Research Article

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Sarcopenia associated with portosystemic shunting is reversed by follistatin

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Background & Aims: The distinct role of portosystemic shunting (PSS) in the pathogenesis of sarcopenia (skeletal muscle loss) that occurs commonly in cirrhosis is unclear. We have previously shown increased expression of myostatin (inhibitor of skeletal muscle mass) in the portacaval anastamosis (PCA) rat model of sarcopenia of PSS. The present study was performed to examine the mechanisms of sarcopenia following PCA.

Methods: In PCA and sham operated pair fed control rats, the phenylalanine flooding dose method was used to quantify the fractional and absolute protein synthesis rates in the skeletal muscle over time and in response to follistatin, a myostatin antagonist. The expression of myostatin and markers of satellite cell (myocyte precursors) proliferation and differentiation were quantified by real-time PCR and Western blot analyses.

Results: The absolute synthesis rate (ASR) was lower at 2, 4, and 6 weeks (p <0.05) and the fractional synthesis rate (FSR) of skeletal muscle protein was significantly lower (p <0.05) at week 2 in the PCA rats compared to control rats. Expression of myostatin was elevated while markers of satellite cell proliferation and differentiation were lower at 4 and 6 weeks after PCA. Follistatin increased skeletal muscle mass, muscle FSR and ASR, decreased expression of myostatin protein, and increased expression of markers of satellite cell function.

Conclusions: Sarcopenia associated with PSS is caused by impaired protein synthesis and reduced satellite cell function due to increased myostatin expression. Confirming these alterations in human patients with cirrhosis will provide novel therapeutic targets for sarcopenia of liver disease.

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Introduction

Sarcopenia or loss of skeletal muscle mass is the most common and potentially reversible complication of cirrhosis with portosystemic shunting (PSS) and adversely affects prognosis [1]. However, the distinct role of PSS in the etiology of sarcopenia is unclear. Sarcopenia results from varying contributions of reduced protein synthesis, increased protein breakdown, and an impaired proliferation and differentiation of skeletal muscle progenitor satellite cells [2]. Satellite cells are myogenically committed precursor cells that contribute to recovery from injury as well as the regenerative response in adult skeletal muscle cells [3]. The portacaval anastamosis (PCA) rat reproduces the nutritional, metabolic, biochemical, hormonal, and behavioral changes that occur in cirrhosis and permits the consequences of PSS to be examined independent of any inflammatory and cellular injury responses of cirrhosis [2,4,5]. Hepatic atrophy develops following PSS and despite the above described changes, cirrhosis, ascites or varices do not develop. We have previously shown that in the initial two weeks following PCA, there is an increase in expression of genes regulating protein breakdown. Subsequently, there is an increased expression of myostatin accompanied by impaired satellite cell function after this initial two week period [6].

Myostatin, a member of the TGFβ superfamily, has been implicated in the pathogenesis of diminished muscle mass by impaired satellite cell function (*in vitro* studies) as well as reduced protein synthesis (both cell culture and *in vivo* animal studies) [7–11]. The relation between changes in myostatin expression and skeletal muscle protein synthesis following portosystemic shunting has not been examined [6,12]. Therefore, we quantified both the fractional synthesis rate (FSR) and the absolute synthesis rate (ASR) of the skeletal muscle protein at 2, 4, and 6 weeks following PCA and in sham operated, pair fed, control rats. We also studied the response to follistatin, a functional antagonist of myostatin, in order to establish the causal role of myostatin in sarcopenia of PSS [13,14].

Materials and methods

Animals

Male Sprague–Dawley rats (post pubertal, 9 weeks old) with an end to side PCA or sham surgery (Charles River, Inc.; Wilmington, DE) were housed individually in a 12 h light/dark cycle. Pair feeding was done by providing the sham operated control rats with the same quantity of standard rat chow (Harland Teklad rodent chow #8604; protein 24.5%, fat 4.4%, 3.93 kcal/g) as had been ingested by a paired PCA rat fed *ad libitum*.

Keywords: Skeletal muscle loss; Liver disease; Portacaval anastamosis; Myostatin.

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Food and water intake were measured daily, and animals were weighed every 3 days. Lean body mass and grip strength were measured weekly until the animals were killed. Shunt patency and size were established by our previous criteria of elevated plasma ammonia, low spleen weight, and plasma amino acid concentrations and anatomic evaluation at necropsy [2]. Rates of protein synthesis were measured at 2, 4, and 6 weeks after surgery in PCA and control rats ($n = 5$ each).

A separate group of male Sprague–Dawley rats (8–9 weeks age) subjected to PCA or sham surgery ($n = 8$ each) were randomized to receive either follistatin in phosphate buffered saline in the dose of 10 μg/100 g or vehicle alone. Follistatin or vehicle was administered three times a week intraperitoneally at weeks 3 and 4 after surgery. This time interval was chosen based on our previous observations that the skeletal muscle expression of myostatin is elevated at weeks 3 and 4 after PCA [6]. The dose was based on previously published data [14–16]. Food, water intake, lean body mass and forelimb grip strength were measured. In a subgroup ($n = 5$ in each of the four groups) of these rats, skeletal muscle protein synthesis rate was quantified after completion of follistatin administration. All studies were approved by the Institutional Animal Care and Use Committee.

