Skeletal muscle wasting in liver cirrhosis is a prominent and potentially reversible complication that is associated with a decreased quality of life and a poor outcome before and after liver transplantation (23). The mechanism(s) of skeletal muscle wasting in cirrhosis is not well understood. Skeletal muscle atrophy may result from a perturbation in protein breakdown, protein synthesis, muscle cell regeneration by satellite cells, or a combination of these processes (6, 19, 20, 26). Proliferation and differentiation of satellite cells (mononuclear precursor stem cells in skeletal muscle) into mature myocytes has been shown to be responsible for muscle growth and repair (12). Attenuated satellite cell proliferation and differentiation into mature skeletal muscle may limit regeneration after atrophy (5, 10, 12). Decreased satellite cell function occurs with skeletal muscle atrophy of aging and immobilization (4, 12, 27). The role of muscle cell regeneration in the development of skeletal muscle atrophy in cirrhosis has not been examined.

Several growth factors and hormones have been identified that affect skeletal muscle regeneration via the satellite cells (5, 18). These include myostatin and insulin-like growth factor 1 (IGF1) (10). Myostatin, which is a member of the transforming growth factor-β superfamily, inhibits muscle growth as well as skeletal muscle regeneration in a paracrine manner (16, 18). Increased expression of myostatin has been demonstrated in human immunodeficiency virus and immobilization-associated atrophy (11, 21). Myostatin inhibits protein synthesis as well as negatively regulates satellite cell activity (18).

Circulating and locally expressed IGF1 is decreased in cirrhosis. IGF1 regulates muscle mass by promoting synthesis of muscle protein and by stimulating recruitment of satellite cells (3, 28). Myostatin and IGF1 have opposing effects on proliferation and differentiation of satellite cells (4, 18).

Proliferation of satellite cells is associated with the expression of PCNA, and their differentiation is associated with the expression of myogenic regulatory transcription factors (myoD, myf5, and myogenin) (8). These factors are used as markers of satellite cell function in intact adult muscles. Myostatin impairs satellite cells by inhibiting the expression of myoD and myogenin (14, 22). Myostatin also increases the expression of cyclin-dependant kinase inhibitor (CDKI p21), which inhibits differentiation of satellite cells into myoblasts and myotubes (18). In contrast, IGF1 increases the expression of CDKI p21 and stimulates satellite cell differentiation (15).

We hypothesized that the muscle wasting in cirrhosis may be related to impaired satellite cell function of skeletal muscle modulated by altered expression of myostatin and IGF1. We tested this hypothesis in the portacaval anastomasis (PCA) rat model, shown to mimic many of the growth and metabolic abnormalities of human cirrhosis (7, 24). With the use of both real-time PCR and Western blot assay, the expression and quantification of myostatin, IGF1, and myogenic regulatory factors were measured in the skeletal muscle.

**METHODS**

**Animals.** Male Sprague-Dawley rats (6 wk old; n = 8) were randomly allocated to an end-to-side PCA or sham surgery. They were maintained for 4 wk while being fed a standard rat chow diet ad libitum. All animals were killed at the end of 4 wk, and the lower extremity muscles and liver were harvested. The tissues were provided very kindly by Dr. Andrew Blei at Northwestern University (Chicago, IL). All organs were then snap frozen in liquid nitrogen and stored at −80°C.

**Tissue extraction.** The lower extremity muscles were dissected to isolate the gastrocnemius muscle. Muscle samples were homogenized,
and subsequently, RNA was extracted using the TRI reagent per manufacturer protocol (Sigma-Aldrich, St. Louis, MO) protocol. Briefly, with the use of a Brinkman tissue homogenizer, 100 mg of gastrocnemius muscle were homogenized in 5 ml of TRI reagent (Sigma-Aldrich). Debris was removed by centrifugation, and RNA isolated and resuspended in diethyl pyrocarbonate-treated water, and the final concentration was determined by measuring absorption at 260 and 280 nm. Ten micrograms of total RNA from each sample were separated on a 1.2% formaldehyde agarose gel to verify the quality of the RNA. First-strand cDNA synthesis was performed using 1 μg of total RNA using BD Clontech kit (Hercules, CA) per manufacturer protocol. Oligonucleotide primers (Table 1) for myostatin, myoD and myogenin (myogenic transcription factors), CDK1 p21, IGF1, IGF1 receptor-α (IGF1Rα), and PCNA as a marker of satellite cell proliferative activity were designed using OLGIO software with a product size 150–600 bp. Primers for proteasome C3, C5, C9, and ubiquitin ligase E3 (atrogin) were used as markers of the ubiquitin proteasome pathway.

