Skeletal muscle atrophy is associated with an increased expression of myostatin and impaired satellite cell function in the portacaval anastamosis rat

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Running head: Muscle regeneration in portacaval anastamosis rat

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Financial support: This work was supported in part by the Institutional Research Award from MetroHealth Medical Center and NIH DK61732
Abstract

Proliferation and differentiation of satellite cells are critical in the regeneration of atrophied muscle following immobilization and aging. We hypothesized that impaired satellite cell function is responsible for the atrophy of skeletal muscle seen in cirrhosis also. Myostatin and insulin like growth factor 1 (IGF1) have been identified to be positive and negative regulators respectively, of satellite cell function. Using a rat model of cirrhosis (portacaval anastomosis or PCA) and sham operated controls, we examined the expression of myostatin, its receptor activinR2b and its downstream messenger, cyclin dependant kinase inhibitor p21 (CDKI p21) as well as IGF1 and its receptor in the gastrocnemius muscle. Expression of proliferating cell nuclear antigen (PCNA), a marker of proliferation, and myogenic regulatory factors (myoD, myf5 and myogenin), markers of differentiation of satellite cells were also measured. Real time polymerase chain reaction for mRNA and western blot assay for protein quantification were performed. PCA rats had lower body weight and gastrocnemius weight compared to sham animals (p<0.05). PCNA and myogenic regulatory factors were lower in PCA rats (p<0.05). Myostatin, activinR2b and CDKI p21 were higher in the PCA animals (p<0.05). The expression of insulin like growth factor 1 and its receptor were lower in liver and skeletal muscle of PCA animals (p<0.05). These data suggest that skeletal muscle atrophy seen in the portacaval shunted rats is a consequence of impaired satellite cell proliferation and differentiation mediated, in part, by higher myostatin and lower IGF1 expression.

Keywords: Myostatin, satellite cell, insulin like growth factor, cirrhosis.
Introduction

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 TGFβ 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 (HIV) and immobilization associated atrophy (11,21). Myostatin inhibits protein synthesis as well as negatively regulates satellite cell activity (18).

Circulating and locally expressed insulin like growth factor 1 (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 proliferating cell nuclear antigen (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) that inhibits differentiation of satellite cells into myoblasts and myotubes (18). In contrast, IGF 1 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 anastomosis (PCA) rat model, shown to mimic many of the growth and metabolic abnormalities of human cirrhosis (7,24). Using both real time polymerase chain reaction and western blot assay, the expression and quantification of myostatin, insulin like growth factor 1 and myogenic regulatory factors were measured in the skeletal muscle.
Methods

Animals

Male Sprague Dawley rats (6 weeks old; n=8) were randomly allocated to an end to side portacaval anastamosis or sham surgery. They were maintained for 4 weeks while being fed a standard rat chow diet ad libitum. All animals were sacrificed at the end of 4 weeks 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 extracted using the TRIZOL reagent per manufacturer protocol (Sigma Aldrich, St Louis, MO) protocol. Briefly, using a Brinkman tissue homogenizer 100 mg of gastrocnemius muscle was homogenized in 5 ml of Tri reagent (Sigma Aldrich, St Louis, MO). Debris was removed by centrifugation and RNA isolated, resuspended in diethyl pyrocarbonate treated water and the final concentration determined by measuring absorption at 260 nm and 280 nm. Ten micrograms of total RNA from each sample was separated on a 1.2% formaldehyde agarose gel to verify the quality of the RNA. First strand cDNA synthesis was performed using 1 microgram of total RNA using BD Clontech kit (Hercules, CA) per manufacturer protocol. Oligonucleotide primers (table 1a) for myostatin, activinR2b (myostatin receptor), myoD and myogenin (myogenic transcription factors), CDKI p21, IGF1, IGF1 receptor alpha and proliferating cell nuclear antigen (PCNA) as a marker of satellite cell proliferative activity were designed using OLIGO® software with a product size
150 to 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 polymerase chain reaction**

Real time polymerase chain reaction 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 beta actin and the product of interest. Results were calculated as relative differences in target 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 are 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; St. Louis, MO) with addition of activated sodium orthovanadate (Sigma Aldrich; St. Louis, MO) using a Brinkman tissue homogenizer. The homogenate was subsequently centrifuged at 10,000g at 4°C for 30 minutes to remove the tissue debris. The protein concentration of the supernatant was then quantified using a BioRad DC protein Assay using BSA as a standard per the manufacturer protocol (BioRad; Hercules, CA). The protein extract was then boiled for 5 minutes with 1:1 volume of Laemmeli loading buffer (125 mM Tris HCl, pH 6.8; 20% glycerol, 4% SDS and 10% β mercaptoethanol; 0.05% bromophenol blue). 20 µg of protein from each sample was then separated using SDS PAGE 4-
20% gradient gel under reducing conditions. After overnight electro transfer to PVDF membranes (BioRad; Hercules, CA), the membranes were stained with Ponceau S to confirm equal loading and uniformity of transfer and then de-stained in Tris Buffered saline (TBS) with Tween (TBST) [0.05M Tris, pH 7.4; 0.1M NaCl, 0.1% Tween 20 (Sigma Aldrich; St. Louis, MO)] and blocked in TBST containing 10% non fat milk at room temperature for 4 hours.