Methods

Protein synthesis rates were measured using the flooding dose technique by an intravenous injection of L-[4-^{3}H]phenylalanine [17]. In brief, L-[4-^{3}H] phenylalanine with unlabeled phenylalanine (150 mM in sterile water) 50 μCi/ml was injected (1.0 ml/100 g body weight) through the tail vein. Twenty minutes later the rats were killed with intraperitoneal pentobarbital and the gastrocnemius muscle and liver rapidly harvested, blotted dry of blood, weighed, and frozen in liquid nitrogen.

A precisely weighed amount (~0.1 g) of frozen muscle was pulverized, precipitated using ice cold 2% (w/w) perchloric acid, and centrifuged. Free amino acids in the supernatant were separated using HPLC (Agilent Technologies, Santa Clara, CA) and the specific radioactivity (SA) of phenylalanine measured. The precipitate containing labeled phenylalanine bound to muscle protein was quantified by hydrolyzing the protein in 5 ml of 6 M HCl for 24 h at 110 °C.

Fractional rate of protein synthesis

The FSR of protein (K_s) was obtained by

$$K_s = S_B \times 100/S_A \times t$$

where t is the incorporation time in hours, S_B is the specific radioactivity of phenylalanine in the protein, S_A the specific radioactivity of phenylalanine in the tissue, and the unit of K_s is % per hour.

The ASR was calculated as the FSR × total protein content of the tissue and expressed as mg/h. The total protein in the skeletal muscle was measured using the BioRad DC assay (BioRad, Hercules, CA). Food efficiency was calculated as previously described [2,18].

Generation of follistatin

Follistatin is a potent antagonist of myostatin and at least three isoforms of follistatin have been identified [19]. The FST303 expression construct was prepared from the FST315 cDNA by deleting the sequence 3′ to codon 303 up to the start of the Myc-His tag using standard PCR methods. The resulting C-terminal Myc-His-tagged cDNA constructs were transfected into HEK-293-F cell suspension cultures in freestyle serum-free medium (Invitrogen). Recombinant proteins were purified by nickel–Sepharose affinity chromatography (QIAGEN, Valencia, CA) and concentrated by centrifugal dialysis into Dulbecco's PBS.

Measurement of lean body mass

Lean body mass was obtained weekly using the TOBEC® body analyzer for small animals (SA-3000) fitted with the 114 × 318 mm measuring chamber (SA-3114) (EC Systems, Inc., Springfield IL), as described by us [6].

Grip strength

Forelimb grip strength was measured by a single operator using a computerized rat grip strength meter model 1067CSX (Columbus Instruments, Columbus, OH) as described earlier [6].

Table 2. Protein synthesis rate in skeletal muscle.

	FSR %/hr	ASR mg/hr	Protein Synthesis/ gm muscle mg/hr/gm
PCA			
Week 2	0.2 ± 0.02	11.2 ± 2.4	18.2 ± 4.9
Week 4	0.18 ± 0.03	11.9 ± 2.6	17.7 ± 7.4
Week 6	0.13 ± 0.02	6.3 ± 1.3	12.0 ± 6.5
SHAM			
Week 2	0.36 ± 0.1[a]	34.1 ± 11.9[c]	39.4 ± 23.2[d]
Week 4	0.17 ± 0.02[b]	24.7 ± 3.3[c]	25.3 ± 7.6[d]
Week 6	0.13 ± 0.0[b]	23.5 ± 4.2[c]	19.4 ± 6.0[d]

Mean ± SEM. FSR, fractional synthesis rate; ASR, absolute synthesis rate.
a: p <0.05 PCA vs. sham FSR; b: p <0.05 FSR in sham at week 2 compared to weeks 4 and 6; c: p <0.01 PCA vs. sham ASR; d: p <0.01 PCA vs. sham protein synthesis/g muscle.

Table 1. Characteristics of PCA and sham rats.

	Preoperative Weight (g)	Weight at kill (g)	Lean body mass (g)	Gastrocnemius Weight (g)	Grip Strength (lb)	Arterial Ammonia (μmol)
PCA						
Week 2	259.2 ± 11.8	261.2 ± 36.2[a]	234.9 ± 25[a]	0.6 ± 0.1[a]	0.65 ± 0.002	396.0 ± 35.6[a]
Week 4	261.0 ± 7.6	282.6 ± 45.8[a]	248.5 ± 29.6[a]	0.7 ± 0.1[a]	0.79 ± 0.002	308.6 ± 14.3[a]
Week 6	260.8 ± 10.6	298.6 ± 57.4[a]	263.3 ± 39.8[a]	0.5 ± 0.1[a]	1.28 ± 0.01	246.6 ± 12.4[a]
SHAM						
Week 2	261.0 ± 8.3	344 ± 10.7[b]	290.3 ± 16.7[b]	0.8 ± 0.1[b]	1.7 ± 0.004	109.8 ± 14.4[d]
Week 4	258.0 ± 7.2	388.0 ± 12.0[c]	317.1 ± 8.2[c]	1.0 ± 0.2[c]	2.06 ± 0.003	80.8 ± 6.6[d]
Week 6	262.3 ± 9.3	452 ± 26.3[d]	369.3 ± 20.3[d]	1.2 ± 0.2[e]	5.16 ± 0.008	80.7 ± 4.3[d]

Mean ± SEM $n = 5$ in each group.
a–b, a–c, c–e, b–c: p <0.05.
a–d, c–d, b–d: p <0.01.
c–f: p <0.001.

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