Quantitative real-time PCR. Real-time PCR for quantification of RNA was carried out using a SYBR protocol on the fluorescence temperature cycler (Light cycler; Roche Molecular Diagnostics, Indianapolis, IN). The reaction conditions were optimized at different temperature ranges and magnesium concentrations. Real-time reactions were carried out in duplicate, and amplicons were analyzed by generating melting curves with continuous measurement of fluorescence. External standard curves were generated by amplification of 10-fold dilutions of β-actin and the product of interest. Results were calculated as relative differences in target threshold cycle (Ct) values normalized to β-actin. All real-time PCR products were then separated on a 1.5% Tris-acetic acid agarose electrophoresis to confirm product presence and size. A list of primer sequences and expected product size is shown in Table 1.

Western blot analysis. Muscle samples (100 mg each) were homogenized in 1 ml of lysis buffer with protease inhibitor cocktail (Sigma-Aldrich) with addition of activated sodium orthovanadate (Sigma-Aldrich) using a Brinkman tissue homogenizer. The homogenate was subsequently centrifuged at 10,000 g at 4°C for 30 min to remove the tissue debris. The protein concentration of the supernatant was then quantified using a Bio-Rad DC protein assay using BSA as a standard per the manufacturer protocol (Bio-Rad, Hercules, CA). The protein extract was then boiled for 5 min with 1:1 volume of Laemmli loading buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, and 0.05% bromophenol blue). Twenty micrograms of protein from each sample were then separated using SDS-PAGE 4–20% gradient gel under reducing conditions. After overnight electrottransfer to polyvinylidene difluoride membranes (Bio-Rad), the membranes were stained with Ponceau S to confirm equal loading and uniformity of transfer, and then they were destained in Tris-buffered saline (TBS) with Tween (TBST; 0.05 M Tris, pH 7.4, 0.1 M NaCl, 0.1% Tween 20 (Sigma-Aldrich)) and blocked in TBST containing 10% nonfat milk at room temperature for 4 h.

The primary antibodies for myostatin were provided by Dr. Paralkar (Pfizer, Groton, CT). This was a polyclonal antibody raised in rabbits against the COOH-terminal domain (residues 360–365). The purified COOH-terminal domain of human myostatin-secreted component (US Biologicals, Swampscott, MA) was used as a positive control. To ensure selectivity of the antmyostatin antibody, blocking assays were performed using the peptide against which the antibody had been raised. This antibody has been previously reported to detect bands at 15, 30, and 42 kDa (Fig. 1) (2). These represent the active secreted component that has undergone cleavage from the propeptide, incompletely reduced cleaved protein, and the unprocessed monomeric from of the propeptide, respectively.

Myosin heavy-chain monoclonal antibody was obtained from Up-state Biotechnology (Charlottesville, VA). This antibody is directed against 220-kDa myosin heavy chain. All other primary and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A list of the primary antibodies and the dilutions at which they were used for Western blot analysis are shown in Table 2. Membranes were incubated in primary antibody in TBST at 1:200 to 1:1000 concentrations overnight at 4°C followed by four washes for 15 min each in TBST. Subsequently, incubation was done with the secondary antibody at 1:10,000 conjugated to peroxidase for 1 h at room temperature. Membranes were washed again four times in TBST and horseradish peroxidase activity was detected using enhanced chemiluminescent reagent (Amersham Biosciences, Piscataway, NJ). Band intensities were quantified by densitometry (Bio-Rad GL710) using multianalyst software (Bio-Rad).

Myosin heavy-chain protein quantification using Western blot analysis was done as a marker for loss of type 2 fast fiber in skeletal muscle. PCNA is a cofactor for DNA polymerase that is expressed during the S phase of the proliferative cycle and is expressed in activated satellite cells (6). We used PCNA expression to indicate

### Table 1. List of primers and their sequences used in the study

<table>
<thead>
<tr>
<th>Product</th>
<th>Sequence</th>
<th>Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myo D</td>
<td>5′TCAGAGGGGACTGAGAGC3′</td>
<td>565</td>
</tr>
<tr>
<td>Actin</td>
<td>5′GAGCTTCGAAGAAGGAGATGCT3′</td>
<td>233</td>
</tr>
<tr>
<td>PCNA</td>
<td>5′GAAAGAAGCTCCCAAGTTGCG3′</td>
<td>100</td>
</tr>
<tr>
<td>IGF1</td>
<td>5′GGAGAAAGACCTGTTAAGCACCTA3′</td>
<td>167</td>
</tr>
<tr>
<td>IGF1Rα</td>
<td>5′GAATGAGGTCGCTGCTGATG3′</td>
<td>245</td>
</tr>
<tr>
<td>Myogenin</td>
<td>5′TGGGGAGCCTCCTGCTC5′</td>
<td>233</td>
</tr>
<tr>
<td>P21</td>
<td>5′CCCTGGAAGGATGCTGGT3′</td>
<td>233</td>
</tr>
<tr>
<td>Myosin</td>
<td>5′AGGAGGCCAGAGGATGTA3′</td>
<td>226</td>
</tr>
<tr>
<td>Proteasome C3</td>
<td>5′CATCTGGCCTGCTAACTG5′</td>
<td>196</td>
</tr>
<tr>
<td>Proteasome C5</td>
<td>5′CTCTGGGATCAGTGCGCTC5′</td>
<td>159</td>
</tr>
<tr>
<td>Proteasome C9</td>
<td>5′AAAGCTGAGAGAGGGCGCTG3′</td>
<td>155</td>
</tr>
<tr>
<td>Ubiquitin Ligase</td>
<td>5′CCCTGGAAGGATGCTGGT3′</td>
<td>172</td>
</tr>
</tbody>
</table>

