and enhancers of genes involved in the regulation of immune or cellular proliferation/differentiation responses (2). In skeletal muscle, the TNF- $\alpha$ /NF- $\kappa$ B signaling can inhibit myogenesis or induce protein breakdown (22, 32, 35). Moreover, Li et al. (34) have reported recently that NF- $\kappa$ B is essential for increased ubiquitin conjugation activity induced by TNF- $\alpha$  in cultured myoblasts. Although it is generally thought that NF- $\kappa$ B activation by most physiological signals is a transient response, a recent study reports that TNF- $\alpha$ -induced NF- $\kappa$ B activity can persist (31). Our observation of increased NF- $\kappa$ B DNA binding activity in skeletal muscle in a BDL-induced cirrhosis model appears to support that finding.

Several studies have reported that biliary tract obstruction can induce endotoxemia and bacterial translocation by altering intestinal mucosal integrity and thus predispose to infection (45). Because either bacterial infection or administration of endotoxin can stimulate skeletal muscle proteolysis (5, 49), muscle wasting seen in BDL rats could conceivably have been a consequence of BDL-associated infection and endotoxemia rather than of BDL-induced cirrhosis. However, for a number of reasons this possibility seems unlikely. First, previous studies have shown that rats with BDL do not suffer from any negative nitrogen balance during the first 2–3 wk after ligation,

when biliary obstruction-associated endotoxemia and/or sepsis usually occur (7, 20, 45). In contrast, 5 wk after BDL, when biliary cirrhosis has fully developed, muscle proteolysis is increased, as found in this study. In addition, another cirrhotic model, induced by carbon tetrachloride, in which endotoxemia and bacterial systemic infection are less prominent (17, 33), also shows similarly increased muscle catabolism (3, 53). Second, compared with the blood endotoxin level observed in septic rats [ $\sim$ 420 pg/ml to 30 ng/ml (40)], the circulating endotoxin value after BDL [ $\sim$ 15–90 pg/ml (7)] may be too low to elicit activation of the ubiquitin-proteasome system in skeletal muscles. Although the effect of chronic low-level endotoxemia on muscle proteolysis could not be excluded with certainty, the major source of circulating endotoxin at the later stage of BDL-induced liver cirrhosis derives from decreased hepatic clearance rather than from biliary duct obstruction itself. Third, superimposed bacterial infection in animals with biliary obstruction is usually fatal, with marked body weight loss and anorexia before death (23). In contrast, the BDL-induced cirrhotic rats in this study had food intake comparable to that of sham-operated controls throughout the induction period (except during the 1st wk) with apparent increase in body weight. In addition, negative bacterial cultures and microscopic examination of visceral organs argue against infection. Together, these factors support the effects of liver cirrhosis rather than biliary obstruction-associated endotoxemia and/or infection upon skeletal wasting in these BDL rats.

There were some limitations to our study. We did not provide any direct evidence that TNF- $\alpha$  caused muscle protein loss by way of the ubiquitin-proteasome system activation. It is possible that increased TNF- $\alpha$  in liver cirrhosis induces other unknown effectors, such as glucocorticoids, or acts in conjunction with other cytokines known to be elevated in cirrhosis, such as interleukin or interferon, which have been shown to be involved in skeletal muscle proteolysis (1, 24, 41). Pharmacological interventions, such as administration of anti-TNF- $\alpha$  antibodies, will be necessary to elucidate the precise role of TNF- $\alpha$  in skeletal muscle wasting in liver cirrhosis.

In summary, our study showed that muscle wasting in BDL-induced cirrhotic rats resulted mainly from a sustained increase of protein breakdown despite favorable food intake. The coordinated upregulation of gene and protein expression of various components of the ubiquitin-proteasome system associated with increased ubiquitin-protein conjugates suggests a major role of this pathway in muscle wasting in BDL rats, most likely through the activation by TNF- $\alpha$ /NF- $\kappa$ B signaling. The results of our study indicated that the ubiquitin-proteasome pathway as well as TNF- $\alpha$  might be potential therapeutic targets for maintaining muscular protein balance in subjects with liver cirrhosis.

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