The primary antibodies for myostatin were provided by Dr. Paralkar (Pfizer; Groton, CT). This was a polyclonal antibody raised in rabbits against the carboxy terminal domain (residues 360-365). The purified carboxy terminal domain of human myostatin secreted component (US Biologicals; Swampscott, MA) was used as a positive control. To ensure selectivity of the antimyostatin 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 kD (Figure 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 Upstate Biotechnology (Charlottesville, VA). This antibody is directed against 220 kD 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 blots 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 4 washes for 15 min each in TBST. Subsequently, incubation was done with the secondary antibody at 1:10,000 conjugated to peroxidase for 1 hour at room temperature. Membranes were washed again 4 times in TBST and HRP activity by chemiluminescence using ECL reagent (Amersham Biosciences; Piscataway,
NJ). Band intensities were quantified by densitometry (BioRad GL710) using multianalyst software (BioRad; Hercules, CA).

Myosin heavy chain protein quantification using western blots was done as a marker for loss of type 2 fast fiber in skeletal muscle. PCNA is a co factor 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 overall changes in proliferation in skeletal muscle and measured the expression of MRFs (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 alpha were studied because of their trophic effect on satellite cell activity (3).

Analyses

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

All data are expressed as mean ± SEM. 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 2 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 2 groups were similar but at the end of the 4 weeks 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 to sham animals (p= 0.02). As shown in Figure 2, there was a significant loss of the fast fibers (type 2) in PCA animals compared to sham animals as demonstrated by lower levels of myosin heavy chain protein (p=0.02).

Markers of myogenic differentiation

Protein expression of proliferating cell nuclear antigen (PCNA) was significantly lower in PCA animals than sham animals (p=0.01) (Figure 3). The three markers involved in differentiation of satellite cells: myoD, myf5 and myogenin were significantly lower in PCA animals compared to sham animals (p=0.02) (Figure 4).

Myostatin expression

As displayed in Figure 5, the myostatin protein expression was 3 fold higher in the PCA compared to the sham animals (p=0.02). The expression of the myostatin receptor, activinR2b was also higher in the PCA compared to 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) while the mRNA for PCNA, myoD, myf5 and myogenin were lower(p=0.02) in PCA compared to sham animals. These changes observed in the mRNA levels were consistent with the changes in protein expression displayed in Figures 2-5.
Insulin like growth factor 1 pathway

IGF1 protein was lower (p=0.01) in the liver of PCA rats (Figure 7). However, in the gastrocnemius muscle, it was not significantly lower in PCA animals compared to control animals (p=0.08). The mRNA of IGF1 and its receptor were lower (p=0.02) in the muscle of the PCA animals (Figure 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 Figure 9. As shown, that the changes in the ubiquitin pathway were not significantly different in the PCA rats compared to 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 was 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 to 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 maybe secondary to changes in the hormone and cytokine levels. These include, decreased androgens and growth hormone levels, increased glucocorticoids and tumor necrosis factor alpha, 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 hind limb 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 IGF1 receptor in skeletal muscle. The importance of this finding is emphasized by previous reports 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 (28).

Myostatin has been shown to inhibit protein synthesis while 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 have suggested that the major mechanism of muscle loss in cirrhosis is related to increased protein breakdown (19). 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 cause 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.
Acknowledgment

The authors thank Mrs. Joyce Nolan for her secretarial support.
References:


Legends to figures.

**Figure 1.** Myostatin isoforms in rat gastrocnemius muscle. Western blot was performed on protein extracts from the gastrocnemius muscle. 20 µg of total protein was resolved on 4-20% gradient SDS PAGE under reducing conditions. Rabbit polyclonal antibody against myostatin peptide was used. The bands at 15 kD, 30 kD, and 42 kD are shown. These represent the secreted component, the incompletely reduced and the unprocessed monomeric species of the protein respectively.

**Figure 2 A.** Western blots of myosin heavy chain in the gastrocnemius muscle protein extract of the PCA (indicated by arrows) and sham animals. The monoclonal antibody against myosin heavy chain detected only a single band at 220 kD. Beta actin was used as loading control. 20 µg of total protein was resolved in 4-20% SDS PAGE.

**Figure 2.B.** Histogram showing the mean and SEM of the arbitrary densitometric readings of the myosin heavy chain normalized for beta actin in the PCA and sham animals. N=4 in each group, *p=0.02.