IGF1, insulin-like growth factor 1; IGF1Rα, IGF1 receptor-α.
overall changes in proliferation in skeletal muscle and measured the expression of myogenic regulatory factors (myoD, myogenin, myf5) as evidence of differentiation of the satellite cells into myotubes. Myostatin and CDKI p21, which inhibit satellite cell proliferation and differentiation into mature myocytes, were also measured (25).

The expression of IGF1 in the skeletal muscle and liver as well as its receptor IGF1R were studied because of their trophic effect on satellite cell activity (3).

Analyses. Optical density data from Western blot analyses were compared between the anastamosis and sham animals after normalizing for loading, using cytoskeletal β-actin protein. Real-time PCR data were expressed as arbitrary units of the ratio of the cross-over temperatures of the gene of interest to β-actin. Both Western blot analyses and real-time PCR were done randomly on samples in a masked manner. Unmasking was done after final analysis.

All data are expressed as means ± SE. Results were compared using a standard statistical package (SPSS 11.5). Student’s t-test was used to compare the independent group samples. The sample size was estimated based on an expected difference of at least 10-fold in the myostatin RNA expression in the two groups based on our preliminary data on the expression of myostatin in fasted animals.

RESULTS

Total body weight, weights of the liver and gastrocnemius muscle, as well as the organ-to-body weight ratios are shown in Table 3. The initial body weights of the two groups were similar, but at the end of the 4 wk following the anastamosis, PCA animals weighed less than the sham animals. The muscle-to-body weight and the liver-to-body weight ratios were both lower in the PCA animals compared with sham animals (*P = 0.02). As shown in Fig. 2, there was a significant loss of the fast fibers (type 2) in PCA animals compared with sham animals as demonstrated by lower levels of myosin heavy-chain protein (*P = 0.02).

Table 3. Body and organ weights of the two groups

<table>
<thead>
<tr>
<th></th>
<th>PCA</th>
<th>Sham</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preoperative wt, g</td>
<td>338.5±1.1</td>
<td>337.5±0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Postoperative wt, g at 4 weeks</td>
<td>381.5±6.1</td>
<td>489.5±4.3</td>
<td>0.02</td>
</tr>
<tr>
<td>Liver wt, g</td>
<td>8.5±0.1</td>
<td>16.8±0.1</td>
<td>0.02</td>
</tr>
<tr>
<td>Liver-to-body wt ratio, %</td>
<td>2.2±0.00</td>
<td>3.4±0.00</td>
<td>0.02</td>
</tr>
<tr>
<td>Gastrocnemius wt, mg</td>
<td>80.8±0.4</td>
<td>129±1.3</td>
<td>0.01</td>
</tr>
<tr>
<td>Gastrocnemius to body wt ratio, %</td>
<td>0.021±0.004</td>
<td>0.026±0.003</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Values are means ± SE for n = 4 rats per group. PCA, porta caval anastomosis.
Markers of myogenic differentiation. Protein expression of PCNA was significantly lower in PCA animals than sham animals ($P < 0.01$; Fig. 3). The three markers involved in differentiation of satellite cells (myoD, myf5, and myogenin) were significantly lower in PCA animals compared with sham animals ($P < 0.02$; Fig. 4).

Myostatin expression. As displayed in Fig. 5, the myostatin protein expression was threefold higher in the PCA compared with the sham animals ($P = 0.02$). The expression of the myostatin receptor, activinR2b, was also higher in the PCA compared with the sham animals ($P = 0.02$). CDKI p21, which mediates the effect of myostatin, was higher in the PCA animals ($P = 0.01$). Figure 6 summarizes the quantitative changes in the mRNA expression in the myostatin and the pathways it regulates. The mRNA for myostatin, activinR2b, and CDKI p21 were all higher ($P = 0.02$), whereas the mRNA for PCNA, myoD, myf5, and myogenin were lower ($P = 0.02$) in PCA compared with sham animals. These changes observed in the mRNA levels were consistent with the changes in protein expression displayed in Figs. 2–5.
IGF1 pathway. IGF1 protein was lower ($P = 0.01$) in the liver of PCA rats (Fig. 7). However, in the gastrocnemius muscle, it was not significantly lower in PCA animals compared with control animals ($P = 0.08$). The mRNA of IGF1 and its receptor was lower ($P = 0.02$) in the muscle of the PCA animals (Fig. 8). These changes were also reflected in the changes in their protein expression.

Ubiquitin proteasome pathway. The results of the quantitative PCR of the key genes in the ubiquitin pathway are shown in Fig. 9. As shown, the changes in the ubiquitin pathway were not significantly different in the PCA rats compared with the sham animals ($P > 0.1$).

DISCUSSION

We used the PCA rat, because it shares many of the nutritional and metabolic changes with those that occur in human cirrhosis including muscle wasting (7, 24). In addition to a decreased ratio of muscle weight to body weight, evidence of muscle atrophy was also demonstrated by lower levels of myosin heavy-chain protein, the predominant skeletal muscle-specific structural protein present in type 2 fibers. These data confirm previous observations documenting loss of type 2 fiber in cirrhosis (9).

The proliferation and differentiation of satellite cells is an important physiological mechanism for the growth and maintenance of muscle mass. An increased proliferation and differentiation of the satellite cells into mature myocytes contributes to protein accretion (5, 10). In the present study, lower PCNA levels in the gastrocnemius muscle of PCA rats suggest impaired function of satellite cells. Consistent with this finding were the decreased levels of the myogenic transcription factors (myoD, myf5, and myogenin) (13). These observations suggest that impaired satellite cell function may be responsible for the skeletal muscle atrophy in the PCA rat.

Myostatin and IGF1 are important regulators of satellite cell proliferation and differentiation (5, 10, 13, 18). Myostatin inhibits satellite cell function by first binding to its receptor, activinR2b, and then increasing the expression of a cell cycle regulator, CDKI p21 (18). In the PCA rat, both myostatin mRNA and protein were higher in the gastrocnemius muscle compared with sham animals. The expression of activinR2b was also higher in the PCA rats. Expression of CDKI p21, which inhibits proliferation and differentiation of satellite cells, was also increased in the PCA rat. CDKI p21 inhibits cyclin-dependant kinases, a family of proteins that are responsible for the proliferation and differentiation of satellite cells (18). Thus, in the PCA rat, satellite cell function may be inhibited by...
increased myostatin and CDKI p21. Increased expression of myostatin in the PCA rat may be secondary to changes in the hormone and cytokine levels. These include decreased androgens and growth hormone levels, increased glucocorticoids, and TNF-α that may influence the expression of myostatin by binding to the promoter binding site of myostatin gene (17).

The other key regulator of satellite cell proliferative activity is IGF1, which is predominantly synthesized in the liver as well as in the skeletal muscle itself (1). IGF1 has also been shown to increase the regenerative capacity of quiescent satellite cells in aging or hindlimb immobilization (3). In the present study, the IGF1 expression in the liver was decreased in the PCA animals. Additionally, there was a decrease in the IGFR1-α in skeletal muscle. The importance of this finding is emphasized by previous reports (28) of low IGF1 levels in other models of liver disease and that administration of IGF1 improves muscle mass in a rat model of cirrhosis with muscle wasting.

Myostatin has been shown to inhibit protein synthesis, whereas IGF1 promotes protein synthesis in skeletal muscle cell cultures. We did not measure protein synthesis in this study. However, muscle regeneration and protein synthesis are closely associated. A combination of decreased protein synthesis and diminished regeneration may well contribute to the muscle wasting in this model, and investigation of the combined abnormality warrants future studies.

Previous studies (19) have suggested that the major mechanism of muscle loss in cirrhosis is related to increased protein breakdown. This conclusion was drawn predominantly from studies using stable isotopic tracers in human cirrhosis with or without muscle wasting. Further analysis of these studies suggests that decreased protein synthesis may also be a contributory factor to the muscle wasting (26). The observations of insignificant changes in the mRNA of the ubiquitin pathway in the present study are consistent with these data.

In summary, our data indicate that skeletal muscle atrophy in the PCA rat model is associated with impaired proliferation and differentiation of satellite cells mediated via increased myostatin and decreased IGF1 expression. The mRNA of the ubiquitin proteasome-mediated proteolytic pathway is not significantly altered. These combined data suggest that impaired regeneration and decreased protein synthesis rather than increased protein degradation are the major causes of muscle wasting in the PCA rat. Understanding the regulatory factors involved in these abnormalities may provide new therapeutic strategies for the management of skeletal muscle wasting in cirrhosis.

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GRANTS

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