**Figure 3.A.** Western blots of proliferating cell nuclear antigen (PCNA) in gastrocnemius muscle protein from PCA (indicated by the bold arrows) and sham animals. Beta actin was used as loading control.

**Figure 3.B.** Histogram showing the mean and SEM of the densitometric readings of PCNA normalized for beta actin in the PCA (n=4) and sham (n=4) animals. *p = 0.01

**Figure 4. A.** Western blots of myogenic regulatory factors (myoD, myf5 and myogenin) in the gastrocnemius muscle protein of PCA (indicated by the bold arrows) and sham animals. Beta actin was used as loading control.
Figure 4.B. Histogram showing the mean and SEM of the arbitrary densitometric readings of the myosin regulatory factors normalized for beta actin in the PCA and sham groups. N=4 in each group, *p=0.02

Figure 5.A. Western blots of myostatin (30 kD) and its receptor, activinR2b and cyclic dependent kinase inhibitor p21 in the PCA (indicated by the bold arrows) and sham animals. Beta actin was used as loading control.

Figure 5.B. Histogram showing the mean and SEM of the arbitrary densitometric readings of myostatin, activinR2b and p21 in the PCA and sham groups. N=4 in each group, *p=0.02, #p = 0.01

Figure 6. Histogram showing the quantitative changes (mean and SEM) by real time PCR in the mRNA of the various myogenic genes studied in the PCA animals compared to the sham animals. Bars extending above the baseline show an increase and those extending below this show a decrease in expression of the mRNA. *p=0.02.

Figure 7.A. Western blots of insulin like growth factor (IGF1) and its receptor in the liver and skeletal muscle of the PCA (indicated by the bold arrows) and sham animals. Beta actin was used as loading control.

Figure 7.B. Histogram showing the mean and SEM of the arbitrary densitometric readings of the IGF1 and its receptor in the PCA and sham groups. N=4 in each group; *p = 0.01  #p = 0.03.

Figure 8. Histogram showing the quantitative changes (mean and SEM) by real time PCR in the mRNA of the IGF1 and IGF1 receptor alpha genes in the PCA animals compared to the sham animals. Bars extending above the baseline show an increase and those extending below this show a decrease in expression of the mRNA. *p=0.02.
Figure 9. Histogram showing the quantitative changes (mean and SEM) by real time PCR in the mRNA of atrogin (ubiquitin ligase E3), and of proteasome C3, C5, and C9 in the PCA animals compared to the sham animals. Bars extending above the baseline show an increase and those extending below this show a decrease in expression of the mRNA. (p>0.1 for all the mRNA)
Figure 1

<table>
<thead>
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<td></td>
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Figure 2

A

Myosin heavy chain
Beta actin

B

Units

PCA  Sham

*
Figure 3

A

B

![Image of a gel showing PCNA and Beta actin bands]

![Bar graph showing units comparison between PCA and Sham with an asterisk (*) indicating a significant difference]
Figure 4

A

M 1 2 3 4 5 6 7 8

Myf5

MyoD

Myogenin

Beta actin

B

Units

0 0.2 0.4 0.6 0.8 1 1.2 1.4 1.6

MyoD Myf5 Myogenin

PCA Sham

*
Figure 5

A

Myostatin
p21 protein
ActivinR2b receptor
Beta actin

B

Myostatin
Activin 2B
p21
Figure 6
Figure 7A

1 2 3 4 5 6 7 8

- IGF1 muscle
- IGF1 receptor in muscle
- IGF1 liver
- Beta actin

Figure 7B

Muscle IGF1 | Liver IGF1 | Muscle IGF1 Receptor

- PCA
- Sham

Unit

0 0.5 1 1.5 2 2.5 3

* #
Figure 8

- IGF1 muscle
- IGF1 liver
- IGF1 receptor muscle

Fold change
Figure 9

Fold change in mRNA

Atrogin  Proteasome C3  Proteasome C5  Proteasome C9
**Table 1.** List of primers and their sequences used in the study

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<th>Sequence</th>
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<td>100</td>
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<tr>
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<td>P21</td>
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<td>233</td>
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Table 2. Primary antibodies used for the western blot assays.

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<th>1º Antibody Used</th>
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<td>β-Actin</td>
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<td>MyHCF</td>
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<td>PCNA</td>
<td>Mouse monoclonal</td>
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<td>IGF1Rα</td>
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MyHCF: Myosin heavy chain; CDKI: cyclin dependant kinase inhibitor;
PCNA: proliferating cell nuclear antigen; IGF: insulin like growth factor; IGFR1α: Insulin like growth factor 1 receptor alfa.
Table 3. Body and organ weights of the 2 groups

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<th>Sham (n=4)</th>
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<td>Postoperative weight (gm) at 4 weeks</td>
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